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Photophysical Characterization and BSA Interaction of Direct Ring Carboxy Functionalized Symmetrical squaraine Dyes

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Abstract: A series of far-red sensitive symmetrical squaraine dyes bearing direct –COOH functionalized indole ring were synthesized, characterized and subjected to photophysical investigations. These symmetrical squaraine dyes were then subjected to investigate their interaction with bovine serum albumin (BSA) in Phosphate buffer solutions. All the squaraine dyes under investigation exhibit intense and sharp optical absorption mainly in the far-red wavelength region from 550 nm -700 nm having very high molar extinction coefficients from $1.3 \times 10^5 \, \text{dm}^3.\text{mol}^{-1}.\text{cm}^{-1}$. A very small Stokes shift of 10-17 nm indicates the rigid conformational structure of squaraine chromophore. Interaction of these dyes with BSA leads to not only enhanced emission intensity but also bathochromically shifted absorption maximum due to formation of dye-BSA conjugate. These dyes bind strongly with BSA having about an order of magnitude higher binding constant as compared to the reported squaraine dyes. Amongst the symmetrical squaraine dyes investigated in this work one bearing substituents like trifluorobutyl as alkyl chain at N-position of indole ring and carboxylic acid on benzene ring at the terminal (SQ-26) exhibited highest association with the BSA having very high binding constant $8.01 \times 10^6 \, \text{M}^{-1}$.

1. Introduction

Biomedical diagnosis is a fast growing area in order to afford high quality of life by early disease diagnosis. In recent years there has been dramatic development towards biosensors due to their applications in chemical analysis and medical diagnosis. Optical imaging has emerged as most common imaging practice in medical research due to its high sensitivity, low cost and non-invasive nature [1]. Near infrared (NIR) fluorescence probes have their potential attraction towards non-invasive in vivo imaging due to their highly diminished auto-fluorescence from the biological fluids and deep tissue penetration [2]. NIR probes have drawn attention in several areas of the biomedical field, including tissue perfusion, vascular mapping, inflammation monitoring and tumor diagnosis. The squaraine dyes belongs to the interesting organic chromophoric system, where oxocyclobutenolate core is linked by aromatic or heterocyclic components at both ends leading to donor–acceptor–donor molecular motif. Sensitizing dyes belonging to squaraine family are of considerable interest as fluorescent labels/probes for biological and pharmaceutical research owing to their tunable absorption/emission from visible to NIR wavelength region, high molar absorptivity and reasonably good quantum yields [3-4]. By logical molecular design, the absorption and emission behaviour of
these dyes can be tuned from visible to IR wavelength making them suitable for fluorescence imaging since they are exterior to the self-absorption and auto-fluorescence regions of biological matrices [5]. The unique chemical and physical properties of squaraine dyes find their versatile applications in the area as NIR fluorescent probes, environmental sensors [6], molecular sensors [7-8], bio imaging and biochemical labelling [9-10] which have gathered huge attentions from the scientific community.

The present work deals with the synthesis of four direct carboxy ring functionalized symmetrical NIR squaraine dyes (SQ-3, SQ-26, SQ-56 and SQ-57) from their respective quaternary heterocyclic ammonium salt containing N-alkylated group. The presence of direct ring functionalized carboxy groups provide the capability of covalent coupling with biomolecules such as peptides, oligonucleotides and proteins to enhance their application potential as fluorescence probes. In order to explore their potential application as fluorescent probes to sense protein in phosphate buffer solution (PBS), these dyes were subjected to investigate their interaction using Bovine serum albumin (BSA) as a model protein to observe the influence of alkyl chain with varying functional groups of the dyes.

2. Materials and Methods

1-Iodobutane, 1,1,1-trifluoro-4-iodobutane, 1,3-propanesultone, Ethyl-4-bromobutanoate used in the present synthesis were purchased from Tokyo Kasei Co. Ltd. Solvents (reagent grade, Wako chemical company) and Squaric acid was purchased from Alfa Aesar and used as received. Synthesized synthesized squaraine dyes and dye intermediates were analysed by high performance liquid chromatography (HITACHI) equipped with chromolith analytical column (RP – 18e, φ 4.6 mm × 100 mm) using methanol-water solvent gradient. Fig. 1 indicates the purity of final symmetrical squaraine dyes used for present investigation which were verified by HPLC. Mass of the intermediates as well as final SQ dyes were confirmed by MALDI-TOF-mass or fast ion bombardment (FAB) mass in positive ion monitoring mode. For final symmetrical squaraine dyes, high resolution FAB-mass (HR-MS) in positive ion monitoring mode was also measured. Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL JNM A500 MHz spectrometer in CDCl3 or d6-DMSO with reference to TMS for structural elucidation.

2.1 BSA Interactions

The protein (BSA)-dye interactions were conducted using PBS (0.1 M at pH 7.4) and squaraine dye solutions (2μM), prepared by the addition of 100 μl of 0.1 M dye solution in DMF to the various concentrations of PBS/BSA solutions in the concentration range of (0-10 μM). The final solutions were stirred at room temperature before recording the respective electronic absorption and fluorescence emission spectra. To compare the interactions between protein and dye, the apparent binding constants (K_a) were also calculated from the following titrations of dyes at 25°C. Considering BSA/dye association in 1:1 ratio, the constant (K_a) was calculated using the equation 1 [11].

\[
\frac{1}{(F_x - F_0)} = \frac{1}{(F_\infty - F_0)} + \frac{1}{K_a [\text{BSA}]} \frac{1}{(F_\infty - F_0)}
\]

(1)

Where, \( F_0 \), \( F_X \), and \( F_\infty \) are the fluorescence intensities of dyes in the absence, presence of BSA and at a concentration of complete interaction, respectively, while \([\text{BSA}]\) is the protein concentration. Equation [1] can be modified as

\[
\frac{(F_\infty - F_0)}{(F_X - F_0)} = 1 + \frac{1}{K_a [\text{BSA}]}
\]

(2)
The binding/association constant ($K_a$) values for the interaction between the BSA and Squaraine dyes were calculated from the slopes of the corresponding plots between the $(F_x - F_0)/(F_X - F_0)$ as a function of $[\text{BSA}]^{-1}$ as per the equation (2).

**2.2 Synthesis of SQ dyes and dye intermediates**

Aromatic ring carboxy functionalized indole 2,3,3-trimethyl-3H-indole-5-carboxylic acid was synthesized following the method reported [12]. Symmetrical squaraine dyes and dye intermediates of 5-carboxy-2,3,3-trimethyl-indole have been synthesized following the method as shown in Scheme 1.

![Scheme 1: Synthesis of symmetrical squaraine dyes.](image)

**2.2.1. Synthesis of 2,3,3-trimethyl-3H-indole-5-carboxylic acid (1)**

In a round bottom flask fixed with condenser, 4-hydrazinobenzoic acid (5.0 g; 32.85 mmol), glacial acetic acid (80 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, 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 off white powder in 56% yield. HPLC analysis of product suggests that compound was 100% pure. FAB-mass (measured 203; calculated 203.09) and $^1$H NMR (500 MHz, CDCl3): 7.99 (s,
H-4), 7.93 (d, J = 8.0 Hz, H-6), 7.59 (d, J = 8.0 Hz, H-7), 2.26 (s, 3H, H-10), 1.28 (s, 6H, H11, 12) confirms the successful synthesis of the compound.

2.2.2. Synthesis of 5-carboxy-2,3,3-trimethyl-1-alkyl-3H-indolium iodide (2-5)

2,3,3-trimethyl-3H-indole-5-carboxylic acid (1, 1 equiv.) and (a, 3 equiv, in Acetonitrile, 95°C), (b, 3 equiv, in propionitrile, 100°C), (c, 3 equiv, in DCB, 135°C), (d, 3 equiv, in propionitrile, 100°C), were added and reaction mixture was refluxed for (a: 48 h, b: 24 h, c: 72 h, d: 24 h) under nitrogen atmosphere to give corresponding 5-carboxy-N-alkyl-indoium iodides (2-5). After completion of the reaction as monitored by HPLC, solvent was evaporated and the crude product was washed with ample diethyl ether giving the titled compound. The physical and spectral data of N-alkyl-indoium iodides (2-5) are as follows.

2.2.3. Synthesis of 1-butyl-5-carboxy-2,3,3-trimethyl-3H-indolium (2): Yield 77% with 97% purity as confirmed by HPLC. FAB-mass (measured 260.0; calculated 260.16) confirms successful synthesis of the compound [13].

2.2.4. Synthesis of 5-Carboxy-2,3,3-trimethyl-1-Trifluorobutyl-3H-indolium iodide (3): Yield 46% with 99% purity as confirmed by HPLC. FAB-mass (measured 441.0; calculated 441.04) confirms the successful synthesis of the compound [14].

2.2.5. Synthesis of 5-carboxy-2,3,3-trimethyl-1-(3-sulfopropyl)-3H-indol-1-ium (4): Yield 59% with 99% purity as confirmed by HPLC. FAB-mass (measured 326.1066; calculated 326.1057) confirms the successful synthesis of the compound [15].

2.2.6. Synthesis of 5-carboxy-1-(3-ethoxy-3-oxopropyl)-2,3,3-trimethyl-3H-indol-1-ium (5): Yield 59% with 99% purity as confirmed by HPLC. FAB-mass (measured 318.1698; calculated 318.1700) confirms the successful synthesis of the compound [16].

2.2.7. Synthesis of symmetrical squaraine dyes

Symmetrical SQ Dyes (SQ-3, 26, 56 and 57) were synthesized using corresponding carboxy functionalized trimethyl-indolium iodide salt 2-5 ((2 equiv.) and squaric acid (1 equiv.)) in 1-butanol: benzene mixture. Reaction mixture was refluxed for 18 h using Dean–Stark trap for azeotropic removal of water. 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 dyes are as follows;

2.2.8. Synthesis of N-butyl substituted squaraine dye SQ-3:

Yield 64% with HPLC purity 100%. MALDI-TOF-mass (calculated 596.29 and observed 597.25 [M+H]+). HR-MS (calculated 596.288 and observed 596.296 [M]+). 1H NMR (500 MHz, d6-DMSO): δ/ppm = 12.85 (b, –COOH), 8.04 (dd, H-6), 7.96 (dd, H-4), 7.43 (dd, H-7), 5.90 (s, H-10), 4.13 (q, 2H, H-13), 1.76 (m, 2H, H-14), 1.70 (s, 6H, H11 + 12), 1.40 (m, 2H, H-15), 0.95 (t, 3H, H-16) confirms the successful synthesis of the dye.

![Figure 1 HPLC chromatogram of symmetrical Squaraine dyes](image-url)
2.2.9. Synthesis of N-Trifluorobutyl substituted squaraine dye SQ-26: Yield 71% with HPLC purity 100%. FAB-mass (calculated 704.23 and observed 705.0 [M+H]+) confirms the identity of the compound.

2.2.10. Synthesis of N-sulfopropyl substituted squaraine dye SQ-56: Yield 42% with HPLC purity 100%. FAB-mass (calculated 728.17 and observed 729.00 [M+H]+) confirms the identity of the compound.

2.3.11. Synthesis of N-butoxybutyl substituted squaraine dye SQ-57: Yield 42% with HPLC purity > 80%. FAB-mass (calculated 769.9040 and observed 769 [M]+) confirms the identity of the compound.

3. Results and Discussion

3.1. Photophysical Characterization

After the successful synthesis and purification, these far-red sensitive dyes were subjected to photophysical investigations pertaining to the electronic absorption and fluorescence emission spectroscopy. The results thus obtained have been summarized in the table 1. Fig. 2 exhibits the solution state electronic absorption and fluorescence emission spectra of symmetrical squaraine dyes in the dimethylformamide (DMF) solution.

![Figure 2](image)

**Figure.** 2 Electronic absorption (solid line) and fluorescence emission (dashed line) spectra of dyes in DMF solution (5 μM). The inset shows the molecular structure of squaraine dyes.

It can be observed that position of absorption maxima (λ_max) in the electronic absorption and fluorescence emission spectra are slightly affected by the different substituents on indole ring. The λ_max of symmetrical squaraine dyes ranges from 650 to 656 nm with high molar extinction coefficient ranging from 2.34 × 10^5 to 1.45 × 10^5 dm^3 M^-1 cm^-1. The intense optical absorption of these dyes are associated with π−π* electronic transitions. The fluorescence emission spectra were measured slightly
below (about 12 to 16 nm) to the resultant $\lambda_{\text{max}}$ of absorption spectra. The dyes exhibit main fluorescence emission band where the peak maxima varies from 662 to 672 nm with a small stoke shift of 10 to 17 nm. This small Stokes shift represents the rigidity of the molecules without having any conformational changes after the photoexcitation.

**Table 1.** Photophysical properties of squaraine dyes in DMF solution

<table>
<thead>
<tr>
<th>Sensitizing Dyes</th>
<th>$\lambda_{\text{max}}$ (Absorption)</th>
<th>$\lambda_{\text{max}}$ (Emission)</th>
<th>Stoke Shift</th>
<th>$(\varepsilon)$ $(\text{dm}^3\text{ M}^{-1}\text{ cm}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ-3</td>
<td>650 nm</td>
<td>662 nm</td>
<td>12 nm</td>
<td>$2.2\times10^5$</td>
</tr>
<tr>
<td>SQ-26</td>
<td>652 nm</td>
<td>664 nm</td>
<td>12 nm</td>
<td>$1.4\times10^5$</td>
</tr>
<tr>
<td>SQ-56</td>
<td>656 nm</td>
<td>666 nm</td>
<td>10 nm</td>
<td>$1.5\times10^5$</td>
</tr>
<tr>
<td>SQ-57</td>
<td>655 nm</td>
<td>672 nm</td>
<td>17 nm</td>
<td>$2.3\times10^5$</td>
</tr>
</tbody>
</table>

3.2. **Interaction of dyes with BSA**

To investigate the interactions between dyes and proteins, PBS has been used as a choice of buffer, which is most widely used in imaging applications for protein folding and ligand binding mechanism [17]. Non-covalent labelling of biomolecules has caught much attention to investigate the interactions between dye and protein molecules. Therefore, in order to avoid the purification steps and chemical reactions of biomolecules with dyes towards the application of optical imaging, non-covalent labelling has been employed [18]. Frank Welder et al were studied the noncovalent labelling of both Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) with NIR Squarylium dyes using absorption detection techniques [19]. Bovine serum albumin (BSA) is a globular protein which has been prominently used as protein model to investigate the interactions between dye and protein owing to its high homology with HSA in the amino acidic sequences [20]. Therefore, dyes used in this work have also been subjected to investigate their interaction with BSA as a model protein.

![Figure 3](image)

**Figure.** 3 Electronic absorption spectra of SQ-3 in 0.1 M PBS at different concentrations of BSA for fixed dye concentration of 2 $\mu$M.
Fig. 3 depicts the absorption spectra of one of the representative squaraine dyes (SQ-3) in the presence and absence of BSA. It can be seen that there are two different sets of prominent electronic absorption bands in the wavelength region of 250 nm - 300 nm and 620 nm to 680 nm associated with the absorption of BSA and dye SQ-3, respectively. Increase in the BSA concentration led to the gradual increase in intensity of absorption between 250 nm - 300 nm, which was associated with electronic absorption of the protein BSA. However, the interaction of BSA with SQ-3 exhibits slight red-shift from 639 nm to 649 nm upon increasing concentration of BSA. There was initial decrease (hypochromism) in absorption intensity which continues up to 4-6 μM along with the presence of isobestic point and then remains constant upon the further addition of BSA. The isobestic point indicates that the equilibrium between the free and bound protein is simple in all the dyes under investigation. This observed red shift and hypochromicity could be attributed to the presence of hydrophobic environment provided by BSA to the SQ-3 which is an indication for the formation of squaraine dye-BSA conjugate. It is well known that squaraine dyes are prone to dye aggregate formation in the solution owing to their flat molecular structure. Upon interaction with BSA first of all there is dye-BSA conjugate formation leading to disruption of dye aggregates resulting in to red-shifted absorption maximum. At the same time, compaction of the effective π-electron density decreases due to dye-BSA conjugate formation leading to the hypochromicity. Once conjugate formation is finished, further increase in the BSA (> 6 μM) does not affect the decrease in the intensity of SQ-dye rather than only red-shift. This is attributed to the fact that now added additional BSA is involved mainly in the prevention of dye aggregation rather than conjugate formation. Similar behaviour of interaction between squaraine dyes with HSA has also been noticed by Jisha et al during their investigation [21]. It is worth to mention that similar behaviour has also been observed for the other squaraine dyes such as (SQ-26, SQ-56, and SQ-57) but SQ-56 shows rather enhanced side vibronic shoulder associated with the dye-aggregation owing to its strong hydrophilic nature due to presence of two highly hydrophobic -SO3H groups in the side chain.

![Figure 4](image_url)

**Figure. 4** Fluorescence emission spectra of SQ-3 with varying concentrations of BSA for a fixed dye concentration of 2 μM.

Changes in the fluorescence behaviour of dyes on interactions with biomolecules forms the fundamental basis for fluorescence bio-imaging. Spectroscopic behaviour of squaraine dyes have been reported to be highly sensitive to the environmental conditions due to their self-aggregation [22]. In common, fluorophores bind with protein covalently or non-covalently and non-covalent binding or
labelling shows preference for hydrophobic binding sites, where it finds potential application in biomedical science in order to investigate the changes in the conformation of proteins and their binding with drugs [18]. The fluorescence emission spectra of SQ-3 in the presence and absence of BSA is shown in the Fig. 4. It can be seen from Fig. 4 that SQ-3 exhibits an increase in fluorescence intensity along with slight red shift of peak maxima upon the addition of increasing amounts of BSA. The increase in fluorescence intensity could be attributed to the possible noncovalent interactions between the dye and BSA. The bathochromic shift of 10 nm with SQ-3 depicts that BSA microenvironments are less polar than that of PBS due to presence of hydrophobic groups on surface and interiors of BSA. It can be argued that why there was continues increase in the fluorescence intensity of dye upon the addition of BSA where absorption maximum saturates up to 6 μM of BSA concentration. As discussed earlier, BSA is not only involved in the dye-BSA conjugate formation but also prevention of dye aggregation. It has also been reported that there is quenching in the fluorescence upon the dye aggregate formation [23]. This is the reason why addition of the BSA with concentration > 6 μM leads to further enhancement in the fluorescence intensity of dye by preventing the dye aggregation. Similar results were also observed with the other dyes (SQ-26, SQ-56 and SQ-57) with relatively quenched fluorescence intensity for SQ-56. This quenching in the fluorescence emission for SQ-56 could be associated with strong dye aggregation providing additional pathway for non-radiative decay.

BSA concentration dependence of the fluorescence intensity for various symmetrical Squaraine dyes have been shown in the Fig. 5. A linear correlation between fluorescent intensity of dyes as a function of BSA concentration was observed up to 6 μM concentrations of BSA and attained saturation. Though, these dyes exhibit interactions on further addition of BSA, the emission intensity seems to follow nonlinearity, where the change in fluorescence intensity is comparatively less as it attained saturation at 6 μM of BSA. This increase in fluorescent intensity along with red shift of $\lambda_{\text{max}}$ can be attributed to the noncovalent interaction between dyes and protein for the formation of BSA-dye conjugates. It has been widely accepted that drugs or probes interact differently with the protein under consideration and their ability to bind depends on protein concentration [24]. In order to mark the successful application of dyes as a probe, it is necessary that there should not be any damage to the structure of the protein on interaction with dyes, which basically governs the protein function and activity. The conformational changes in protein may occur at the point where probe is located. As the dye concentration increases, there may be more damage in its activity. Therefore, a very low dye concentration of (2 μM) has been used for investigation of the dye-BSA interaction with all the dyes under investigation.
Figure. 5 Plot of florescence emission intensity at peak maxima for Squaraine dyes as a function of BSA concentration. Dye concentration was constant (2 μM) for each of the dyes.

To compare the ability of binding and relative association of dyes with BSA quantitatively, apparent binding constant ($K_a$) was calculated using equation (2) by plotting $(F_\infty - F_0)/(F_X - F_0)$ as function of inverse of BSA concentration as shown in the Fig. 6. The value of ($K_a$) was calculated from the slopes of the curve which was found to be $6.2 \times 10^6$ M$^{-1}$, $8.0 \times 10^6$ M$^{-1}$, $0.3 \times 10^6$ M$^{-1}$ and $3.9 \times 10^6$ M$^{-1}$ with the SQ-3, SQ-26, SQ-56 and SQ-57, respectively. Therefore, symmetrical squaraine dye bearing trifluorobutyl as alkyl chain substituent at `N` position of the indole ring exhibits highest binding affinity and estimated ($K_a$) is about an order of the magnitude higher as compared to that reported squaraine dyes [25]. Relatively moderate hydrophobicity/hydrophilicity of the SQ-26 is responsible to show the high binding ability to the protein and hence high binding constant. The dye SQ-56 exhibits least binding ability towards BSA, the enhanced hydrophilicity or least hydrophobicity along with pronounced dye aggregate formation might be responsible for the least interaction with protein. BSA possess distinct hydrophobic and hydrophilic active sites site I and Site-II [26]. Upon interaction with the ligands (drug or dyes) the relative hydrophobicity of ligands plays an important role in controlling their interactions with the BSA specifically with Site-I. A perusal of the molecular structures of the dyes under investigation reveals that all of dyes possess nearly same main π-framework, and –COOH functional group apart from the varying alkyl substituents on N-position of the indole unit. The relative hydrophobicity of the molecules are in the order SQ-57 > SQ-3 > SQ-26 > SQ-56 as confirmed by the HPLC retention time shown in the Fig. 1. Since other structural factors are constant balanced hydrophilic and hydrophobic interactions with Site-I and Site-II of the BSA could be attributed to its strongest affinity with BSA.

Figure. 6 Plot of $(F_\infty - F_0) / (F_X - F_0)$ as function $[\text{BSA}]^{-1}$ at a fixed dye concentration of 2 μM.

4. Conclusions

Carboxy functionalized symmetrical squaraines with varying substituents were synthesized with satisfactory yields and characterized. The dyes were subjected to photophysical investigations in order to explore their applicability as fluorescent probes. Interactions of these dyes with BSA as a protein model suggested the formation of dye-BSA conjugates, which led to the enhancement in fluorescence
Squaraine dyes can be used as good fluorophores for designing NIR-FRET systems. Interaction between dyes with protein was found to be (SQ-26 > SQ-3 > SQ-57 > SQ-56) while hydrophobicity of the dyes was found to be in the order (SQ-57> SQ-3 > SQ-26 > SQ-56) as confirmed by HPLC retention time. The dye SQ-26 showed highest binding ability with BSA hence showed high binding constant (K<sub>a</sub>) which can be attributed to its possible interactions with both the binding sites of BSA owing to its moderate hydrophilicity as well as hydrophobicity. The above photophysical characterization reveals that Squaraine dyes can be used as good fluorophores for designing NIR-FRET systems.

References