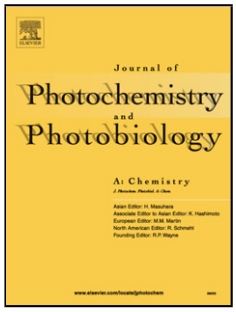

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Novel synthetic approach to Near-Infrared Heptamethine Cyanine Dyes and Spectroscopic characterization in Presence of Biological Molecules

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Graphical abstract

Highlights

- Novel synthetic approach to Near-Infrared heptamethine cyanine dyes.
- Evaluation of photophysical properties in organic solvents.
- Association of the dyes **AK7** in presence of dsDNA.
- Binding of the dyes **AK7** to Bovine Serum Albumine (BSA).
- Partition of the dyes **AK7** with lipid membranes (PC).

Abstract

Two near-infrared symmetric heptamethine cyanine dyes, containing integrated chlorosubstituted cyclohexenyl ring were obtained via a novel synthetic approach, using mild conditions at room temperature. UV-Vis and fluorescence properties of the dyes were evaluated in organic solutions varying solvent polarity. Further spectral studies illustrating their application as potential labels for biological molecules, were reported in the presence of deoxyribonucleic acid (DNA), bovine serum albumin (BSA), and phospholipids (PC). The dyes undergo H-type intramolecular complexation between the two cyanine subunits when free in buffer. Absorption studies show that binding of cyanines to DNA, BSA and lipid membranes leads to the red shift in the H-aggregates peak with appearance of monomeric and dimeric dye species pointing to the disaggregation of the dyes under study in the presence of biological molecules. The fact that the decrease of liposome-induced cyanine aggregation was more pronounced in the zwitterionic phosphatidylcholine bilayer, suggests that the disruption of highly organized molecular arrangements are most likely electrostatically-controlled.

Keywords: Heptamethine cyanine dyes; H-aggregates; DNA; Bovine serum albumin; Phospholipids.

1. Introduction

Among a wide variety of fluorescent dyes currently used in biomedical research and industry, considerable attention is given to near-infrared (NIR) cyanine probes, which have found numerous analytical applications in enzymatic and immuno-assays [1, 2], drug displacement studies [3], DNA sequencing [4] and cancer targeting applications[5], as optical imaging agents, labels and stains [6-8], to name only a few. Undeniably, NIR cyanine dyes have been extensively used due to their advantageous photophysical properties, namely absorption and fluorescence spectrum in the NIR region (670-1100 nm). Another essential feature of cyanine dyes is very high polarizability of the π-electron system along the polymethine group in the ground state, which give rise to strong dispersion forces between two cyanine molecules in solution [9]. These dispersion forces are thought to control the formation of extended aggregates of cyanines in solutions [10-12]. Since cyanine dyes undergo self-organization, they have been found to be an invaluable tool used to study molecular organization in proteomics [13], optics [14] and nanoscience [15].

Depending on the angle of molecular slippage, α, self-association of cyanine dyes results in the formation of molecular aggregates of different structures, known as H-and J-aggregates [16, 17]. Large molecular slippage ($\alpha<32^o$) led to the self-organization of "brickwork" arrangements (J-aggregates), while small one ($\alpha>32^o$) is characterized by "card-pack" structures (H-aggregates). Dictated by the geometry of the molecules within the aggregate, H- and J-aggregation patterns possess unique electronic and spectroscopic properties and can be easily identified by spectroscopic methods. According to Kasha [18] exciton model for molecular aggregates, the interactions between neighboring transition dipoles of tightly packed molecules generates a splitting of the excited state into excitonic levels that are shared between all the

molecules within arrangement. When two dipoles are face-to-face (H-aggregates), the energy of the allowed state corresponding to in phase transition dipoles is increased by repulsive electrostatic interactions between the transition dipoles [18]. In contrast, the lower exciton level is observed for molecules with end-to-end packing (J-aggregates), which is manifested in red shift in the absorption spectrum.

Trends of cyanine dye aggregation are well described now, and it has been found that the structure of the dye, particularly the presence of substituents in heterocyclic residues and the N-alkyl chain length, directly dictates the type of aggregates formed [11, 19, 20]. The other factors contributing to the formation of highly organized molecular arrangements are probe concentration [21], solvent polarity [22], temperature [23], presence of polyelectrolytes [24, 25], polymers [26], Langmuir-Blodgett films [19], etc. The self-association have been shown to be inhibited also by high ionic strength since at high salt concentration the aggregates are more energetically stable then the monomeric dye due to the formation of contact ion pairs between the cationic dyes and counterions [10]. An additional agent affecting the formation of ordered nanoassemblies of noncovalently coupled probes is surfactants [11, 27, 28]. Numerous studies indicate that the addition of surfactants can provoke the aggregation of dyes, in certain cases changing the morphology of arrangements [11, 28]. Recently, it was concluded that the presence of biomolecules, such as proteins and nucleic acids, have a significant impact on aggregation properties of organic dye molecules [15, 20, 21, 29-31]. Specifically, DNA was shown to serve as a template for the growth of helical arrays of cyanine dyes [15, 21, 29]. The assembly of these supramolecular structures occurs by a cooperative, chain-growth mechanism and is essentially promoted by DNA [15, 29]. The role of proteins as scaffold for cyanine aggregation or disaggregation was also described in a number of works [20, 31].

The aim of the present paper was threefold: i) to utilize a novel synthetic approach to NIR heptamethine cyanine dyes containing integrated chloro-substituted cyclohexenyl ring (Figure 1), ii) to investigate photophysical properties of the cyanines in organic solvents with varying polarity and iii) to examine the impact of DNA, bovine serum albumin (BSA) and liposomes (PC) on aggregation properties of the examined dyes.

2. Materials and methods

2.1. Materials

Unless otherwise stated, reagents and solvents used in the synthesis and analysis of the dyes were obtained from Sigma-Aldrich, Organica Feinchemie GmbH Wolfen, Alfa-Aesar and Deutero GmbH, as commercial products of analytical grade and used in the synthesis of all target products without further purification. The solvents used for the spectrophotometric and spectrofluorimetric analyses were supplied by Macron Fine Chemicals TM. Double stranded deoxyribonucleic acid from salmon testes (DNA), bovine serum albumin (BSA), 2-Oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PC) and Tris-HCl were purchased from Sigma.

2.2. Analysis methods and equipment

All products including dyes and intermediates were characterized using various spectroscopic techniques. Recrystallization from methanol was carried out as a main method of purification in order to obtain analytical samples of the target products. NMR spectra were recorded on Brucker Avance III HD, 500 MHz instrument (^{1}H-NMR 500 MHz, ^{13}C-NMR 125 MHz) in DMSO-d_6 at 25 ^{o}C. The Coupling constants J were expressed in Hz. ESI mass spectra of both positive and negative ionization modes were recorded on triple quadrupole mass spectrometer Agilent 6410 coupled with HPLC Agilent 1200 Series. Melting point temperatures were determined on a Kofler bench and are uncorrected. UV-visible spectra of the dyes were recorded in various

solvents of spectroscopic grade on a Cecil Aurius CE 3021 UV-Vis spectrophotometer at room temperature. The corresponding emission spectra were recorded on Perkin Elmer LS45 spectrofluorimeter at room temperature using 10-mm path-length quartz fluorescence cuvettes. The dye stock solutions (1 mM) were prepared in DMSO, which were diluted with the corresponding organic solvent. UV-visible spectra of heptamethine dyes in the presence of DNA, BSA and liposomes were measured in the range 500-900 nm with Cecil Aurius CE 3021 UV-Vis spectrophotometer at room temperature using 10-mm path-length quartz cuvettes.

2.3. Synthesis of intermediates 2a, 2b

2-methyl benzothiazole **1** (1.27 mL, 10 mmol) and a small excess of the corresponding alkylating reagent R-Br (11 mmol) were dissolved in 2-methoxyethanol (2 mL). The reaction mixture was heated at reflux for 2 hours. The reaction outcome was monitored by TLC. After the reaction was completed, and the reaction mixture was cooled down to room temperature, N-quaternary product **2** was precipitated with diethyl ether (20 mL). The precipitate formed was filtered off, washed with diethyl ether, and stored in a desiccator (Scheme 1). Purification of the benzothiazolium salts was held by recrystallization from methanol.

3-isopentyl-2-methylbenzo[d]thiazol-3-ium bromide (**2a**): (pale brown solid), yield = 29 %, m.p. = 190-193 ^{o}C; ^{1}H-NMR (DMSO-d$_6$, 500 MHz) δ/ppm: 1.00 (6H, d, J 6.6, 2 x CH_3- CH), 1.71-1.76 (2H, m, CH_2), 1.86 (1H, sept., CH), 3.27 (3H, s, CH_3), 4.72-4.76 (2H, m, CH_2-N), 7.78-7.82 (1H, m, ArH), 7.88-7.91 (1H, m, ArH), 8.31 (1H, d, J 8.5, ArH), 8.53 (1H, d, J 8.2, ArH); ^{13}C-NMR (DMSO-d$_6$, 125 MHz) δ/ppm: 17.4; 22.6; 26.0; 36.5; 48.4; 117.2; 125.3; 128.4; 129.6; 129.8; 141.0; 177.3;

3-benzyl-2-methylbenzo[d]thiazol-3-ium bromide (**2b**): (pale pink solid), yield = 72 %, m.p. = 190-193 ºC; m.p. = 246-249 ºC, lit. m.p. = 255-256 ºC [33]; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ/ppm: 3.30 (3H, s, CH_3), 6.13 (2H, s, CH_2-N), 7.33-7.42 (5H, m, 5 x ArH), 7.78-7.86 (2H, m, 2 x ArH), 7.23 (1H, d, J 8.3, ArH), 8.55 (5H, m, 5 x ArH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ/ppm: 17.9; 52.4; 117.6; 125.4; 127.5; 127.5; 128.7; 129.0; 129.6; 129.7; 130.0; 133.3; 141.4; 178.9;

2.4. Synthesis of heptamethine cyanine dyes AK7 via novel method.

2-methylbenzothiazolium salt **2** (3 mmol) and 2-chloro-3-((phenylamino)methylene) cyclohex-1-en-1-yl)methylene) benzenaminium chloride **3** (1.5 mmol) were suspended in methanol (5 mL) in a mortar, and grinded with a pestle at room temperature (Scheme 2), followed by addition of sodium acetate (3 mmol) upon grinding. After 10 seconds a dark blue color is developed, followed by the simultaneous crystallization of the reaction mixture. The crude product was dissolved in 10 mL methanol and transferred to a beaker containing 100 mL of an aqueous solution of potassium iodide. After 15 minutes the precipitated dyes were filtered off and washed with diethyl ether. Purification of the heptamethine cyanine dyes was held by recrystallization from methanol.

2-((E)-2-((E)-2-chloro-3-((Z)-2-(3-isopentylbenzo[d]thiazol-2(3H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3-isopentylbenzo[d]thiazol-3-ium iodide (**AK7-5**): (green crystals), yield = 90%, m.p. = 236-237 ºC; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ/ppm: 1.00 (12H, d, J 6.6, 4 x CH_3-CH); 1.61 (4H, q, J 7.1, 2 x CH_2); 1.78 (2H, sept., J 6.6, 2 x CH); 1.83-1.89 (2H, m, CH_2); 2.64-2.70 (4H, m, 2 x CH_2); 4.40-4.48 (4H, m, 2 x CH_2-N); 6.45 (2H, d, J 12.1, 2 x CH); 7.40-7.46 (2H, m, 2 x ArH.); 7.57-7.60 (2H, m, 2 x ArH.); 7.71-7.73 (2H, m, 2 x ArH.); 7.82 (2H, d, J

12.1, 2 x CH); 7.99 (2H, d, J 7.7, 2 x ArH.); ESI: m/z: Found [M+] 575.2 $C_{34}H_{40}ClN_2S_2^+$ requires [M+] 575.23; Found [I^-] 126.8 requires [I^-] 126.91; UV/VIS (methanol): λ_{max} = 795 nm, ε = 189.500 $M^{-1}cm^{-1}$, λ_{fl} = 812 nm; (DMSO): λ_{max} = 810 nm, ε = 138.800 $M^{-1}cm^{-1}$, λ_{fl} = 824 nm; (chloroform): λ_{max} = 809 nm, ε = 230.600 $M^{-1}cm^{-1}$, λ_{fl} = 821 nm;

3-benzyl-2-((E)-2-((E)-3-((Z)-2-(3-benzylbenzo[d]thiazol-2(3H)-ylidene)ethylidene)-2-chlorocyclohex-1-en-1-yl)vinyl)benzo[d]thiazol-3-ium iodide (**AK7-6**): (green crystals), yield = 84% (greenish crystals), m.p. = 219-220 ºC; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ/ppm: 1.73-1.78 (2H, m, CH_2); 2.53-2.57 (4H, m, 2 x CH_2); 5.78 (4H, s, 2 x CH_2-N); 6.62 (2H, d, J 13.3, 2 x CH); 7.27-7.29 (4H, m, 4 x ArH.); 7.33 (2H, d, J 7.0, 2 x ArH.); 7.37-7.43 (6H, m, 6 x ArH.); 7.52-7.55 (2H, m, 2 x ArH.); 7.69-7.71 (2H, d, J 8.2, 2 x ArH.); 7.84 (2H, d, J 13.2, 2 x CH); 8.01 (2H, d, J 7.9, 2 x ArH.); ESI: m/z: Found [M+] 615.2 $C_{38}H_{32}ClN_2S_2^+$ requires [M+] 615.17; Found [I^-] 126.8 requires [I^-] 126.91; UV/VIS (methanol): λ_{max} = 802 nm, ε = 170.800 $M^{-1}cm^{-1}$, λ_{fl} = 824 nm; (DMSO): λ_{max} = 817 nm, ε = 133.900 $M^{-1}cm^{-1}$, λ_{fl} = 834 nm; (chloroform): λ_{max} = 815 nm, ε = 225.100 $M^{-1}cm^{-1}$, λ_{fl} = 833 nm;

2.5. Study of DNA, BSA, and PC liposomes interactions.

Stock solutions (1 mM) of heptamethine cyanine dyes **AK7** were prepared immediately before the measurements by dissolving the dyes in DMSO. Stock solutions of DNA and BSA were prepared in 10 mM Tris-EDTA buffer, pH 7.4. The concentrations of dyes, DNA and BSA were determined spectrophotometrically using their molar absorptivities and ε_{260} = 6.4 × 10^3 $M^{-1}cm^{-1}$, ε_{276} = 4.25 × 10^4 $M^{-1}cm^{-1}$ for DNA and BSA, respectively. Working solutions of the heptamethines were prepared by dilution of the dye stock solution in buffer. To determine the absorption of cyanine dyes in the presence of biomacromolecules and lipid membranes,

appropriate amounts of the stock solution of DNA, BSA or liposomes were added to each dye in buffer.

Lipid vesicles composed of PC were prepared using the extrusion technique. A thin lipid film was first formed from the lipid mixtures in chloroform by removing the solvent under a stream of nitrogen. The dry lipid residues were subsequently hydrated with 20 mM HEPES, 0.1 mM EDTA, pH 7.4 at room temperature to yield lipid concentration of 1 mM. Thereafter, lipid suspension was extruded through a 100 nm pore size polycarbonate filter (Millipore, Bedford, USA). In this way, lipid vesicles containing PC. The phospholipid concentration was determined according to the procedure of Bartlett [32].

3. Results and discussion

*3.1. Synthesis and structural analysis of **AK7** dyes*

Herein we report a novel synthetic approach for the synthesis of near-infrared symmetric heptamethine cyanine dyes **AK7-5** and **AK7-6** [33], containing integrated chlorosubstituted cyclohexenyl ring, under mild reaction conditions. The target cyanine dyes were obtained via a simple environmentally green approach, involving a condensation reaction which proceeds through grinding for few seconds the starting 2-methylbenzothiazolium quaternary salts **2** and 2-chloro-3-((phenylamino)methylene)cyclohex-1-en-1-yl)methylene)benzenaminium chloride **3** in a mortar. In order to exchange the anion of the target cyanine dyes, they were dissolved in a minimum amount of methanol and transferred to a beaker containing an aqueous solution of potassium iodide.

The main advantages of this novel synthetic approach are: i) Reactions for the synthesis of the target heptamethine cyanine dyes were carried out at room temperature without need of any heating sources. ii) a mild basic reagent, such as sodium acetate was used in order to generate

methylene group on the benzothiazolic chromophore. iii) The total amount of the solvent involved in the synthesis is minimized to few mL, which is a semi-solid state approach. iv) The condensation reaction yielding the target **AK7** dyes proceeds within 10 seconds according to our method which compared to reported methods [33, 34] the reaction time varies from 3.5 hours to overnight. This accounts for over 10^3 times faster reaction. v) The target heptamethine cyanine dyes were isolated with excellent yields.

The title heptamethine cyanine dyes **AK7** were evaluated by melting point temperatures, ^{1}H NMR spectroscopy, and ESI mass spectrometry of both positive and negative ionization modes.

*3.2. Photophysical properties of the studies dyes **AK7** in various solvents*

The cyanines under study are symmetric heptamethine cyanine dyes, containing integrated chloro-substituted cyclohexenyl ring. The dyes are cationic in nature due to the delocalized positive charge of the chromophore. Spectroscopic characteristics of the two **AK7** dyes in organic solvents with different polarities are presented in Table 1.

Upon varying the solvent polarity from DMSO to chloroform a small shift of absorption and fluorescence maximum positions was observed without almost any significant change in spectrum shape as illustrated for dye **AK7-5** (Figure 2). In the absorption spectra of the studied heptamethine cyanine dyes, the band corresponding to the dye monomer form is observed in the range 795-810 nm, and 802-817 nm for **AK7-5** and **AK7-6**, respectively. Fluorescence spectra of the dyes are typical for heptamethines with emission maxima around 812-824 nm and 824-834 nm for **AK7-5** and **AK7-6**, respectively.

3.3. Quantum yield calculation

Quantum yields of the examined dyes were calculated using Indocyanine green (IR-125) as a standard according to the formula:

The relative fluorescence quantum yield of the examined dyes **AK7** were determined in organic solvents with different polarities using the equation:

$$\Phi_{F(\mathrm{x})} = \frac{m_x}{m_s}\frac{{n_x}^2}{{n_s}^2}\Phi_{F(s)}$$

where, Φ_F is the fluorescence quantum yield, *m* is the slope obtained from the plot of the integrated fluorescence intensity vs absorbance, *n* is the refractive index of the solvent used in the measurement, and the superscripts *s* and *x* represent the reference and unknown parameters respectively. Indocyanine green (IR-125) was used as a reference standard, which has a quantum yield of 0.106 in DMSO [35]. The results depict lower relative quantum yields compared to Indocyanine green (IR-125), which is most likely due to the rigidified polymethine chain.

3.4. Photophysical studies of free dyes AK7 in buffer solutions

Figure 3 represents the absorption spectra of the examined cyanine dyes in buffer solution at different concentration ranging (1.0-20.0) × 10^{-6} M. In aqueous medium at the initial dye concentration the absorption spectra of the heptamethines were blue-shifted by ~ 200 nm relative to those in organic solvents, with the maximum being centered at 589 nm and 641 nm for **AK7-5** and **AK7-6**, respectively. This implies that H-aggregates are the dominating species of the dyes in a buffer solution. Increasing heptamethine concentration resulted in the change of the absorption spectrum from 'one-peak' to 'three-peak' which is more pronounced for **AK7-5**, indicating that these dyes are represented by monomers, H-dimers and higher class of H-aggregates. It is known that the aggregation properties of cyanines are controlled by the balance

between the van der Waals interactions, dispersion forces within the cyanine backbone, the forces of the alkyl chain of entropic and hydrophobic nature together with H-bonding and the electrostatic interactions of the ionic groups [11]. The appearance of dimer and monomer peaks in the absorption spectrum of dyes at the increasing heptamethine concentration is attributed to the dual nature of these dyes. Initially, at low dye concentration in buffer solution the polarizability and hydrophobicity of the dye molecules drive aggregation, overcoming the Coulombic repulsion between the two dye molecules. Further increase in dye concentration change the balance of forces, and electrostatic repulsion between heptamethine molecules led presumably to the occurrence of dimeric and monomeric dye species.

3.5. Photophysical properties of free dyes at 1 × 10^{-6} M, and studies in presence of NaCl

Additional studies of the heptamethine cyanine dyes were performed at lower concentrations (1 × 10^{-6} M) when free in buffer solution, and in presence of sodium chloride in order to examine the behavior of the aggregates.

In the presence of salt, the absorption spectra of the dyes indicate mainly a decrease of the maxima around 613-637 nm representing higher class of H-aggregates. Furthermore, a bathochromic shift of 20 nm is observed for dye **AK7-6**, whereas in the case of **AK7-5** almost no significant changes can be observed. This suggests that the aggregation forces are greater in the case where the dye contains pure aliphatic substituents on the nitrogen atoms of the two chromophores. This red shift of the maxima is also followed by appearance of new peak at 711 nm. This phenomena indicates either disaggregation of the higher class of H-aggregates and

subsequently formation of H-dimers, or transition to different type such as J-aggregates, as a consequence from the presence of salt.

*3.6. Asocciation of the dyes **AK7** with dsDNA*

Next step of the study was directed towards the evaluation of possible biological applications of the examined cyanine dyes. Figure 5 shows the representative absorption spectra of **AK7** dyes in the absence and presence of dsDNA. The complexation of heptamethine cyanine dyes with DNA resulted in attenuation of the intensity due to dilution of the sample concentration, and almost no significant change in the shape of the spectra.

It is known, that depending on dye structure, cyanine dyes exhibit a variety of DNA binding motifs including intercalation, minor groove binding and aggregation. The intercalation between the base pairs is the predominant binding motif for the dyes with a single methine bridge at low dye:base pair concentrations [36,37-39]. Groove binding becomes prevalent for cyanines with more than one methine bridge [30, 40, 41]. Mikheikin et al. [40] demonstrates that trimethine cyanine dyes bind to minor groove of DNA as monomers and dimers. The same binding mode was also observed for tri- and penthamethines [41, 42].

Though, in the heptamethine cyanine dyes under study, the organization of the dye molecules to higher class of H-aggregates most likely appears to be too bulky even for groove binding. In addition to, the corresponding fluorescence studies depict no significant change in terms of fluorescence enhancement (Figure 6), even at high ratios of [DNA]:[AK7] such a 80:1.

3.7. *Asocciation of the dyes **AK7** with Bovine Serum Albumine (BSA)*

In the following, the spectral properties of the examined heptamethine cyanine dyes were analyzed upon their binding to bovine serum albumin. The interactions were monitored by

evaluating the changes in absorbance at a fixed concentration of dye with increasing protein concentration (Figure 7). The changes in absorption spectrum at the presence of protein can be summarized as follows: i) association of heptamethines with BSA led to the diminishing of the peak, attributed to the dye H-aggregates (588 and 634 nm for **AK7-5** and **AK7-6**, respectively) and appearance of the new peaks around 811 and 818 nm, characteristic of the monomer absorption. The observed effect assumes that dye-protein complexation breaks heptamethine aggregates, and the dyes undergo the transition into the monomeric state. However, the contribution of H-aggregate band in the total spectrum was still prevailing over the other dye forms. ii) The reduction of H-aggregates peak is accompanied by red-shift of absorption maximum from 588 nm to 645 nm and from 633 nm to 658 nm for **AK7-5** and **AK7-6**, respectively. iii) The formation of protein-dye complexes resulted in the broadening of H-aggregates absorption spectrum, coupled with appearance of a shoulder around 708-715 nm.

Together with the absence of isosbestic point between aggregated and monomer dye forms, these findings indicate that the heptamethine-protein interaction leads to the concomitant rupture of H-aggregate, appearing in buffer solution and the subsequent formation of dimeric and monomeric dye molecules. Most probably, the binding strength of heptamethines with BSA was strong enough to cause the dissociation of aggregates with aggregation potential strongly reduced after formation of the dye-BSA complexes. The disruption of plane-to-plane arrangements followed by the formation of randomized monomers in the presence of BSA was reported also for merocyanine 540 [43], and heptamethine cyanine dyes [20, 44] and was interpreted as arising from higher strength of interactions between the dye and the BSA compared to the coupling between the cyanine monomeric species in the aggregate. The possible explanation of disassembly of H-aggregates in the presence of BSA in our study could lie in the strong

electrostatic interaction between cyanine dyes and protein. The isoelectric point of BSA is ~4.7 [44, 45], so at the pH 7.4 this protein bears a negative electric charge. It can be seen from the structure of **AK7-5** and **AK7-6** that this dyes are cationic in nature, therefore the binding of heptamethines with BSA is likely to involve electrostatic attraction. In addition, crystal structure of BSA shows that the regions of ligand-binding sites are located in hydrophobic cavities in BSA subdomains, referred to as site I and site II according to terminology proposed by Sudlow et al. [46]. Since heptamethine cyanine dyes under study contain hydrophobic groups in their structure, we can hypothesize that hydrophobic interactions along with electrostatic attractions serve as driving forces to transfer the dye molecules from their aggregate states to the monomeric form. Previously, Patonay and coworkers [47] characterized the hydrophobic binding pockets of the albumin molecule and observed that the binding affinity of cyanine dyes is attributed to the substitution of hydrophobic moieties at the heterocyclic ring of the cyanine dyes. Numerous studies indicate that cyanine dyes generally exhibit a high specificity to the binding site II of albumins [3, 20], so one can hypothesize that the same mode of interactions is realized in our systems.

The disaggregation of the dyes in the presence of BSA and appearance of a monomeric peak lead to multiple enhancement of the fluorescence signal at 818 nm and 824 nm respectively for **AK7-5** and **AK7-6** upon excitation λ_{ex} = 780 nm (Figure 8).

*3.8. Binding of cyanine dyes **AK7** to lipid vesicles.*

Cyanine dyes have been widely employed to trace the processes occurring in biological assemblies such as micelles and vesicles, since their photophysical behavior is strongly depend on the properties of the surrounding medium [22]. Therefore, to get further insights into the

interactions of cyanine dyes with biological objects, their behavior in lipid environment have been studied. Presented in Figure 6 are the absorption spectra of the studied dyes in the presence of liposomes. Association of heptamethines with the PC lipid vesicles (Figure 9) led to the decrease of the absorption peak, attributed to the H-aggregates and appearance of two bands, characteristic of the H-dimer and monomer absorption. The position of H-dimer peak was around 713-720 nm, while monomers were the most absorptive at 819-826 nm, respectively. These findings suggest that upon transition to the lipid environment plane-to-plane arrangement is disrupted, thereby favoring the randomized monomer arrangement. The results presented in Figure 9, indicate that partition of heptamethine cyanine dyes into the liposomes is two-stage cooperative process. Initially, only H-aggregates are present. At the first stage, the addition of the liposomes in relatively low concentrations stimulates the dimer formation. The observed dimerization is most probably arises from the decrease of polarity of the aggregates environment. It is highly probable that the dispersion forces become incapable to govern the staking orientation of the molecules in H-aggregates in low-polar solvents, so we observed the accumulation of dimers in the polar region of lipid bilayer. At the second stage, increase in lipid concentration promotes the appearance of monomeric dye form, and, finally, the contribution of monomeric band in the total spectrum prevails over the other dye forms. The driving force of this process involves, most likely, the hydrophobic interactions. One may suppose that it will be energetically more favorable for hydrophobic moieties at the heterocyclic ring of heptamethines dyes to penetrate dipper into the lipid bilayer orienting parallel to the lipid acyl chains. These hydrophobic interactions led to further decrease of the polarity of lipid dye microenvironment, since it is known that the polarity profile is change dramatically from polar to hydrophobic core of liposomes [48].

Moreover, additional argument comes from the spectral response of a number of environment-sensitive fluorophores [49-51]. It is noted that the changes in hydration extent may considerably affect molecular organization of a lipid bilayer. In particular, increase of water content in headgroup region was reported to modify the alignment of choline-phosphate dipole and lateral packing of hydrocarbon chains [52]. In addition to, as follows from ^{2}H-NMR spectroscopic studies, the presence of PC bilayers alters the orientation and mobility of adjacent choline headgroups [53, 54].

In terms of fluorescence the disaggregation of the dyes in the presence of phospholipids and appearance of a strongly expressed monomeric peak (7.8 and 3.3 fold increase for **AK7-5** and **AK7-6** respectively) lead to multiple enhancement of the fluorescence signal at 828 nm and 832 upon excitation at λ_{ex} = 770 nm and 780 nm respectively (Figure 10).

3. Conclusions

To summarize, the present study was focused on synthesis and spectral characteristics of two near infrared heptamethine cyanine dyes, referred to here as **AK7-5** and **AK7-6**. Based on the results of absorption studies, it was concluded:

The free dyes in aqueous solutions exist mainly in the form of “card-pack” structures (H-aggregates) with the absorption maximum being centered at 588 nm and 639 nm for **AK7-5** and **AK7-6**, respectively. The molecular distribution of the dye in buffer solution is dependent on concentration.

Upon binding to DNA, heptamethines show almost no significant difference in terms of disaggregation. Same observation applies to the fluorescence investigations on the studied dyes.

Heptamethine cyanine dyes form the complexes with BSA mainly in dimeric and monomeric form, binding presumably to the II Sublow center. The possible explanation of dissagregation of

cyanine dyes under study lie in higher strength of interactions between the dye and the BSA compared to the coupling between the cyanine monomeric species in the aggregate.

Association of heptamethines with the lipid vesicles led to the decrease of absorption peak, attributed to the H-aggregates and appearance of two bands, characteristic of the H-dimer and monomer absorption.

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Figure Captions

Figure 1. Studied dyes **AK7**.

Figure 2. Normalized absorption (A) and fluorescence (B) spectra of **AK7-5** in various organic solvents.

Figure 3. Absorption spectra of the dyes **AK7** in buffer solution at increasing concentration.

Figure 4. Absorption spectra of **AK7-5** (A) and **AK7-6** (B) at dye concentration of 1μM recorded in the presence of NaCl.

Figure 5. Absorption spectra of **AK7-5** (A) and **AK7-6** (B) of concentration 10μM recorded in the presence of dsDNA.

Figure 6. Fluorescence spectra of **AK7-5** (A) and **AK7-6** (B) of concentration 10 μM recorded in the presence of dsDNA.

Figure 7. Absorption spectra of AK7-5 (A) and AK7-6 (B) in the presence of bovine serum albumin. AK7-5 and AK7-6 concentrations were 2 μM and 6 μM, respectively.

Figure 8. Fluorescence spectra of AK7-5 (A) and AK7-6 (B) in the presence of bovine serum albumin. AK7-5 and AK7-6 concentrations were 2 μM and 6 μM respectively, λ_{ex} = 780 nm.

Figure 9. Absorption spectra of heptamethine dyes in the presence of pure PC liposomes for AK7-5 (A) and AK7-6 (B), respectively. AK7-5 and AK7-6 concentrations were 2 μM and 6 μM, respectively.

Figure 10. Fluorescence spectra of heptamethine dyes in the presence of pure PC liposomes for AK7-5 (A, λ_{ex} = 770 nm) and AK7-6 (B, λ_{ex} = 780 nm), respectively. AK7-5 and AK7-6 concentrations were 2 μM and 6 μM, respectively.

Scheme 1. Synthesis of N-quaternary 2-methylbenzothiazolium salts **2a, 2b**.

Scheme 2. Synthetic approach to dyes **AK7**.

Tables

Table 1. Spectral characteristics of heptamethine cyanine dyes in different organic solvents.

Dye	Methanol			DMSO			Chloroform		
	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{fl}^{*} (nm)	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{fl}^{*} (nm)	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{fl}^{*} (nm)
AK7-5	795	189.500	812	810	138.800	824	809	230.600	821
AK7-6	802	170.800	824	817	133.900	834	815	225.100	833

$^{*}\lambda_{ex}$ = 730 nm

Table 2. Quantum yields of heptamethine cyanine dyes measured in different solvents

Dye	Methanol	DMSO	Chloroform
AK7-5	0.085	0.095	0.077
AK7-6	0.065	0.078	0.043