

Research Article

Monofunctional and Bifunctional Boronic Acid Functionalized Squarylium Dyes as Non-Covalent Protein Labels: A Fluorimetric Study

Mahmoud M. Sebaiy^{1,2*}, Abdullah A. El-Shanawany², Mohamed M. Baraka², Lobna M. Abdel Aziz² and Christa L. Colyer¹

¹Department of chemistry, Wake Forest University, USA

²Department of Medicinal Chemistry, Zagazig University, Egypt

***Corresponding author**

Mahmoud Sebaiy, Department of chemistry, Department of Medicinal Chemistry, Wake Forest University, Winston-Salem, North Carolina, 27109, USA, Tel: 336-473-0805; Fax: 336-758-4656; Email: sebaiym@gmail.com

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Abstract

A fluorimetry comparison study has been established for the labeling of human serum albumin (HSA) using two boronic acid functionalized squarylium dyes, one is monofunctional boronic acid dye (SQ-BA4) while the other one is bifunctional boronic acid dye (SQ-DBA2). The spectral properties of these two squarylium dyes have been studied under a variety of solutions to explore the optimal conditions for non-covalent protein labeling. Both dyes exhibited very low fluorescence intensity in aqueous solutions presumably due to H-aggregate formation in absence of the protein but showed a significant enhancement in the fluorescence intensity with the addition of HSA in the optimal acidic citrate buffer. Stability constant studies were conducted and SQ-BA4 dye was shown to have much stronger binding affinity with HSA than SQ-DBA2. Also, the stoichiometries of non-covalent complexes were found to be (1:1) for SQ-BA4 and different (1:2) for SQ-DBA2 with HSA. These studies are claimed to be the optimal for CE-LIF work which will be progressed in our continuing project.

INTRODUCTION

The development of novel methods and techniques for protein determination is very important in clinical and analytical applications. Spectral detection methods are widely used due to their high sensitivity and selectivity [1]. Analysis can usually be carried out using absorbance detection. However, laser induced fluorescence (LIF) detection has become a more attractive detection method [2]. This is due to the lower background, increased sensitivity, and increased selectivity that accompanies this method compared to UV-Vis absorbance [2-4]. LIF detection requires analytes (protein) to be made fluorescent by some labeling procedure if they are not already natively fluorescent at the wavelengths associated with the excitation laser sources [2,5]. Analytes can be rendered fluorescent through one of two derivatization reactions: covalent labeling and non-covalent labeling reactions [6]. Non-covalent labeling is an attractive option, because it requires less sample preparation and less analysis time than covalent labeling procedures [7,8]. Two main

classes of dyes that demonstrate fluorescence in the NIR region have been explored as non-covalent protein labels: cyanine and squarylium dyes [2,7-10].

Squarylium dyes were first synthesized over fifty years ago through a condensation reaction of squaric acid with two aromatic and/or heterocyclic compounds, which produces the 1,3-disubstituted compound [11]. They are characterized by their ability to exhibit effective light absorption with long excitation and emission wavelengths, high fluorescence quantum yields, and resistance to photodegradation [9]. Structure modifications of the dye by incorporating boronic acid functional groups is claimed to improve the affinity of these dyes towards the target protein analyte as these boronic acid-based dyes had showed excellent selectivity towards monosaccharides [12,13] and gram-positive bacteria [14,15] as previously established in our laboratory.

Human serum albumin (HSA) will be studied as a model binding protein. HSA, being the most abundant protein of

blood plasma, has many important physiological functions like regulating colloidal osmotic pressure, and transporting numerous endogenous compounds such as fatty acids, hormones, bile acids, amino acids, metals and toxic metabolites [16,17]. Additionally, there is a wide variety of drugs that are delivered to their targeting organs/tissues by binding with HSA and so, it does not only protect the bound drugs against oxidation and influences the *in vivo* drug distribution, but also alters the pharmacokinetic and pharmacodynamic properties of drugs [18]. Binding properties between HSA and ligands have been studied for several decades using a variety of methods [19–23] but all of them were non-fluorescence based studies. As such, the aim of current work is to compare the optical spectroscopic properties of two related squarylium dyes, an asymmetric monofunctional boronic acid-based dye (SQ-BA4) and a symmetric bifunctional boronic acid-based dye (SQ-DBA2) while both dyes have the same squaraine core structure (see Figure 1). The absorbance and emission properties of the dyes in a variety of solvents and aqueous buffers at different pHs were determined prior to titration of fixed quantities of the dyes with HSA. Also, stability constant studies and stoichiometric ratio calculations were applied to give us an overall idea about the nature of such protein-dye interactions. The solution conditions found to yield the most favorable fluorescence emission response in these studies are supposed to be optimal for determination and separation of three model proteins by CE-LIF technique in our continuous project.

MATERIALS AND METHODS

Reagents, buffer, and sample preparation

Squarylium dyes, SQ-BA4 and SQ-DBA2 were supplied by Prof. H. Nakazumi (Osaka Prefecture University, Japan) [13,24]. Both stock solutions of 4.00×10^{-4} M SQ-BA4 and 3.50×10^{-5} M SQ-DBA2 were prepared by weighing out an appropriate amounts of the solid dyes and dissolving in methanol (GJ Chemicals, Newark, NJ, USA) and dimethylsulfoxide, DMSO (Sigma Aldrich, St. Louis, MO, USA), respectively. These two stock solutions were stored in the dark at 4°C while not in use, under which conditions they are stable for several months or longer. Dilute working stock solutions were prepared just before use from the original stock solutions by dilution with additional methanol or DMSO to a concentration of 1.00×10^{-5} M, and these solutions were also

stored in the dark at 4°C when not in use. **Human serum albumin (HSA)** was purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received. Stock solution was freshly prepared weekly to a concentration of 1.00×10^{-5} M in MilliQ distilled, deionized water (Millipore, Bedford, MA, USA), and was stored in the dark at 4°C while not in use. Dilute protein working stock solution was prepared from the original stock solution by dilution with additional MilliQ water to a concentration of 1.00×10^{-6} M, and was stored in the dark at 4°C when not in use. Four buffers were used in this study to span the pH range from acidic to basic. Appropriate quantities of **citric acid** (Sigma Aldrich, St. Louis, MO, USA), **tris (hydroxymethyl) aminomethane** (AMRESCO, Solon, OH, USA), **ammonium carbonate** (Sigma Aldrich, Buchs, Switzerland), and **ammonium dihydrogen phosphate** (JMC, Royston, Hertfordshire, UK) were dissolved in MilliQ distilled, deionized water prior to pH adjustment by the addition of 1 M NH_4OH or 1 M HCl (Fisher Scientific, Pittsburgh, PA, USA). All buffers were stored in high density poly ethylene plastic bottles at room temperature for 1-2 weeks, and were filtered through 0.20 μm nylon syringe filters (VWR International, Houston, TX, USA) prior to use. Protein-dye solution mixtures were prepared by adding 5.00 μL of the 1.00×10^{-5} M solution of SQ-BA4 or SQ-DBA2 to a 500 μL quartz cuvettes. Some volumes of protein working stock solution were then added to this aliquot of dye, along with sufficient buffer to achieve the final desired concentrations of both protein and dye. The final volume for all solutions was 500 μL . The total organic solvent content in aqueous buffer solutions was kept below 2% (v/v).

Synthesis and characterization of squarylium dyes

SQ-BA4 was synthesized according to the method described previously by Ouchi K *et al.* [13], who also presented a detailed characterization of the dye. Briefly, SQ-BA4 was synthesized by condensation of both 2,3,3-Trimethyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)-3H-indole and 3-(4-dibutylaminophenyl)-4-hydroxy-3-cyclobuten-1,2-dione, which were synthesized according to the literature [25]. The crude product was purified by two stages of column chromatography followed by recrystallization from $\text{CHCl}_3/\text{hexane}$, yielding the pure compound SQ-BA4 as a brown solid in 18% yield. The full synthesis and characterization of the SQ-DBA2 dye is to be detailed in a future publication by T. Maeda *et al.*, but the dye was

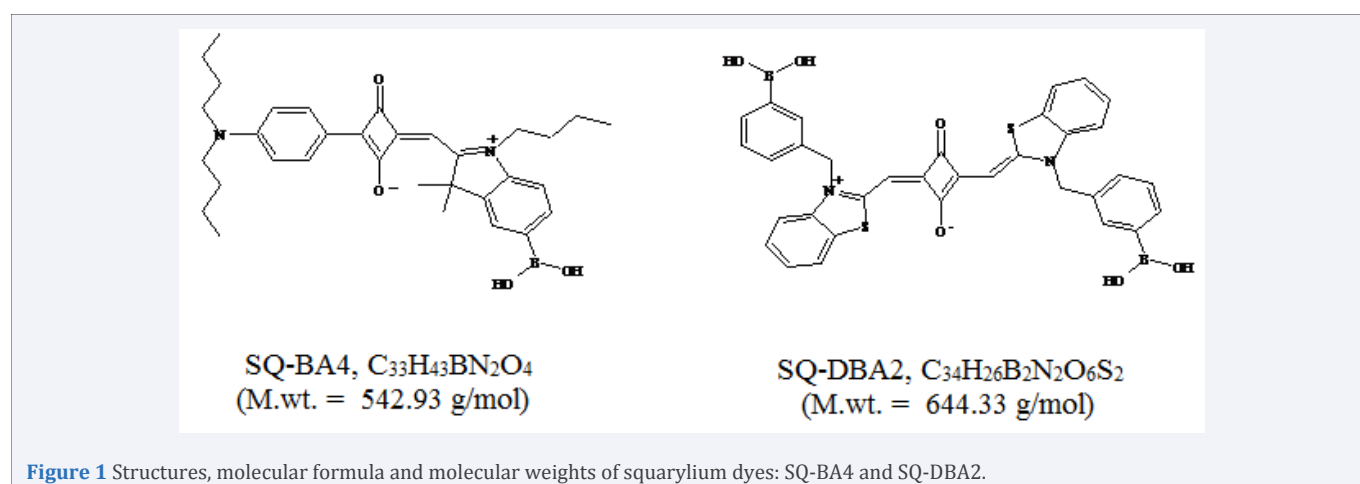


Figure 1 Structures, molecular formula and molecular weights of squarylium dyes: SQ-BA4 and SQ-DBA2.

used here to provide a useful comparison with the behavior of the previously published dye SQ-BA4 towards model proteins.

Instrumentation

Absorbance studies were conducted over a scan range of 400-800 nm with an integration time of 0.5 sec and an interval of 1 nm using a **Hewlett Packard HP8453 UV-Vis spectrometer** (Waldbronn, Germany) and 1-cm, semi-micro (total volume 500 μ L) quartz cuvettes (Perkin-Elmer, Shelton, CT, USA).

Fluorimetry studies were conducted using a **Perkin-Elmer LS50B luminescence spectrometer** (Shelton, CT, USA). Excitation and emission slit widths were 10 nm, the excitation wavelength (as specified in Table 1) was selected according to the wavelength of maximum absorbance for the sample or solution under consideration and the emission wavelength range was scanned from 630 nm to 800 nm at a rate of 250 nm/min. Fluorescence spectra of both free dyes and the protein-dye mixtures were measured using a 1-cm, semi-micro (500 μ L) quartz cuvette (Perkin-Elmer, Shelton, CT, USA).

RESULTS AND DISCUSSION

Spectral properties of SQ-BA4 & SQ-DBA2 alone and protein-dye complex

Spectroscopic analysis of boronic acid-based squarylium dyes were conducted under various solution conditions in order to evaluate their suitability as fluorescent probes for proteins. Also, these studies will help us to better understand the hydrophobic interactions, electrostatic interactions, and hydrogen bonding involved in the formation of non-covalent complexes between the dyes and protein targets. The exact nature and combination of these interactions can be difficult to determine, but evidence of interaction is provided by a change in the emission of the protein-dye complex relative to that of the free, uncomplexed dye, and can be easily monitored by fluorimetric studies [24]. Since

SQ-BA4 is a monofunctional boronic acid-based dye while SQ-DBA2 is a bifunctional boronic acid-based dye, it was expected that differences in their complexation with proteins could be attributed to the boronic acid moieties in both structures, thus providing insight into future design and planning for squarylium dye structures tailored to particular analytical needs. Additionally, this understanding proved to aid in the development of a CE-LIF protocol for the separation of various protein-dye complexes, with the promise of extension to protein assays more generally. UV-Vis absorbance and fluorescence emission studies of the dyes (1.00×10^{-7} M SQ-BA4 & SQ-DBA2) were conducted in acidic, neutral, and basic solution conditions, in the presence and absence of HSA in order to supposedly determine the optimal buffer conditions for further CE-LIF analysis. A summary of absorbance and emission properties of the dyes under various solution conditions, both with and without added protein, is presented in Table 1.

The absorbance of both SQ-BA4 and SQ-DBA2 dyes in aqueous buffer solutions was blue shifted relative to their values in methanol or DMSO, respectively. The blue shift in case of SQ-BA4 was between 18-36 nm while in case of SQ-DBA2, it was between 39-54 nm with a noticeable more shift in case of neutral and acidic buffers for both dyes. Also the band shape was changed where a weak broad absorbance band (around 500 – 730 nm in case of SQ-BA4 and 520 – 800 nm in case of SQ-DBA2) was observed for both dyes in acidic aqueous conditions, which suggests that they all form water-soluble aggregates (Figure 2b, red trace). Two possible types of aggregates-referred to as J and H- are possible. J-aggregates arise from the head-to-tail alignment of monomers, while H-aggregates are aligned in a head-to-head (or parallel) arrangement. Fukuda and Nakahara reported that squarylium dyes showed a strong absorbance band from 630-640 nm in chloroform with a blue shift for H-aggregates and a red shift for J-aggregates [26]. Figure 2 (b & a, red vs. blue traces) also shows the blue shift in acidic aqueous conditions relative to non-

Table 1: Spectral properties for SQ-BA4 & SQ-DBA2 with and without added HSA in organic solvents or various aqueous buffer systems (25 mM). Absorbance maxima for protein-dye complex were used as excitation wavelengths for fluorescence emission studies. Dyes and HSA concentrations were 1.00×10^{-6} M and 1.00×10^{-7} M for absorbance and fluorescence measurements, respectively.

Solvent or Buffer	SQ-BA4 alone		SQ-BA4 with HSA			SQ-DBA2 alone		SQ-DBA2 with HSA		
	λ_{ex} (nm)	λ_{em} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Fluorescence Enhancement Factor	λ_{ex} (nm)	λ_{em} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Fluorescence Enhancement Factor
Methanol or DMSO	628	661	N/A	N/A	N/A	679	683	N/A	N/A	N/A
Ammonium Citrate (pH= 3.50)	595	659	621	644	49.59	628	663	657	675	84.95
Tris-HCl (pH=7.50)	592	661	623	651	8.74	625	665	648	672	9.38
Ammonium Carbonate (pH= 9.80)	601	661	618	652	5.65	630	660	653	672	6.05
Ammonium Phosphate (pH= 11.30)	610	662	618	655	2.62	640	660	640	663	2.81

aqueous conditions. Under non-aqueous conditions (methanol or DMSO), it is assumed that the dyes would exist predominantly in their monomeric forms with a relative much stronger and sharper band as seen in Figure 2a (around 500–670 nm in case of SQ-BA4 and 570–720 nm in case of SQ-DBA2). This would indicate that both dyes in this study were most likely forming H-aggregates in aqueous solution, in the absence of protein. Upon the addition of an equimolar amount of our model protein, HSA, λ_{ex} for each dye was red shifted relative to λ_{ex} for each dye alone (in the absence of protein) under all buffer conditions as seen in Table 1. Figure 2c (green trace) shows the change in absorbance for each dye upon the addition of HSA under acidic aqueous conditions. These spectral changes are indicative of a non-covalent complex being formed between each dye and the protein. Also, variations in the extent of shifts are indicative of differing strengths of protein–dye interactions under different solution conditions. In addition, these spectral changes imply disaggregation, since the λ_{ex} of the protein–dye complex closely resembled that of the dye in methanol or DMSO, where it is presumed to exist in its monomeric state. Furthermore, these longer wavelength absorbances can be beneficial because absorbances at longer wavelengths, in general, allow the optical detection to be shifted away from the region of native protein absorbance bands, therefore reducing interference from the bands with the absorbance signal of the protein–dye complex. This reduces background and in turn can be beneficial in improving detection limits.

Regarding the fluorescence studies, the changes in wavelength of maximum fluorescence emission (λ_{em}) for dye alone and upon the addition of HSA are also shown in Table 1. SQ-BA4 showed a blue shift in λ_{em} while SQ-DBA2 showed a red shift in λ_{em} upon the addition of HSA, but to varying extents under different solution conditions. In case of SQ-BA4, a larger blue shift in λ_{em} was seen in the acidic buffer (15 nm) which decreases gradually once pH increases in neutral or basic buffers. On the other hand, SQ-DBA2 showed the largest red shift in λ_{em} in both acidic (pH 3.50) and basic (pH 9.80) conditions (12 nm),

followed by a shift of 7 nm in neutral conditions and a shift of 3 nm in highly basic (pH 11.3) conditions. In both cases, it is noted that upon the addition of HSA to the dyes in aqueous buffered media, there was a shift in the wavelength of maximum emission, which confirms that a non-covalent complex was being formed for both dyes as was seen in the absorbance spectra. A larger shift in wavelength is desired since the wavelength for the complex is then further separated from that of the dye alone, reducing possible spectral interferences and this will ultimately improve fluorescence sensitivity. Determining the optimal buffer (in terms of analytical response) for each dye also required a study of the emission intensity of the protein–dye complexes in order to discover which buffer would provide the greatest sensitivity for determination of the complex.

According to Table 1, acidic buffer was the optimal for maximum fluorescence enhancement in case of both dyes when complexed with HSA. **Fluorescence emission increased by a factor of 49.59 and 84.95 (highlighted in bold) in the case of SQ-BA4 and SQ-DBA2, respectively, upon the addition of equimolar amount of the protein under acidic solution conditions.** The fluorescence enhancement factor is calculated as the difference between emission intensities in the presence and absence of analyte divided by the emission intensity of dye alone. SQ-DBA2 complex with the protein has a much higher enhancement factor than SQ-BA4 (with a ratio of 1.71 times more sensitive) and this may be attributed to the extra boronic acid moiety in the dye structure. However, both dyes showed a very sharp decrease in the fluorescence enhancement factor upon addition of protein in neutral and basic conditions as mentioned in Table 1 while the lowest enhancement factor was recorded at pH 11.30. Since the isoelectric point (pI) of HSA is 5.30 [27] (which means that HSA at this pH is neutral and carries no net electrical charge and so at a pH below its pI, it carries a net positive charge while above its pI, carries a net negative charge), both neutral and basic conditions cause HSA in addition to the dyes to be negatively charged, leading to electrostatic repulsion

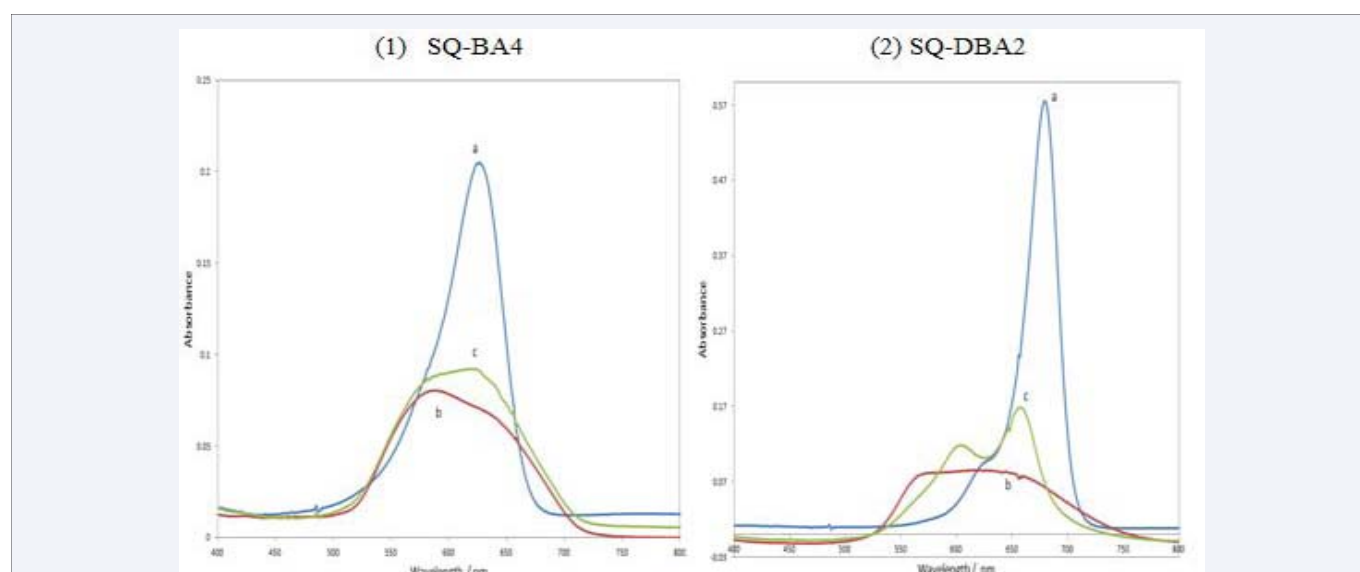


Figure 2 Absorption spectra of 1.00×10^{-6} M squarylium dyes (1) SQ-BA4 and (2) SQ-DBA2. (a) in methanol or DMSO (blue), (b) in 25 mM ammonium citrate buffer, pH 3.50 (red) and (c) in the presence of 1.00×10^{-6} M HSA in 25 mM ammonium citrate buffer, pH 3.50 (green).

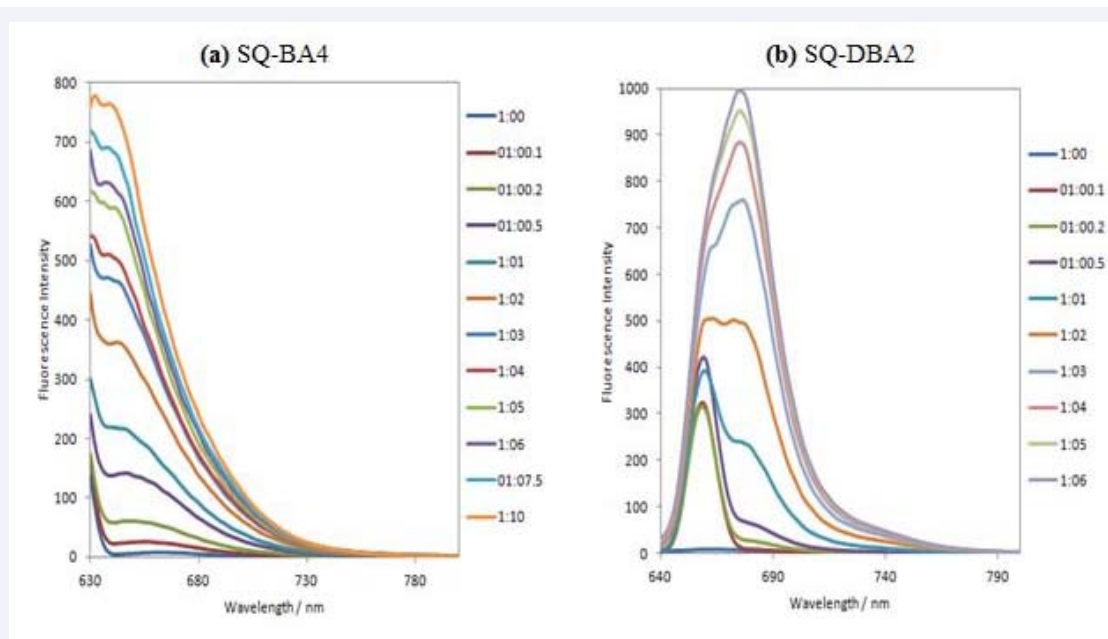


Figure 3 Fluorescence emission spectra corresponding to the titration of squarylium dyes with HSA. Dye:protein ratios are specified in the Figure. **(a)** 1.00×10^{-7} M SQ-BA4 in 25 mM citrate buffer (pH 3.50), $\lambda_{ex} = 621$ nm, **(b)** 1.00×10^{-7} M SQ-DBA2 in 25 mM citrate buffer (pH 3.50), $\lambda_{ex} = 657$ nm.

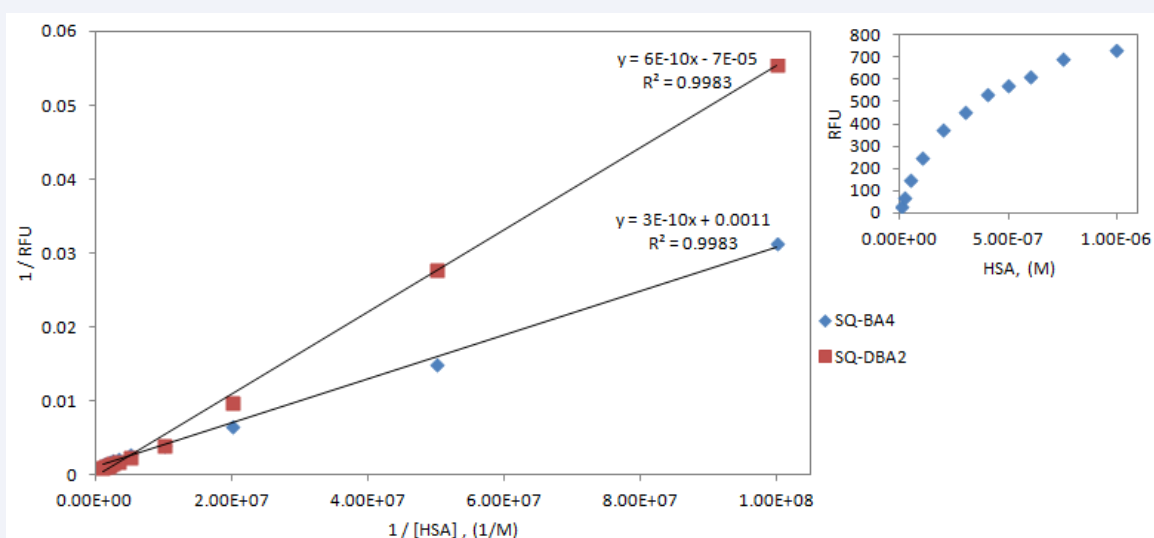


Figure 4 Double reciprocal plots of $1/\text{Fluorescence}$ versus $1/[\text{HSA}]$ for the determination of K_s for SQ-BA4 (blue diamonds) & SQ-DBA2 (red squares). Inset (right top): An example of saturable non-linear plot of fluorescence versus $[\text{HSA}]$ in case of SQ-BA4 due to dye saturation after 1:1 ratio. Fluorescence values are extracted from Figure 3 and experimental conditions are listed in the same Figure.

which would suggest an unfavorable condition for interaction at these pHs, and so such kind of hydrogen bonding, intercalation or hydrophobic interactions must contribute to the formation of the protein-dye complex. It should be noted also that this explanation is consistent with the results in Table 1 where the fluorescence enhancement factor is getting much lower values once pH increases from neutral to basic to a highly basic for both dyes. The concentration of buffer solution, however, had an impact on fluorescence enhancements where the optimal concentration of ammonium citrate (pH 3.50) was found to be between 10 up to 25 mM while increasing the concentration to 50, 75 or even 100

mM showed a less enhancement of the fluorescence emission for both dyes complexes. Through the spectroscopic analysis of these two dyes, it can be concluded that SQ-BA4 and SQ-DBA2 show an optimal fluorescence with HSA in 25 mM citrate buffer (pH 3.50).

A protein titration (with a constant dye concentration and increasing protein concentrations) was also conducted for both dyes in citrate buffer. Figure 3a shows the titration of SQ-BA4 (1.00×10^{-7} M) with HSA from 1:0 (dye with no added HSA) up to a 1:10 dye: protein ratio (beyond this ratio the emission intensity is saturated and a clear emission peak is not detected).

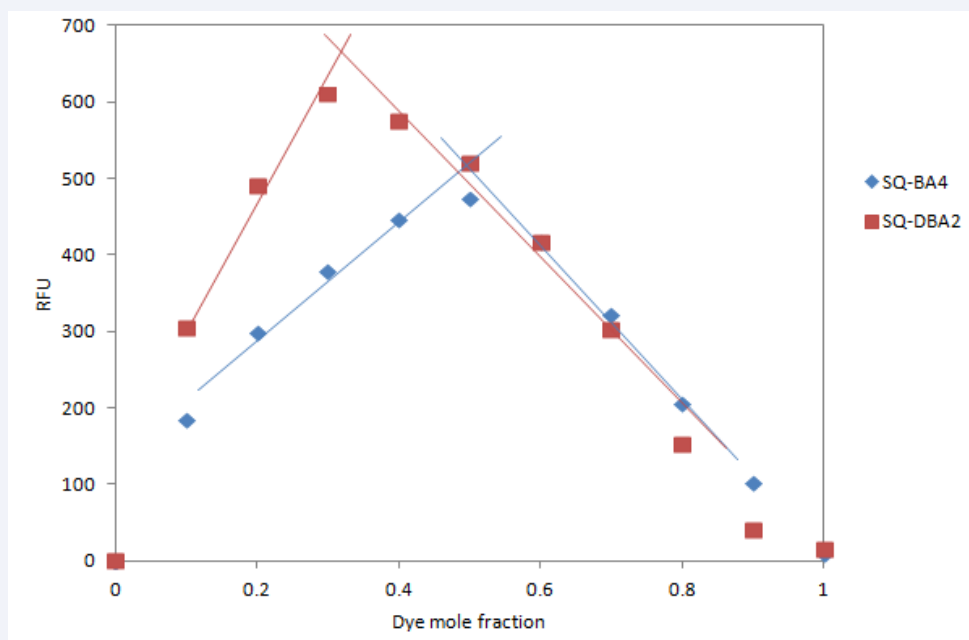


Figure 5 Job's plots for SQ-BA4 & SQ-DBA2 with HSA protein in 25 mM citrate buffer (pH 3.5). $\lambda_{ex} = 621 \text{ nm}$, $\lambda_{em} = 644 \text{ nm}$ for SQ-BA4 (blue diamonds) & $\lambda_{ex} = 657 \text{ nm}$, $\lambda_{em} = 675 \text{ nm}$ for SQ-DBA2 (red squares). Total concentration of dye plus protein held constant for both systems, $[SQ] + [HSA] = 2.00 \times 10^{-7} \text{ M}$.

Table 2: Calibration curves based on lines of best fit from the titration of SQ-BA4 and SQ-DBA2 ($1.00 \times 10^{-7} \text{ M}$) dyes with HSA. Experimental conditions are listed in Figure 3.

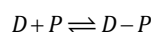
Dye	Range of HSA concentration (μM)	Slope	Intercept	R ²
SQ-BA4	$1.00 \times 10^{-8} - 1.00 \times 10^{-7}$	$2 \times 10^{+09}$	16.23	0.9933
SQ-DBA2	$1.00 \times 10^{-8} - 2.00 \times 10^{-7}$	$3 \times 10^{+09}$	-19.47	0.9980

A linear response in emission intensity for SQ-BA4 at 621 nm as a function of added protein concentrations in citrate buffer was seen up to a concentration of $1.00 \times 10^{-7} \text{ M}$ or a 1:1 dye:protein ratio. On the other hand, the protein titration conducted for SQ-DBA2 (Figure 3b) spanned a 1:6 dye: protein ratio and the concentration of the SQ-DBA2 dye used was also $1.00 \times 10^{-7} \text{ M}$. A linear response in emission intensity for SQ-DBA2 at 657 nm as a function of added protein concentration was observed up to a concentration of $2.00 \times 10^{-7} \text{ M}$ or a 1:2 dye: protein ratio. Table 2 compares the calibration curve results for both dyes titrated with HSA, corresponding to the linear portion of the protein titrations and linearity was high in both cases ($R^2 > 0.992$). The sensitivity of the method is defined as the slope of the line of best fit for each calibration curve. The calibration curve (fluorescence emission as a function of protein concentrations) for SQ-DBA2 displayed steeper slope ($3 \times 10^{+09}$) and thus more sensitive than SQ-BA4 ($2 \times 10^{+09}$). A steep slope means that it is easier to see subtle changes in the concentration of analyte since the small changes in concentration lead to large changes in signal (fluorescence emission in this case).

Stability constants for protein-dye complexes

The stability constant (K_s), also known as formation constant or binding constant, is the equilibrium constant governing the formation of a complex between two or more molecules.

It is a measure of the strength of the interaction between the components of a complex. To quantify the strength of interaction between squarylium dyes and HSA, the stability constant (K_s) for non-covalent complexes formed between HSA and each dye was determined by performing protein titrations (recall Figure 3) and applying the method established by Patonay and coworkers [28]. According to this method, the interaction between dye (D) and protein (P) can be expressed as:



From the equilibrium expression, the stability constant K_s are defined as:

$$K_s = \frac{[D - P]}{[D][P]}$$

From the fluorimetric titration data it is possible to calculate K_s according to the following equation:

$$\frac{1}{F} = \frac{1}{K[D]} + \left(\frac{1}{K[D]K_s} \right) \frac{1}{[P]}$$

Where F is the measured fluorescence emission intensity of the complex and k is a constant dependent upon the instrumentation. A saturable non-linear plot of fluorescence versus the protein concentration as in case of SQ-BA4 with HSA was formed due to the dye saturation after 1:1 complex ratio (see the right top inset

in Figure 4). As a result, a double reciprocal plot of $1/F$ versus $1/[P]$ will yield a straight line. The intercept divided by the slope of this straight line equals K_s .

Stability constants thus determined varied greatly for both dyes since the boronic acid moieties affected not only their spectroscopic properties but also their affinities towards protein targets. The protein titration data shown in Figure 3 was used to construct double reciprocal plots for each dye, as shown in Figure 4. The interesting results obtained showed that SQ-BA4 formed a more stable non-covalent complex with HSA, with $K_s = 3.67 \times 10^6 \text{ M}^{-1}$ than SQ-DBA2 ($K_s = 1.17 \times 10^5 \text{ M}^{-1}$). These results (specially for SQ-BA4) are shown to be superior to other stability constants for some squarylium dye-protein complexes, having higher concentrations of both the dye and HSA that have been reported elsewhere. For example, samples containing concentrations of $5.00 \times 10^{-6} \text{ M}$ HSA with either $5.00 \times 10^{-7} \text{ M}$ Red-1a, Red-1b, Red-1c or Red-1d in trizma buffer (pH 7.4) were reported by Nakazumi *et al.* to have association constants of $8.70 \times 10^5 \text{ M}^{-1}$, $1.10 \times 10^6 \text{ M}^{-1}$, $8.00 \times 10^5 \text{ M}^{-1}$ and $1.70 \times 10^5 \text{ M}^{-1}$, respectively [7] while stability constant on the order of 10^5 M^{-1} was obtained by by Welder F *et al.* through the interactions of $5.25 \times 10^{-6} \text{ M}$ HSA with $5.25 \times 10^{-7} \text{ M}$ SQ-3 in citric acid buffer [24]. These reported values, determined by the same method described by Patonay and coworkers, verifies that the boronic acid group in the dye does, indeed, have an impact on its binding affinity towards protein and increased sensitivity at lower concentrations than other relative squarylium dyes. It is important to note that one limitation of this method used to calculate K_s is that it is only strictly valid for 1:1 stoichiometric complexes of dye and protein (as was the case for SQ-BA4 with HSA). However, it is still also used as a tool to support us with related approximate values for the stability constant with other stoichiometries (as for SQ-DBA2 with HSA). Other methods for measuring the association of dye and protein, which are not dependent upon stoichiometry, include frontal analysis (FA) [8] or non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) [29] for more precise measurements.

Dye: protein stoichiometries

The stoichiometries of both protein-dye complexes were calculated through the method of continuous variation (Job's plot) [30]. In our fluorescence studies, a plot of fluorescence emission as a function of mole fraction of dye is constructed for various protein-dye mixtures possessing a fixed total concentration of dye plus protein which was $2.00 \times 10^{-7} \text{ M}$. Two straight lines are formed within the plot and the mole ratio corresponding to the point at which they intersect can be used to determine the predominant stoichiometry of the protein-dye complex.

Under the prescribed optimal acidic buffer condition discussed above, SQ-BA4 and SQ-DBA2 each formed different non-covalent complexes with HSA. For SQ-BA4, the fluorescence maxima in the resulting Job's plot occurred at a dye mole fraction of 0.50, corresponding to a predominant stoichiometry of 1:1 (dye1-HSA1). On the other hand, the fluorescence maxima in the resulting Job's plot in case of SQ-DBA2 occurred at a dye mole fraction of 0.30 corresponding to a predominant stoichiometry of 1:2 (dye1-HSA2), as shown in Figure 5. It can be also seen from the figure that when the amount of protein was greater than that

of the dye, a relative increase in signal was seen. As soon as more dye than protein was present in the solution, a decrease in signal was observed. This may be attributed to the probable chance of dye aggregation in the aqueous solution with its increasing concentration while the protein concentration is relatively decreasing and thus a tangible quenching in the fluorescence of the complex can be observed. Overall, these findings suggest that the monofunctional or bifunctional boronic acid moieties in the dye structure impact the dye binding with the protein may be through H-bonding with the protein functional groups located on its binding sites. Also, we can't neglect the importance of hydrophobic interactions between squarylium dyes and proteins which have been previously shown [14] and the present binding studies support this. As such, the combination of H-bonding, electrostatic and hydrophobic interactions renders these dyes suitable for on-column, precolumn, and post-column protein labeling strategies under different solution conditions for subsequent protein analysis by CE-LIF.

CONCLUSION

The spectral properties of the two squarylium dyes, monofunctional boronic acid-based SQ-BA4 and bifunctional boronic acid-based SQ-DBA2, were explored under a variety of solutions in order to determine the best conditions for non-covalent protein labeling. These dyes exhibit very low fluorescence intensity in aqueous solutions likely due to H-aggregate formation in absence of the protein. However, both dyes showed a significant enhancement in the fluorescence intensity with the addition of HSA in the optimal citrate buffer (pH 3.50). Stability constant studies were conducted and SQ-BA4 was shown to have much stronger binding affinity with HSA than SQ-DBA2, although it demonstrated a lower fluorescence enhancement compared to SQ-DBA2 with HSA. However, the stability constant values were shown to be superior to other values of some reported squarylium dye-HSA complexes. Additionally, the stoichiometries of non-covalent complexes were found to be (1:1) for SQ-BA4 and different (1:2) for SQ-DBA2 with HSA. The variations in stoichiometry indicate the importance of a combination of electrostatic, hydrophobic, and H-bonding interactions between dye and protein, and suggest the suitability of these dyes as candidates for on-column or pre-column protein labeling strategies under different solution conditions for subsequent protein analysis by CE-LIF. However, more studies will need to be conducted to that end. Those upcoming studies should include systematic buffer studies to determine the optimal conditions for protein assays by CE-LIF

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