Photophysical investigations of squaraine and cyanine dyes and their interaction with bovine serum albumin

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Abstract: A model far-red sensitive symmetrical squaraine dye (SQ-3) and unsymmetrical near infra-red sensitive cyanine dye (UCD-1) bearing direct –COOH functionalized indole ring were synthesized, characterized and subjected to photophysical investigations including their interaction with bovine serum albumin (BSA) as a model protein in phosphate buffer solution (PBS). Both of the dyes exhibit strong interaction with BSA in phosphate buffer with high apparent binding constant. A judicious tuning of hydrophobic main backbone with reactive functionality for associative interaction with active site of BSA has been found to be necessary for BSA detection in PBS.

1. Introduction

Biomedical diagnosis is rapidly growing area in order to provide high quality of life by early disease diagnosis. In this context, optical imaging has emerged as one of the potential candidates due to its low cost, low-energy radiation, high sensitivity and non-invasive nature [1]. In line with fast growing imaging technology, near infrared (NIR) fluorescence probes have shown their potential towards non-invasive *in vivo* imaging owing to their low tissue auto-fluorescence and deep tissue penetration [2]. They have generated considerable research interest in several areas of the biomedical field, including vascular mapping, tissue perfusion, inflammation monitoring and tumor diagnosis. Squaraine dyes belong to interesting organic chromophores consisting of a central four-membered ring-based core and a resonance stabilized zwitterionic structure. The oxocyclobutenolate core is linked by aromatic or heterocyclic components at both ends generating donor–acceptor–donor motif. Squaraine dyes were studied extensively in numerous areas, ranging from synthesis and mechanisms to physical and photophysical properties [3-4]. The absorption and emission behaviour of these dyes can be tuned in the visible and IR wavelength region by judicial molecular design making them suitable for fluorescence imaging since they are outside the self-absorption and auto-fluorescence regions of biological matrices [5]. These unique chemical and physical properties of squaraine molecules have led to studies of their application as near-IR fluorescent chromophores and environmental sensors [6], for bio imaging and biochemical labelling [7-8]. Cyanine dyes are another most extensively investigated organic dyes which are composed of two nitrogen-containing heterocycles as charged chromophores, each conjugated to the end of a polymethine that comprises an odd number of carbons [9]. Cyanine dyes find their versatile applications in the area of fluorescent probes for bio-labelling [10], analyte responsive fluorescent probes [11] and in optoelectronics [12]. Wide range fluorescence

spectral tunability and good fluorescent quantum yield enable the cyanine dyes to detect very low concentrations of analytes [13]. Recent study on unsymmetrical cyanine dyes have gained good attention owing to their excellence nucleic acid staining properties [14].

Photophysical properties such as tuneable absorption and emission in the far-red to NIR region, high molar extinction coefficient, considerable quantum yields and photo-stabilities. Squaraine and cyanine dyes are of utmost interest as fluorescent labels/probes [15-16], biomedical assays [17] and imaging applications [18]. Due to the presence of negatively charged oxygen, zwitterionic squaraine fluorophores are considered more polar molecules as compared to open chain cationic cyanine dyes. Therefore, the quantum yield and fluorescence lifetime of squaraine dyes are considerably decreased, but the increase in quantum yield and lifetime upon covalent or non-covalent binding to most widely investigated model protein bovine serum albumin (BSA) has been reported earlier [19]. This paper describes the detailed synthesis and photophysical characterization of representative NIR cyanine (UCD-1) and far-red sensitive squaraine (SQ-3) dyes. In order to explore their potential application as fluorescent probes to sense protein in phosphate buffer solution (PBS), these dyes were subjected to study their interaction using BSA as a model protein. In order to enable covalent coupling with biomolecules such as peptides, oligonucleotides and proteins, the fluorescent probes should contain reactive functional groups. Therefore, we have chosen these type of molecules where direct ring functionalized carboxy groups which enables covalent attachment either to amino or thiol functionalities of biomolecules.

2. Materials and Methods

All the chemicals for synthesis and solvents are of analytical or spectroscopic grade and used as received without further purification. Unsymmetrical cyanine dye (UCD-1), symmetrical squaraine dye (SQ-3) and their intermediates under investigation (scheme 1) were analysed by MALDI-TOF-mass / FAB-mass spectrometry in positive ion monitoring mode for structural elucidation. Electronic absorption and fluorescence emission spectroscopic investigation in solution phase were executed using UV-visible and fluorescence spectrophotometers. In order to investigate, protein dye interactions, the PBS/Cyanine and PBS/squaraine dye solutions (2μM) were prepared by addition of 100 μl of 0.1 M dye solution in DMF to the different concentrations of PBS/BSA solutions (0-10 μM). The final solutions were gently stirred for 1 hour at room temperature. In order to compare the dye-protein interactions quantitatively, apparent binding constant (K_a) was also calculated from fluorescence titration of dyes at 25°C and at a constant dye concentration by dilution of the initial BSA concentration. Considering BSA/dye association in 1:1 ratio, K_a was calculated using the equation 1 [2, 20].

$$\frac{\mathbf{1}}{(\boldsymbol{F_x - F_0})} = \frac{\mathbf{1}}{(\boldsymbol{F_\infty - F_0})} + \frac{\mathbf{1}}{\boldsymbol{K_a[BSA]}} \frac{\mathbf{1}}{(\boldsymbol{F_\infty | F_0})} \qquad (\mathbf{1})$$

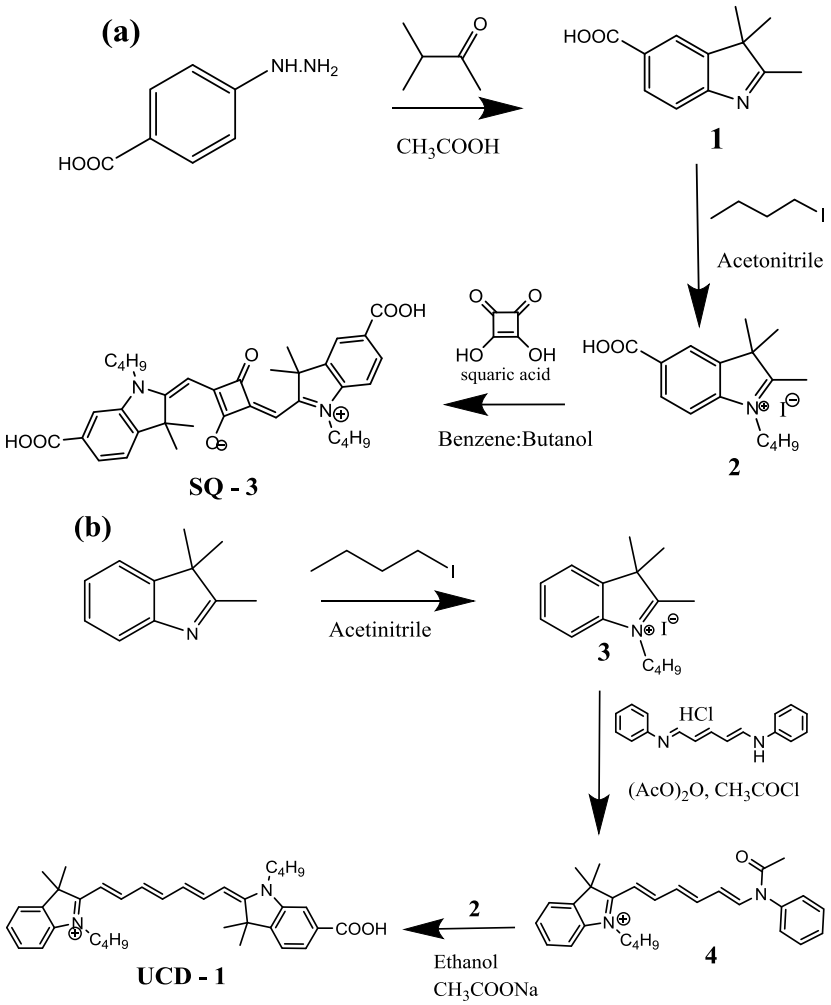
Where, F_0, F_X, and F_∞ are the fluorescence intensities of dyes in the absence of BSA, in the presence of a certain amount of BSA and at a concentration of complete interaction, respectively, while [BSA] is the protein concentration. Equation (1) can be rewritten as

$$\frac{(\mathbf{F_\infty - F_0})}{(\mathbf{F_x - F_0})} = \mathbf{1} + \frac{\mathbf{1}}{\mathbf{K_a[BSA]}} \qquad (\mathbf{2})$$

The binding constant values (K_a) between the BSA and the dyes thus be easily calculated from the slopes of the corresponding plots $(F_\infty - F_0)/(F_X - F_0)$ as a function of $[BSA]^{-1}$ as per the equation (2).

2.1. Synthesis of Squaraine and cyanine dyes and dye intermediates

Model far-red sensitive symmetrical squaraine dye (SQ-3) and unsymmetrical cyanine dye (UCD-1) has been synthesized as per the scheme-1 and reported literature procedures [21-26].



Scheme 1: Synthetic scheme for symmetrical squaraine (SQ-3) dye (a) and unsymmetrical cyanine (UCD -1) dye (b) used for present investigation.

2.1.1. Synthesis of 2,3,3-trimethyl-3H-indole-5-carboxylic acid (1): In a round bottom flask fitted with condenser and N_2 purging, 4-hydrazinobenzoic acid (5.0 g; 32.85 mmol), glacial acetic acid (100 ml), sodium acetate (5.5 g; 67 mmol) and 3-methyl-2-butanone (4.45 g; 51.5 mmol) were added. Reaction mixture was refluxed at 120 °C for 8 h leading to brown suspension. Upon the completion of reaction as monitored by TLC, acetic acid was evaporated followed by addition of 9:1 water methanol mixture on ice-bath leading to precipitation. Residue was filtered and dried giving 3.7 g of titled compound as of white powder in 56% yield. HPLC analysis of product suggests that compound was 100% pure. MALDI-TOF – mass (measured 205.2 $[M+2]^+$; calculated 203.24).

2.1.2. Synthesis of 1-butyl-5-carboxy-2,3,3-trimethyl-3H-indol-1-ium (2): In a round bottom flask one equivalent of (**1**) and 1-Iodobutane (3 eq.) were dissolved in dehydrated acetonitrile and the reaction

was refluxed for overnight to give the corresponding alkyl-3H-indolium iodide. After completion of the reaction as monitored by TLC, solvent was evaporated and the crude product was washed with ample diethyl ether giving the titled compounds in 64% yield having 98% purity as conformed by HPLC. MALDI-TOF – mass (measured 261.13 $[M+H]^+$; calculated 260.36).

2.1.3. Synthesis of 1-butyl-2,3,3-trimethyl-3H-indol-1-ium iodide (3): In a round bottom flask, one equivalent of commercially available 2,3,3-trimethyl indole and 1-Iodobutane (3 eq.) were dissolved in dehydrated acetonitrile and the reaction was refluxed for overnight to give the corresponding alkyl-3H-indolium iodide. After completion of the reaction as monitored by TLC, solvent was evaporated and the crude product was washed with ample diethyl ether giving brown solid in 95% yield having 98% purity as conformed by HPLC. MALDI TOF – mass (measured 217 $[M+H]^+$; calculated 216).

2.1.4. Synthesis of Hemicyanine (4): In a round bottom flask one equivalent of alkyl-3H-indolium iodide (**3**) and glutaconaldehyde dianil monohydrochloride, along with catalytic amount of acetyl chloride were dissolved in acetic anhydride. The reaction mixture was refluxed at 140^0C for 2 hours. After the completion of reaction as monitored by TLC, the reaction mixture was poured on to crushed ice to precipitate the desired compound as black solid. This was filtered, dried and purified by silica gel column chromatography (Ethyl acetate: Hexane = 1:1). The pure compound after column chromatography was obtained as green solid in 60% yield. MALDI-TOF – mass (measured 414.33 $[M+H]^+$; calculated 413.58).

2.1.5. Synthesis of symmetrical Squaraine dye (SQ-3): The dye (**SQ-3**) was synthesized using two equivalents of carboxy functionalized trimethyl indolium iodide salt (**2)** and squaric acid (1 equiv.) in 1-butanol: toluene mixture (1:1, v/v). Reaction mixture was refluxed for overnight using Dean–Stark trap. After completion of reaction, reaction mixture was cooled, solvent was evaporated and product was purified by silica gel column chromatography using chloroform: methanol as eluting solvent. The physical and spectroscopic data of symmetrical SQ-3 dye are as follows; Yield 64% and HPLC purity 98%. MALDI-TOF-mass (calculated 596.29 and observed 597.25 $[M+H]^+$) which confirms the successful synthesis of dye.

2.1.6. Synthesis of Unsymmetrical Cyanine dye (UCD-1): In a round bottom flask one equivalent of hemi cyanine (**4**) was dissolved in 1-butanol: toluene (1:1, v/v). To the above solution one equivalent of Compound **2** and 2 equivalents of sodium acetate were added. The reaction mixture was refluxed at 95^0C for 2 hours. Upon the completion of reaction as monitored by TLC, the solvent was evaporated under reduced pressure and was purified by column chromatography on silica gel to afford titled compound as blue green solid in 43% yield having 95% purity as conformed by HPLC. HRMS (calculated 537.3476 and observed 537.3453 $[M]^+$).

3. Results and Discussion

3.1. *Photophysical Characterization*

Far-red to NIR sensitive dyes SQ-3 and UCD-1 after their successful synthesis and purification were subjected to spectroscopic investigations pertaining to the electronic absorption and fluorescence emission spectroscopy. Figure 1 depicts the electronic absorption and fluorescence emission spectra of both of the dyes UCD-1 and SQ-3 in dimethylformamide (DMF) solution. Characteristic optical parameters for these dyes deduced from Fig. 1 have also been summarized in the table 1. It can be seen from Fig. 1 that both of the dyes SQ-3 and UCD-1 exhibit very sharp and intense light absorption in far-red to NIR wavelength region. Absorption maxima (λ_{max}) of cyanine dye UCD-1 is located at 764 nm with molar extinction coefficient value (ε) (1×10^5 dm^3 M^{-1} cm^{-1}) which is associated with the π-π^* electronic transitions. In the case of squaraine dye SQ -3 ($\varepsilon = 2 \times 10^5$ dm^3 M^{-1} cm^{-1}) observed λ_{max}

is at 650 nm which relatively blue shifted. This is attributed to the reduced extent of p-conjugation in SQ-3 as compared to that of UCD-1. The fluorescent emission spectra for each of the dyes were measured using λ_{max} as excitation wavelength. The emission peaks for UCD-1 and SQ-3 has been observed at 788 nm and 662 nm, respectively with a Stoke shift of 24 nm and 12 nm. This lower Stoke shift for SQ-3 in DMF solution depicts the enhanced rigidity of squaraine dye as compared to that of cyanine dye.

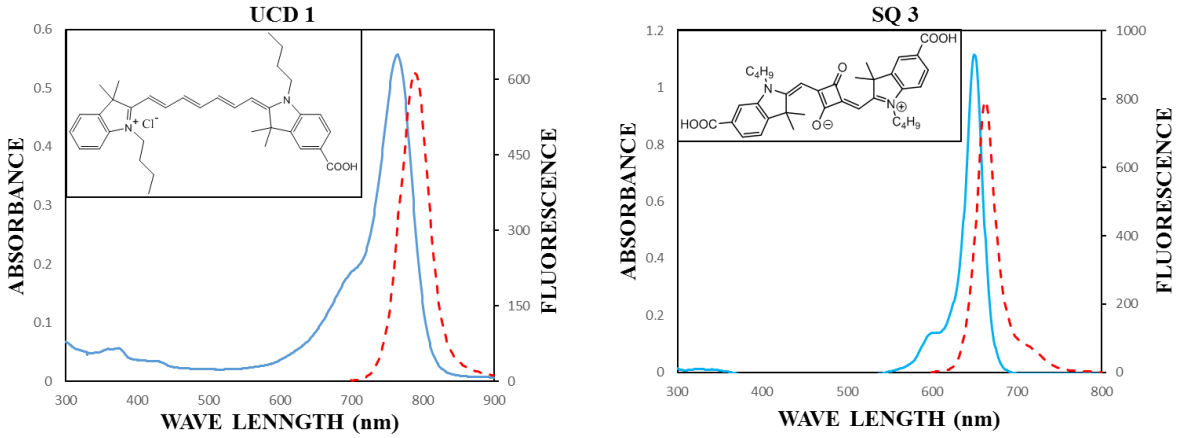


Figure. 1 Electronic absorption (solid line) and fluorescence emission spectra (dashed line) of UCD-1 (left) and SQ-3 (right) in DMF solution (5μM). Molecular structures of dyes are shown in the inset.

Table 1. Spectral properties of sensitizing dyes in DMF and 0.1 M PBS solutions at pH 7.4.

DYE	DMF solution		PBS solution		Stoke Shift		(ε) (dm^3 M^{-1} cm^{-1})
	λ (max) Absorption	λ (max) Emission	λ (max) Absorption	λ (max) Emission	DMF	PBS	
UCD -1	764 nm	788 nm	750 nm	770 nm	24 nm	20 nm	1×10^5
SQ - 3	650 nm	662 nm	639 nm	644 nm	12 nm	5 nm	2×10^5

Phosphate buffer solution (PBS) has been most commonly employed for biological studies especially in order to investigate the interaction between dyes and biomolecules for imaging applications. Keeping this in mind absorption and emission spectra of these dyes were also recorded in the 0.1 M PBS solution at pH 7.4 and shown in the Fig. 2. It is worth mentioning that in the case of phosphate buffer solution (PBS) both cyanine and squaraine dyes although exhibit nearly similar absorption and emission spectral behavior but slightly blue-shifted as compared to that observed in the DMF solution. This blue shift of λ_{max} for both of the dyes in PBS could be attributed to enhanced dye aggregation promoted by the hydrogen bonding between the dye molecules due to the presence of –COOH functional groups. It is well known that both cyanine and squaraine exhibit dye aggregation solution owing to their flat molecular structure [27]. Another interesting feature is that fluorescence emission intensity of cyanine dye is highly decreased (about 10 times) as compared to squaraine dyes for similar concentration and experimental measurement conditions. This suggests that cyanine dyes could be a good candidate as quenching probe while squaraine dyes as fluorescence probe for the design and development of NIR fluorescence resonance energy transfer systems.

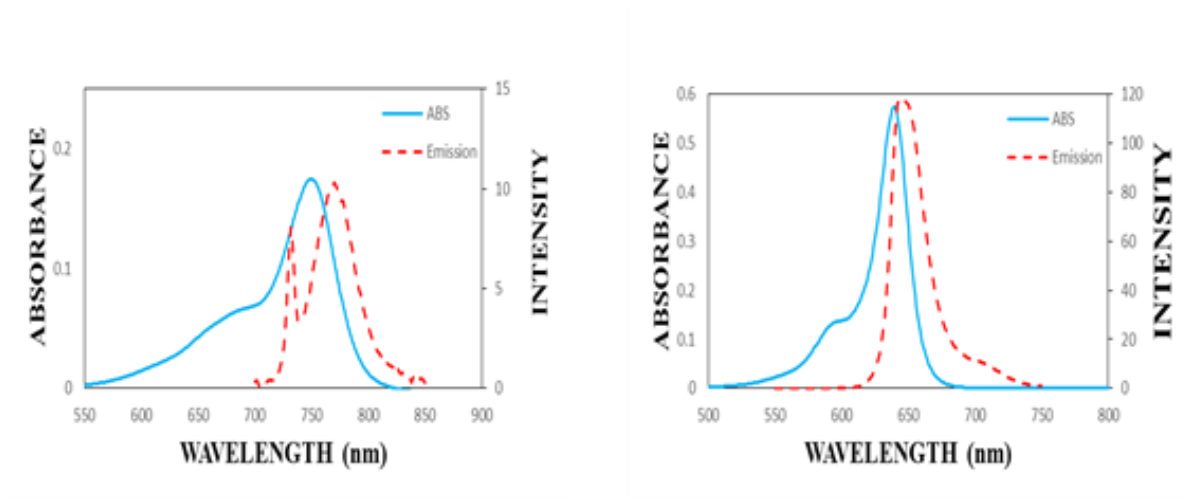


Figure. 2 Electronic absorption and fluorescence emission spectra for 2μM concentration of UCD-1 (left) and SQ-3 (right) in 0.1 M PBS solution.

3.2. Interaction of dyes with BSA

In order to avoid chemical reactions and further purification steps of chemical labelling of biomolecules with dyes in optical bio-imaging, non-covalent labelling is associated with investigation of dye-protein interaction are getting increasing attention. Williams *et al* are amongst one of the pioneer researchers to study about the non-covalent interactions between the NIR dyes and human serum albumin (HSA) [28]. Bovine serum albumin (BSA) is one of the most widely investigated model protein to investigate drug-protein interactions owing to its high sequence identity to that of human serum albumin [29]. Keeping this in mind, interaction of BSA as a model protein has been executed using different amounts of protein. Figure 3 exhibits the electronic absorption spectra of PBS buffer solution of dyes UCD-1 and SQ-3 in the presence and absence of BSA. The spectra show two different sets of electronic absorption transitions associated with absorption regions of the protein BSA (250 to 300 nm) and squaraine/cyanine dyes (650 to 800 nm). The absorption region of squaraine dye is confined in 550 to 700 nm while cyanine dyes absorbs strongly in the 600-800 nm.

It can be observed that increasing BSA concentration leads to gradual increase in the intensity of absorption between 250 to 300 nm which associated with protein absorption. However, the dye absorption region exhibited differential behaviour with UCD-1 and SQ-3. The BSA interaction with cyanine dye (UCD-1) exhibits the random increase and decrease of absorption intensity corresponding to the dye absorption around 750 nm and slight red-shift from 758 nm to 768 nm upon the addition of BSA. Slight red-shift indicates the suppression of dye aggregation by BSA molecules and indicates the presence of interaction between dye and BSA molecules. At the same time, random increase and decrease without any isobestic point indicates that interaction between BSA and UCD-1 is not so simple and a detailed investigation on site specific binding is necessary [14]. A similar kind of complicated BSA-cyanine dye interaction with random increase and decrease absorption intensity at λ_{max} along with red-shift has also been reported by Pisoni *et al* [23].

On the other hand, interaction of BSA with SQ-3 exhibits interesting behaviour apart from slight red-shift from 639 nm to 649 nm upon increasing concentration of BSA. At the fix dye concentration of 2μM, there is an initial decrease in absorption intensity (hypochromism) corresponding to monomeric SQ-3 absorption around 4-5μM along with clear isobestic point and then remains constant upon the further addition of BSA. This red shift and hypochromicity observed for interaction between SQ-3 and BSA could be attributed to the presence of hydrophobic environment

provided by BSA to the hydrophilic SQ-3 and is indicative for the formation of squaraine dye-BSA conjugate. Similar electronic absorption behaviour with clear isobestic point and hypochromism has also been noticed by Jisha et al during investigation of interaction between squaraine dyes with HSA [30].

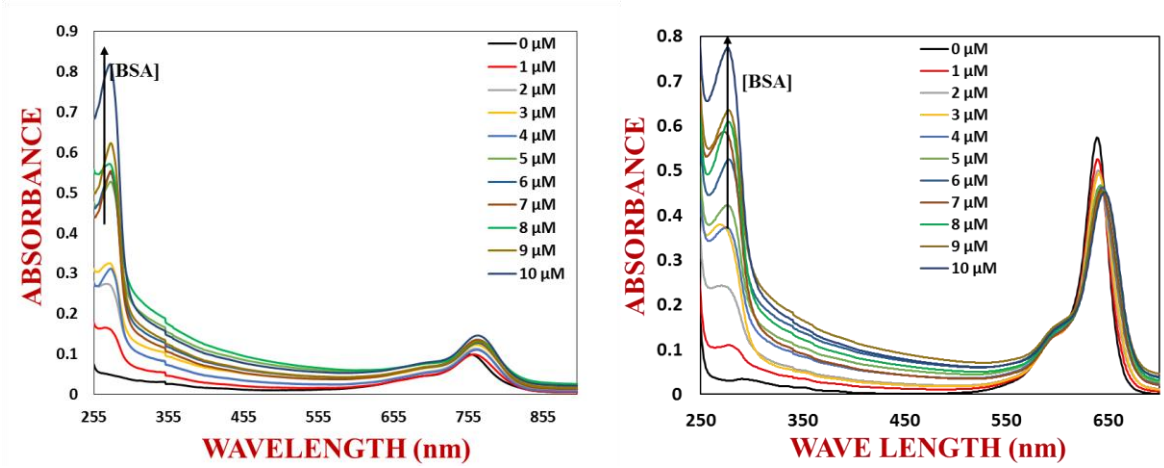


Figure. 3 Electronic absorption of UCD-1 (left) and SQ-3 (right) in 0.1 M PBS at different BSA concentrations for a fixed dye concentration of 2µM.

Changes in the fluorescence behavior of dyes as a result their interactions with biomolecules like proteins, DNA etc. forms fundamental basis for fluorescence bio-imaging. Spectroscopic behaviour of fluorophoric cyanine and squaraine dyes have been reported to be highly sensitive to the environmental conditions owing to their self-aggregation or interaction with other existing molecules in their vicinity [31]. In general fluorophores bind with protein covalently or non-covalently and non-covalent binding finds its application in biomedical science in order to investigate the changes in the conformation of proteins and their binding with drugs [32]. Fluorescence emission spectra of both of the dyes UCD-1 and SQ-3 as function of BSA concentration is shown in the Fig. 4. It can be clearly seen that both of the dyes exhibit increase in the fluorescence intensities of dyes near their respective peak maxima along with the red-shit upon addition of increasing concentration of BSA at fix dye concentration. This increase in the fluorescence intensity along with the red-shift in the peak maxima suggests the interaction between the dye molecules and BSA due to non-covalent BSA-dye conjugate formation. It has been reported that site specific binding of BSA using Dansylproline (DP) and Dansylamide (DNSA) as site specific binder and squaraine dyes as fluorophore leads to decrease in the fluorescence intensity of BSA-dye conjugate as a function of site specific binder concentration [33].

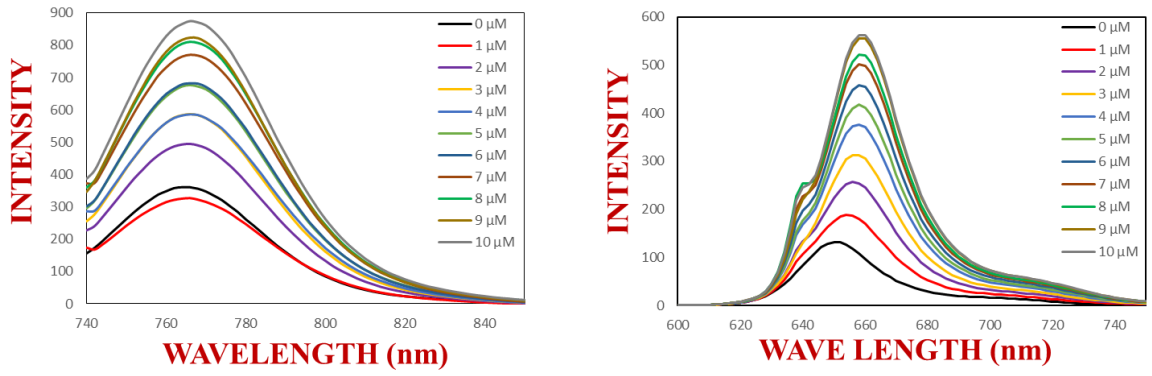


Figure. 4 Fluorescence emission spectra of UCD-1 (left) and SQ-3 (right) in 0.1 M PBS with varied BSA concentrations for a fixed dye concentration of 2µM.

This suggests that in present case also enhancement in fluorescence intensity in the presence of BSA for both of the dyes UCD-1 and SQ-3 is due to non-covalent binding with BSA. To compare the binding ability and relative association of these two dyes with BSA, apparent binding constant (K_a) was estimated using equation (2) mentioned in the experimental section and shown in the Fig. 5. From equation 2, it can be seen that slope gives the value K_a which was estimated to be 7.08 x 10^6 M^{-1} and 6.22 x 10^6 M^{-1} for dyes UCD-1 and SQ-3, respectively. For non-covalent binding with BSA using squaraine dyes, K_a in the similar range has also been reported [30].

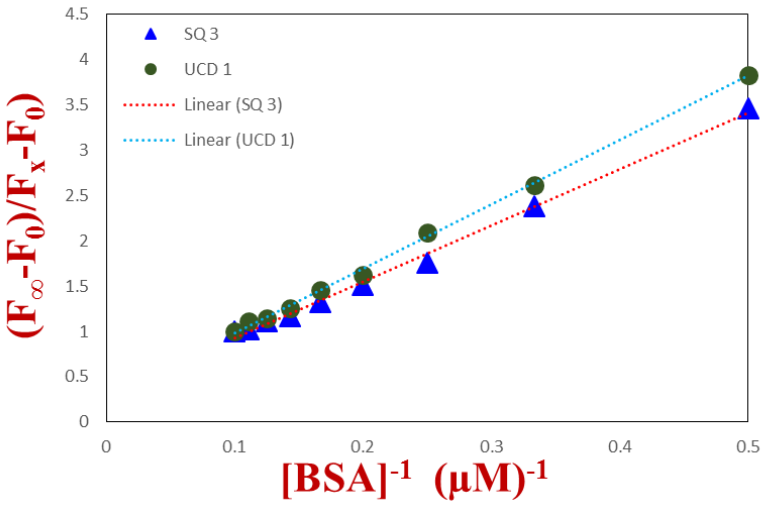


Figure. 5 Plot of $(F_\infty-F_0)/(F_x-F_0)$ as function $[BSA]^{-1}$ at a fixed dye concentration of 2.0×10^{-6} M.

At the same time cyanine dye (UCD-1) not only exhibits the higher binding affinity as compared to squaraine dye (SQ-3) but also an order of higher magnitude K_a had been reported for typical NIR cyanine dyes having non-functionalized hydrophilic groups [23]. In the present case, this could be associated with presence of direct ring substituted –COOH group promoting hydrogen bonding with binding sites of BSA. In this situation, one can argue that why SQ-3 having two such -COOH functionality shows relatively lower binding affinity as compared to UCD-1. Exact reason is although not completely clear but it might be associated judicious balance of main hydrophobic core and presence of active group for promoted binding with the active sites. Actually, active site of model

protein BSA used in this work is covered by hydrophobic amino acid residues to form the binding sites thus hydrophobic region make the outer core for interacting with dye molecules.

4. Conclusions

Direct ring carboxy functionalized far-red to NIR sensitive dyes SQ-3 and UCD-1 has been successfully synthesized and characterized in order to explore their applicability as fluorescent probes. Interaction of these dyes with BSA as model protein leads to enhancement in the fluorescence intensity along with bathochromic shift in the emission maxima suggesting the dye-BSA conjugate formation. Both the dyes show very high affinity for their association with BSA. Site specific binding is still needed to have some more insight about preference of these dyes for the particular binding sites of the BSA.

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