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Symmetric and asymmetric squarylium dyes as noncovalent protein labels: a study by fluorimetry and capillary electrophoresis

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Abstract

Noncovalent interactions between two squarylium dyes and various model proteins have been explored. NN127 and SQ-3 are symmetric and asymmetric squarylium dyes, respectively, the fluorescence emissions of which have been shown to be enhanced upon complexation with proteins such as bovine serum albumin (BSA), human serum albumin (HSA), β-lactoglobulin A, and trypsinogen. Although these dyes are poorly soluble in aqueous solution, they can be dissolved first in methanol followed by dilution with aqueous buffer without precipitation, and are then suitable for use as fluorescent labels in protein determination studies. The nature of interactions between these dyes and proteins was studied using a variety of buffer systems, and it was found that electrostatic interactions are involved but not dominant. Dye/protein stoichiometries in the noncovalent complexes were found to be 1:1 for SQ-3, although various possible stoichiometries were found for NN127 depending upon pH and protein. Association constants on the order of 10^5 and 10^7 were found for noncovalent complexes of SQ-3 and NN127, respectively, with HSA, indicating stronger interactions of the symmetric dye with proteins. Finally, HSA complexes with NN127 were determined by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF). In particular, NN127 shows promise as a reagent capable of fluorescently labeling analyte proteins for analysis by CE–LIF without itself being significantly fluorescent under the aqueous solution conditions studied herein.

Keywords: Squarylium dyes; Non-covalent protein labeling

1. Introduction

The implication of proteins in various biological functions and as markers of various states of disease justifies the need for improved protein assays, and capillary electrophoresis (CE) certainly qualifies as a technique worthy of this task. Typically, CE analysis of proteins is carried out using absorbance detection. However, laser-induced fluorescence (LIF) detection is an attractive alternative, with its advantages of lower background, increased sensitivity and selectivity, and compatibility with integrated, microchip (μ-chip) CE substrates. In most cases, LIF requires the analyte to be rendered fluorescent by way of some labeling procedure. Various review articles summarize fluorescent derivatization and CE–LIF detection schemes [1–6]. Such derivatization often necessitates increased sample preparation, and may

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be difficult to achieve with very dilute samples. Precolumn covalent labeling procedures typically require very specific control of solution pH and temperature, and may result in peak broadening and loss of separation efficiency due to the formation of multiple, differently-labeled species [7,8].

Instead, noncovalent dye–protein interactions can be exploited to facilitate protein determination by CE–LIF, thus offering greater selectivity and sensitivity than is possible with absorbance detection, and entailing a simpler and faster labeling protocol than required by conventional covalent labels [9]. Noncovalent protein–fluorophore interactions can occur by way of a variety of physical mechanisms, including hydrophobic interactions, electrostatic interactions and hydrogen bonding. The exact nature of these interactions is often difficult to determine, but evidence of interaction is clearly provided by a change in the emission of the fluorophore–protein complex relative to that of the free, uncomplexed fluorophore, and this can be easily monitored by regular fluorimetric studies. Although the integrity of noncovalent protein–dye complexes may be compromised by conventional chromatographic methods of analysis (since the dye may have a greater affinity for the chromatographic stationary phase than for the protein analyte [10]), CE provides a suitable alternative by which these sometimes less stable complexes may be studied.

Specifically, this paper will compare the properties of two squarylium dyes as potential noncovalent protein labels for subsequent determination by CE–LIF. As a class of cyanine dyes, squarylium dyes were first synthesized over 30 years ago and are 1,3-disubstituted products of the condensation of squaric acid with two aromatic and/or heterocyclic compounds [11,12]. These dyes exhibit effective light absorption and are resistant to photodegradation, and have found use in applications such as photoconductors in copying devices, organic solar cells, and optical recording media [13–17].

Various squarylium dyes have also found use in optical sensors for metal determination [18–21]. It has been suggested that these mesoionic dyes can serve as π-donor ligands, resulting in increased electron density at the metal center upon electrostatic interaction [18]. As a result, quenching of the fluorescence of the dye was observed and used to determine metal concentration.

In addition to their utility as probe molecules for metal analytes, there is evidence of the utility of squarylium dyes for protein determination. In a work describing the synthesis and spectral properties of various symmetric and asymmetric squarylium dyes, Terpetschnig et al. showed that the interaction of some squarylium dyes with aqueous bovine serum albumin solutions resulted in enhanced fluorescence and increased lifetime of the dye [22]. Likewise, work by Patonay and coworkers has found that two symmetric squarylium dyes, NN127 and NN525 (Fig. 1) exhibit changes in their absorptivity and fluorescence when bound in environments of differing polarity [23,24]. In addition to these noncovalent protein probes, Oswald et al. synthesized and characterized two reactive squaraine dyes for covalent attachment to amino groups of proteins, resulting in enhanced fluorescence lifetimes and quantum yields, which were explained by a shielding effect of the protein [25].

Although the traditional synthesis of squarylium dyes yields symmetrical products, for which variations in types of π-conjugated systems are limited, there have been several recent reports of the synthesis of novel, asymmetrical squarylium dyes [21,22,26–29]. These syntheses have the promise of extending the possible range of fluorophores available, but the effect of dye symmetry on possible interactions with analyte molecules is largely unknown. However, Lakowicz and coworkers did find that symmetry had no effect on the solvatochromic shifts observed for various squarylium dyes [22].

One asymmetric squarylium dye, synthesized by

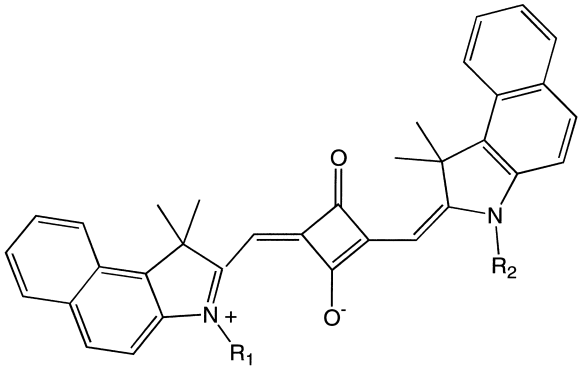

Fig. 1. The molecular structure of a symmetric squarylium dye. For NN127, $R_1 = R_2 = C_2H_5$; and for NN525, $R_1 = C_6H_{11}O_2$ (hexanoic acid) and $R_2 = C_4H_{10}SO_3$ (butyl sulfonate).

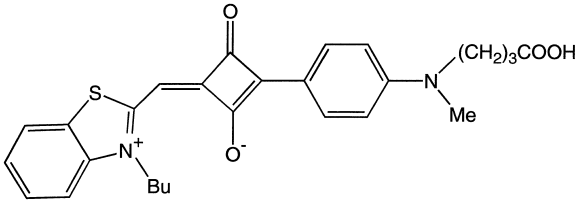

Fig. 2. The molecular structure of asymmetric squarylium dye SQ-3, synthesized as $C_{27}H_{28}N_2O_4S\cdot\frac{1}{2}CHCl_3$.

Yagi [26a], displayed marked enhancements in absorbance in the presence of increasing concentrations of human serum albumin [30], but did not display significant fluorescence in its free or protein-bound state. However, a new asymmetric squarylium dye ''SQ-3'' [31], shown in Fig. 2, displays greatly increased fluorescence intensity when protein-bound in both organic and mostly aqueous media, and so presents itself as a viable noncovalent label for proteins. SQ-3 was prepared from *N*-butyl-2-methylbenzothiazolium iodide and 3-(*N*-[3-ethoxycarbonylpropyl]-*N*-methylamino)phenyl-4-hydroxycyclobut-3-ene-1,2-dione according to a procedure analogous to that reported previously [26a].

This study presents a comparison of symmetric NN127 and asymmetric SQ-3 squarylium dyes (shown in Figs. 1 and 2, respectively) as noncovalent protein labels. As such, we have conducted extensive fluorimetric studies involving the titration of these dyes in various buffers with various model proteins, including human and bovine serum albumin (p*I* 4.8 and 4.9, respectively), trypsinogen (p*I* 9.3), and β-lactoglobulin A (p*I* 5.2). Additionally, the suitability of NN127 as a label for human serum albumin was confirmed by CE–LIF studies. It is hoped that these results will shed some light on the relative affinity of symmetric versus asymmetric squarylium dyes for protein, and will extend the range of protein labeling protocols available to analysts.

2. Material and methods

2.1. Reagents, buffers, and sample solutions

Squarylium dye NN127 was purchased from KPS Technologies (Atlanta, GA, USA) and was used as received. Squarylium dye ''SQ-3'' was prepared as described in Section 2.2. Stock solutions of these dyes were prepared in methanol to a concentration of 1.5×10^{-4} *M* (SQ-3) or 1.5×10^{-5} *M* (NN127), and were stored in the dark at 4 °C when not in use. Dilute stock solutions were prepared from the original stock solutions by dilution to a concentration of 5.25×10^{-5} *M* or 1.5×10^{-6} *M* for SQ-3 and NN127, respectively. Working solutions of the dyes were subsequently prepared just prior to use by dilution to the final desired concentration with one of the aqueous buffers being studied, and/or the addition of protein solution.

Proteins (human and bovine serum albumin, trypsinogen, and β-lactoglobulin A) were purchased from Sigma–Aldrich (St Louis, MO, USA) and used as received. Stock solutions of proteins were prepared to a concentration of 1.5×10^{-4} mol/l in water, and were stored in the dark at 4 °C.

Although many buffers were studied, three predominated and are described in more detail. The first of these, a basic boric acid buffer, was prepared by dissolving boric acid at 25.0 m*M* concentration (J.T. Baker, Phillipsburg, NJ, USA) in Milli-Q (Millipore, Bedford, MA, USA) distilled, deionized water (18 MΩ) and adjusting the pH to the desired value (11.5) by the addition of 0.1 *M* NaOH (Fisher, Pittsburgh, PA, USA). The second, a near-neutral Tris–HCl buffer used in SQ-3 studies, was prepared to a concentration of 20.0 m*M* by dissolution of an appropriate mass of Trizma (''Tris'') hydrochloride reagent (Sigma, St Louis, MO, USA) in water, and adjusting the pH to 7.4 by the addition of 0.1 *M* NaOH. The Tris–HCl buffer employed in the NN127 studies was similarly prepared, but also contained 20 m*M* $CaCl_2$ (Fisher, Fair Lawn, NJ, USA), since this was in use for other protein adsorption studies not related to the present work. The third, an acidic citric buffer, was prepared by dissolving an appropriate mass of citric acid (Mallinckrodt, St Louis, MO, USA) in water to a concentration of 25.0 m*M*, and adjusting the pH to 3.1 by the addition of 0.1 *M* NaOH. All buffers were stored in plastic bottles at room temperature, and were filtered through 0.20-μm nylon syringe filters (Corning, NY, USA) prior to use.

Protein–dye solution mixtures were prepared by adding 200 μl of the 1.5×10^{-6} *M* working solution of NN127 or 15.0 μl of the 5.25×10^{-5} *M* working solution of SQ-3 to a 2000-μl cuvette or a 1500-μl

microcentrifuge tube. Some volume of protein stock solution was then added to this aliquot of dye, along with sufficient buffer as diluent to achieve the final, desired concentration of both protein and dye.

2.2. Synthesis of SQ-3: 3-(N-butylbenzothiazol-2-ylidenemethyl)-4′-(N-[ethoxycarboxypropyl]-N-methyl-amino)phenylsquaraine

A mixture of *N*-butyl-2-methylbenzothiazolium iodide (333 mg, 1 mmol), 3-(*N*-[3-ethoxycarbonylpropyl]-*N*-methylamino)phenyl-4-hydroxycyclobut-3-ene-1,2-dione (289 mg, 1 mmol) (which was prepared by reaction of 3,4-dichlorocyclobut-3-ene-1,2-dione with *N*-(3-ethoxycarbonylpropyl)-*N*-methylaniline followed by hydrolysis), and quinoline (150 mg) in butanol/benzene (2:1, v/v, 15 ml) were refluxed for 1.5 h with removal of water using a Dean-Stark distillation apparatus. After cooling, the solvent was evaporated in vacuo and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 20:1, as eluent). Recrystallization from $CHCl_3$/MeOH (20:1)–hexane afforded a crystal of SQ-3 (30 mg, 0.063 mmol), with the following characterization:

SQ-3: Yield 6.3%; m.p. 242–243 °C; ^{1}H NMR (400 MHz, DMSO) δ=0.95 (t, J=7.3 Hz, 3H), 1.44 (m, 2H), 1.80 (q, J=7.3 Hz, 4H), 2.27 (t, J=7.3 Hz, 2H), 3.00 (s, 3H), 3.43 (t, J=7.3 Hz, 2H), 4.55 (t, J=7.8 Hz, 2H), 6.30 (s, 1H), 6.79 (d, J=9.3 Hz, 2H), 7.51 (t, J=7.3 Hz, 1H), 7.64 (t, J=7.8 Hz, 1H), 7.88 (d, J=8.8 Hz, 1H), 7.92 (d, J=9.3 Hz, 2H), 8.12 (d, J=7.8 Hz, 1H); IR (KBr): ν=3418, 1750, 1587, 1579 cm^{-1}; TOF-MS (m/z) 476(M^+); λ_{max} (buffer/MeOH, 3:7, v/v)=598 nm (ε=1.1×10^5 M^{-1} cm^{-1}); Anal. Calcd. for $C_{27}H_{28}N_2O_4S\cdot\frac{1}{2}$ ($CHCl_3$): C, 61.59; H, 5.36; N, 5.22%. Found: C, 60.72; H, 5.44, N, 5.21%.

2.3. Instrumentation

The capillary electrophoresis experiments involving SQ-3 were carried out on an Agilent HP3D CE (Wilmington, DE, USA) coupled to a Picometrics Zeta-LIF detector (Ramonville, France), which was equipped with a 594-nm He–Ne laser for excitation and a 630-nm cut-off filter for emission. Injection of pre-mixed protein–dye samples was by pressure (at 15 mbar=0.22 p.s.i.) for 2 s; and separations were conducted by applying 15.0 kV across a 50 cm×50 μm I.D. uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with an effective length of 39.5 cm (inlet-to-detector). Prior to sample analysis, the capillary was conditioned by pressure flushing with 0.1 *M* NaOH and water for two, 5-min cycles each, followed by electrokinetic flushing (at 15 kV) with buffer for about 10 min. Capillary, sample, and buffer were maintained at 25 °C throughout the experiments.

A Bio-Rad BioFocus 3000 CE system with LIF detection (Hercules, CA, USA), equipped with an external 650-nm diode laser (Oz Optics, Carp, Ontario, Canada) and a 664-nm cut-off filter was employed for the experiments involving NN127. Samples were pressure injected at 5 p.s.i. for 2 s, and a separation voltage of 15 kV was applied across a 50 cm×50 μm I.D. uncoated fused-silica capillary (Polymicro Technologies), with an effective length of 45.4 cm. Prior to sample analysis, the capillary was conditioned by pressure flushing with 0.1 *M* NaOH and water for 2 min each, followed by pressure flushing with buffer for 3 min. Subsequently, and before each run, the capillary was pressure rinsed with the running buffer for about 25 s. Capillary, sample, and buffer were maintained at 25 °C.

Fluorimetric studies were conducted using a Perkin-Elmer LS50B Luminescence Spectrometer (Shelton, CT, USA). Excitation and emission slit widths were 5 nm (NN127) or 5–10 nm (SQ-3), and the scan rate was 50 nm/min (NN127) or 500 nm/min (SQ-3) unless otherwise stated. Fluorescence spectra of dye and protein–dye solutions were measured using a 1-cm quartz cuvette (VWR, Suwanee, GA, USA).

Absorbance studies were conducted using a Hewlett-Packard HP8453 UV–Vis spectrometer (Waldbronn, Germany) and a 1-cm quartz cuvette.

3. Results and discussion

Enhanced sensitivity in a protein assay is clearly achievable by employing fluorescence rather than absorbance detection methods, since the former

typically afford limits of detection up to several orders of magnitude lower than the latter. Squarylium dyes, which demonstrate changes in their fluorescence properties upon noncovalent interaction with analyte species, are therefore potential tools for protein determination. The spectral properties, photostability, protein binding properties and electrophoretic behavior of two such dyes, symmetric NN127 and asymmetric SQ-3, are presented herein in order to establish their utility as potential protein labels for capillary electrophoretic or other assay methods.

3.1. Spectral properties of SQ-3 and NN127

The absorbance spectrum of SQ-3 showed a rather sharp maximum at 600 nm in methanol, although this maximum was significantly blue-shifted and broadened in aqueous buffer systems ranging in pH from 3.1 (citric acid) to 11.5 (boric acid). Subsequent fluorescence emission was most intense for a methanolic solution of the dye at 658 nm. Relatively small shifts in this emission maximum were observed for predominantly aqueous buffered solutions of SQ-3, although under aqueous conditions, fluorescence intensities were dramatically decreased. It has been suggested that deactivation of the fluorescent state of squarylium dyes in polar solvents involves intramolecular charge transfer and twisting around free bonds [32]. A summary of the absorbance and emission properties of SQ-3 for a variety of solvent systems is presented in Table 1.

A summary of the absorbance and emission properties of the symmetric squarylium dye NN127 is also presented in Table 1. NN127 showed absorbance and emission maxima in methanol at 660 and 671 nm, respectively. Unlike its asymmetric counterpart SQ-3, the absorbance maxima for NN127 in aqueous buffered solutions were slightly red-shifted and broadened, except in boric acid buffer, which resulted in a significant blue shift. Like SQ-3, fluorescence emissions of NN127 were significantly diminished in aqueous systems relative to pure methanol. However, NN127 demonstrated a weak secondary emission at longer wavelengths in all buffers.

The low fluorescence, especially of NN127, in aqueous solutions would seem to preclude its use as a fluorescent label for protein determination. However, as will be shown, the dye is still able to strongly bind to proteins under these conditions, with a concomitant increase in fluorescence. In this respect, NN127 behaves similarly to the cyanine dye, indocyanine green (ICG), which was also found to display fluorogenic behavior under aqueous, acidic buffer conditions [33].

Table 1
Absorbance and emission properties for 5.25×10^{-7} *M* SQ-3 and 3.75×10^{-7} *M* NN127 in various solvent systems

Dye	Solvent[a]	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	Fluorescence (RFU)[b]
SQ-3	Methanol	600	658	1.000
	Water	557	660	0.037
	Citric acid buffer (pH 3.1)	488, 560	652	0.005
	Tris–HCl buffer (pH 7.4)	556	657	0.084
	Boric acid buffer (pH 11.5)	563	655	0.081
NN127	Methanol	660	671	1.000
	Water	685	669.5, 718	0.001
	Citric acid buffer (pH 3.1)	686	662, 717.5	0.001
	Tris–HCl buffer (pH 7.4)	686	663.5, 721.5	0.001
	Boric acid buffer (pH 11.5)	622	668.5, 715.5	0.001

[a] Maximum organic content in aqueous solutions is 2.5% (v/v), due to preparation of aqueous solutions by dilution of methanolic stock solution.

[b] Relative fluorescence emissions (recorded after excitation at λ_{abs}^{max}) have been normalized by dividing the measured fluorescence in aqueous (or buffer) solution by that in methanol.

3.2. Stability of SQ-3 and NN127

Because of the relatively poor solubilities of these squarylium dyes in aqueous solutions, concentrated stock solutions of both SQ-3 and NN127 were first prepared in methanol, and working solutions were subsequently prepared by dilution of aliquots of these stock solutions to the desired final concentration with an aqueous buffer. To determine the stability of these more dilute, primarily aqueous working solutions, we recorded fluorescence emission spectra over a number of days.

In the case of SQ-3, no real change in emission intensity was observed for a 5.25 μM solution in Tris–HCl buffer over the course of 6 weeks. This was not the case, however, for SQ-3 working solutions prepared to the same concentration in citrate and boric acid buffers. In the citric buffer, the fluorescence intensity of the SQ-3 solution quickly decreased to slightly less than 30% of its original value after 48 h, while in the boric acid buffer, fluorescence intensity decreased only slightly to about 95% of its original value within 48 h. However, after 6 weeks, the SQ-3 solutions prepared in citric and boric acid buffers showed 10% or less of their original fluorescence intensity. The addition of an equimolar amount of protein (HSA) to SQ-3 in boric acid served to stabilize the dye, and in fact, resulted in more than a doubling of fluorescence intensity over a period of 4 days of solution aging. This stabilization in the presence of protein is not unlike that seen in other studies, where the addition of protein to the cyanine dye ICG acted to stabilize the dye [10,34–36].

In the case of 1.5×10^{-7} M NN127 in Tris–HCl/$CaCl_2$ buffer (pH 7.4), the addition of protein prevented a decrease in the observed fluorescence signal. In fact, over nearly a 6-day period with a 10-fold excess of HSA present, the fluorescence emission of the NN127 solution more than doubled, which was similar to the trend observed for the 1:1 molar ratio of SQ-3/HSA in boric acid buffer aged over a period of 4 days.

It can be concluded, therefore, that on the timescale of running a protein assay by CE–LIF, degradation of aqueous solutions of SQ-3 and NN127 does not pose a problem. To be certain, however, that working solutions had not undergone any degradation prior to use, they were made fresh daily from more concentrated stock solutions, the latter being stored in the refrigerator, wrapped in aluminum foil, for up to 2 months.

3.3. Dye–protein interactions

The interaction of dye molecules with proteins provides a valuable tool for monitoring the presence and concentrations of those proteins, which makes use of changes in the fluorescence properties of the dye upon binding. In the present work, both SQ-3 and NN127 were found to interact with a variety of model proteins (including human serum albumin (HSA); bovine serum albumin (BSA); trypsinogen; and β-lactoglobulin A), resulting in enhanced fluorescence in each case. By selecting this group of proteins, and studying their interaction with two related yet structurally different squarylium dyes under a wide range of solution pH values, it was hoped that some understanding of the relative importance of electrostatic versus hydrophobic interactions could be qualitatively determined.

3.3.1. SQ-3–protein interactions

SQ-3 interacted differently with HSA in boric acid relative to citric acid and Tris buffers. These data are summarized in Table 2. In boric acid buffer (pH 11.5), there was no substantial change in fluorescence intensity of the dye even after an equimolar amount of HSA had been added. Less than a 4% increase in fluorescence was observed upon the addition of a 1.5 times excess of protein. Substantial increases in fluorescence were not observed in this buffer until at least a five times excess of HSA was added, which led to just greater than a 50% increase in fluorescence, with greater than a 100% increase observed for a 10 times excess of protein in this basic pH solution. The interaction of SQ-3 with BSA in boric acid buffer closely mirrored this behavior, with a substantial increase in fluorescence (of 41%) being observed only after a five times excess of protein had been added. Similar titrations of a fixed concentration of SQ-3 (5.25×10^{-7} M) with varying concentrations of HSA (ranging from 0 to 52.5×10^{-7} M, or a dye/protein concentration ratio ranging from 1:0 to 1:10) in citric and Tris buffers led to significant enhancements in fluorescence even at the

Table 2
Protein titration studies: maximum fluorescence emissions measured at 655 nm (excitation at 594 nm) for 0.525 μ*M* SQ-3 in citric, Tris–HCl, and boric acid buffers with increasing concentrations of added protein (HSA, BSA, β-lactoglobulin A, and trypsinogen)

SQ-3/protein ratio	Buffer	Fluorescence emission (RFU)			
		HSA	BSA	β-Lacto-globulin A	Trypsinogen
1:0	Citric acid	14.3	1.3	14.4	10.0
	Tris–HCl	21.9	34.1	35.4	29.8
	Boric acid	24.5	23.5	22.3	21.1
1:0.1	Citric acid	21.5	6.5	17.2	10.2
	Tris–HCl	26.8	40.9	36.0	30.5
	Boric acid	22.7	24.4	31.2	22.3
1:0.2	Citric acid	25.9	8.1	16.9	8.7
	Tris–HCl	37.0	48.9	39.1	30.8
	Boric acid	22.3	25.5	33.8	21.5
1:0.5	Citric acid	31.6	10.8	19.0	8.7
	Tris–HCl	65.3	54.7	35.0	30.0
	Boric acid	23.5	25.4	29.4	20.8
1:1	Citric acid	66.3	31.5	23.3	10.6
	Tris–HCl	79.6	101.9	35.2	32.3
	Boric acid	23.9	27.5	33.7	20.5
1:1.5	Citric acid	74.7	34.1	20.8	10.0
	Tris–HCl	104.1	117.4	36.1	32.0
	Boric acid	25.5	27.7	28.1	22.3
1:5.0	Citric acid	157.2	59.8	23.3	11.0
	Tris–HCl	193.9	175.7	37.5	35.3
	Boric acid	37.1	33.2	27.2	21.5
1:10.0	Citric acid	258.4	113.2	25.7	14.2
	Tris–HCl	230.1	281.4	40.3	34.2
	Boric acid	51.3	44.6	32.2	21.5

lowest protein concentrations, and led to substantially greater relative fluorescence intensities for all protein concentrations. A representative series of spectra recorded for the titration of SQ-3 with HSA in Tris is shown in Fig. 3.

Some interesting observations can be drawn from this work. Even though a 1:1 mixture of SQ-3/HSA in boric acid did not result in any fluorescence enhancement, the same 1:1 mixture in Tris and citrate buffers led to impressive 263 and 363% increases in fluorescence intensity, respectively. Even with only one-tenth the amount of protein relative to dye in the Tris and citrate buffers, fluorescence enhancements of 22 and 50%, respectively, were achieved. Interestingly, a substantial, non-linear jump in fluorescence was observed for the 1:5 ratio of SQ-3 with BSA, just as had been observed for the 1:5 ratio (and higher) of SQ-3 with HSA in boric acid buffer. Perhaps not surprisingly, the interaction of BSA with SQ-3 in Tris and citrate buffers was very similar to that just described for HSA in these buffers.

Enhancements in fluorescence upon mixing of SQ-3 with different ratios of β-lactoglobulin A or trypsinogen were substantially lower than fluorescence enhancements observed for the albumin-type proteins in all buffers (Table 2). In particular, trypsinogen—a basic protein with a p*I* value of

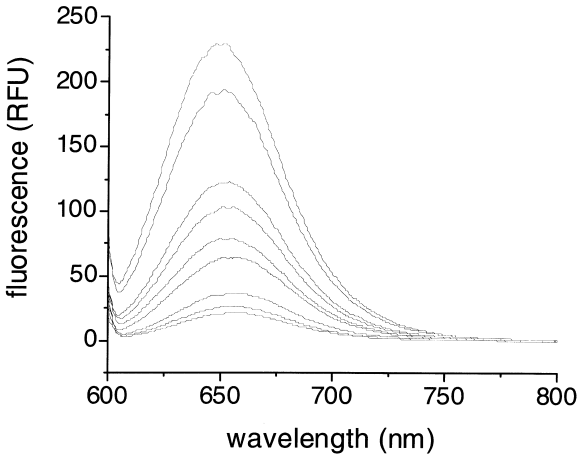

Fig. 3. Fluorescence emission spectra for the titration of $5.25\times 10^{-7}M$ SQ-3 with varying amounts of HSA in Tris–HCl buffer. Excitation at 594 nm. From bottom to top spectrum, fluorescence emissions increase as dye/protein ratios are increased from 1:0, 1:0.1, 1:0.2, 1:0.5, 1:1, 1:1.5, 1:2, 1:5, to 1:10.

9.3—showed no significant interaction with the asymmetric squarylium dye in boric acid, and showed only slight interaction with the dye in citric and Tris buffers upon the addition of at least a five times molar excess of protein relative to dye. β-Lactoglobulin A demonstrated a behavior similar to that of trypsinogen in citric and Tris buffers, but showed a substantial (40%) increase in fluorescence even when mixed in only a one-tenth molar ratio relative to the dye in boric acid buffer. However, this fluorescence enhancement did not significantly improve (and in some cases was diminished) as more β-lactoglobulin A was added (up to a 10-fold excess relative to SQ-3 concentration) in boric acid buffer.

3.3.2. NN127–protein interactions

Protein titrations involving a fixed concentration (3.75×10^{-7} *M*) of the symmetric squarylium dye NN127 with increasing concentrations of HSA, trypsinogen and β-lactoglobulin A (in dye/protein ratios ranging from 1:0 to 1:50) were similarly conducted in three buffer systems similar to those considered for SQ-3. The results of these experiments are summarized in Table 3, and representative fluorescence spectra for NN127/protein mixtures in citric acid buffer are shown in Fig. 4a–c. Because of the near-zero fluorescence of unbound NN127 in aqueous systems, significant fluorescence enhancements were observed upon the addition of just 7.5×10^{-8} *M* protein (yielding a 1:0.5 dye/protein ratio) in each buffer system. However, subsequent additions of more HSA to NN127 in citric and boric acid buffers (up to a 50-fold excess of protein relative to dye) did not result in proportional increases in fluorescence. That is, increases in fluorescence seemed to slow down or even ‘‘level off’’ upon subsequent additions of HSA to NN127, especially in citric and boric acid buffers. This would indicate a saturation, whereby all available dye molecules were protein bound and so the addition of still more protein could result in no further interaction and no further change in fluorescence.

This saturation behavior was not observed for other proteins studied with NN127. Notably, trypsinogen and β-lactoglobulin A mixtures with NN127 resulted in lower absolute fluorescence values than comparable HSA mixtures, but the fluorescence of the trypsinogen and β-lactoglobulin A mixtures continued to increase substantially as protein concentration was increased. For example, the fluorescence of the 1:50 mixture of NN127/trypsinogen exceeded the fluorescence of the 1:0.5 mixture by more than 440% in each of the three buffers (citric, Tris–HCl, and boric acid buffer). Likewise, the fluorescence of the 1:50 mixture of NN127/β-lactoglobulin A exceeded the fluorescence of the 1:0.5 mixture by more than 1250% in each of the three buffers. These results are considerably different from those obtained for SQ-3, which showed less interaction with trypsinogen and β-lactoglobulin A than with BSA and HSA. Clearly, then, changing the symmetry of the squarylium dye significantly affects the nature of its noncovalent interactions with proteins in various buffered, aqueous environments.

3.4. Dye–protein stoichiometries

To determine the predominant stoichiometry of the protein–dye complexes formed in these studies, the method of continuous variation (or Job’s method) was employed [37]. To this end, we plotted fluorescence response versus mol fraction of protein for a

Table 3
Protein titration studies: maximum fluorescence emissions measured at 676 nm (excitation at 650 nm) for 0.375 μ*M* NN127 in citric, Tris–HCl, and boric acid buffers with increasing concentrations of added protein (HSA, β-lactoglobulin A, and trypsinogen)

NN127/protein ratio	Buffer	Fluorescence emission (RFU)		
		HSA	β-Lacto-globulin A	Trypsinogen
1:0	Citric acid	0.5	0.1	0.3
	Tris–HCl	0.3	0.1	0.1
	Boric acid	0.5	0.3	0.3
1:0.5	Citric acid	28.6	2.8	0.5
	Tris–HCl	13.9	1.9	0.3
	Boric acid	55.2	2.7	0.4
1:1	Citric acid	40.2	2.8	0.6
	Tris–HCl	22.1	2.4	0.4
	Boric acid	80.1	3.2	0.6
1:2	Citric acid	56.1	5.0	0.7
	Tris–HCl	33.2	2.9	0.5
	Boric acid	91.9	4.2	0.7
1:5	Citric acid	60.6	9.1	1.3
	Tris–HCl	43.4	4.6	1.2
	Boric acid	90.1	9.0	1.2
1:10	Citric acid	68.3	9.0	1.9
	Tris–HCl	49.4	6.1	1.8
	Boric acid	91.3	9.6	1.9
1:20	Citric acid	65.9	13.6	3.3
	Tris–HCl	53.9	7.2	2.8
	Boric acid	85.0	11.6	3.2
1:50	Citric acid	70.9	16.8	7.0
	Tris–HCl	38.6	10.4	5.1
	Boric acid	88.3	15.9	5.7

number of protein/dye mixtures prepared with a constant total concentration of 4.0×10^{-7} *M* or 5.25×10^{-7} *M* (for NN127 or SQ-3 mixtures, respectively). For HSA with SQ-3, the maxima in the resulting Job's plots occurred at a protein mol fraction of 0.5 (±0.05) in each of the three buffers (data not shown), corresponding to a predominant stoichiometry of $(SQ\text{-}3)_1(HSA)_1$. The Job's plot for HSA with NN127 in Tris–HCl similarly predicted a predominant stoichiometry of 1:1. However, in both citric acid and boric acid buffers, the maxima in the Job's plots for HSA and NN127 occurred at a protein mol fraction of 0.4 (±0.05), corresponding to a predominant stoichiometry of $(NN127)_3(HSA)_2$. This perhaps surprising stoichiometry was also observed in earlier work with the cyanine dye ICG and HSA [34].

3.5. Stability constants

To quantify the strength of interaction between the proteins and SQ-3 and NN127, we determined the stability constant K_s of the various complexes formed, according to the method articulated by Patonay and coworkers [18]. According to this

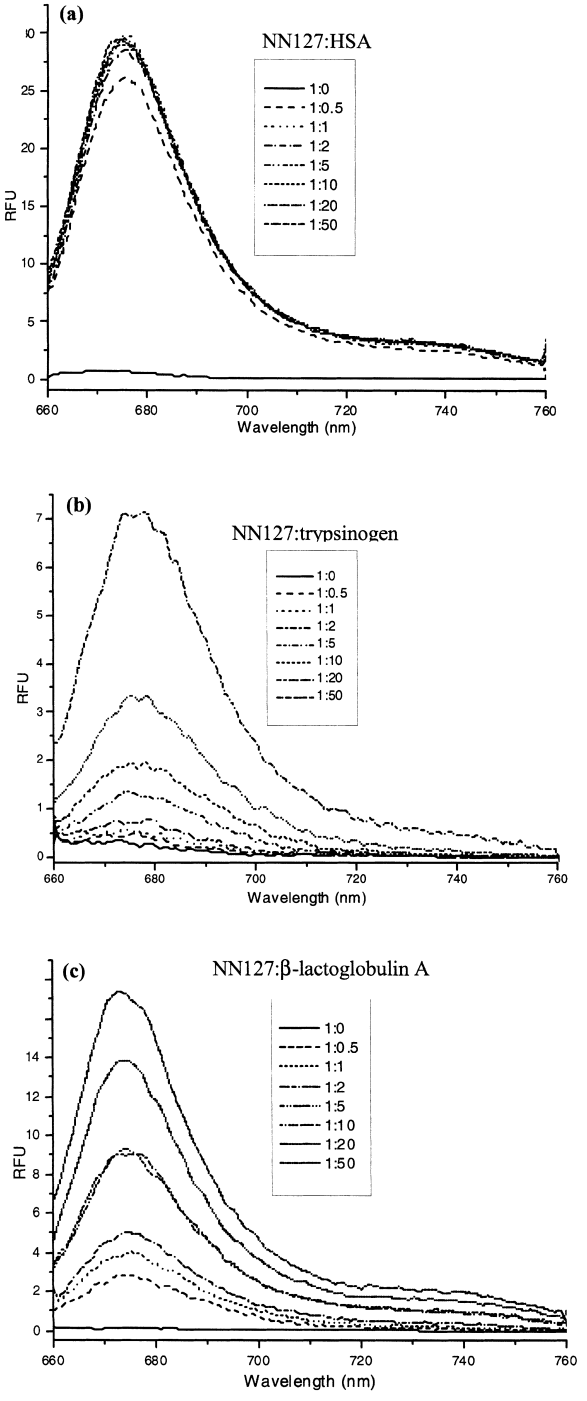

Fig. 4. Fluorescence emission spectra for the titration of 3.75×10^{-7} *M* NN127 in citric acid with varying amounts of protein: (a) HSA; (b) trypsinogen; and (c) β-lactoglobulin A. Excitation at 650 nm. Dye/protein ratios are specified in the figures.

method, the interaction between dye D and protein P, expressed as

$$D + P = DP \tag{1}$$

can be quantified according to the stability constant K_s given by

$$K_s = \frac{[DP]}{[D][P]} \tag{2}$$

The stability constant, in turn, can be determined from the fluorimetric titration data according to the equation

$$\frac{1}{F} = \frac{1}{k[D]} + \left(\frac{1}{k[D]K_s}\right)\frac{1}{[P]} \tag{3}$$

where F is the measured fluorescence emission intensity of the dye/protein mixture; k is a constant dependent upon the instrumentation and quantum efficiency of the process, and all other variables are as previously defined. Thus, a plot of $1/F$ versus $1/[P]$ should yield a straight line, with K_s equal to the intercept divided by the slope of the line. Stability constants on the order of 10^7 were typical of the interactions of NN127 with HSA in Tris and citric buffers, while stability constants on the order of 10^5 were typical of the interactions of SQ-3 with HSA in citric acid buffer. Stronger interactions are characteristic of the symmetric relative to the asymmetric squarylium dye with protein. It should be noted, however, that the relationship defined by Eq. (3) is strictly valid only for protein/dye complexes that are formed with a 1:1 stoichiometry (as was the case for all SQ-3–protein complexes and for NN127–HSA in Tris–HCl). Also, it should be noted that the $1/F$-values for the dye–protein mixtures were not corrected for fluorescence of free dye, although this posed no limitation in the case of NN127 since the fluorescence of this dye in its free, unbound state is negligible compared to its fluorescence when bound to protein. The native fluorescence of SQ-3 is lowest in citric acid buffer and so was assumed to contribute little error to the order-of-magnitude calculation of the SQ-3–HSA stability constant in this buffer.

From these studies, we conclude that neither hydrophobic nor electrostatic interactions are, to the exclusion of one another, entirely responsible for the formation of noncovalent protein–dye complexes, but rather, some combination of these forces are indicated. Electrostatic interactions would be least favorable for trypsinogen with SQ-3 in boric acid buffer, since both the protein and dye would be negatively charged at this pH. Indeed, we saw no linear correlation between $1/F$ and $1/[P]$ in the stability constant study for this combination, indicating poor interaction. Electrostatic interactions would likewise be unfavorable for HSA and β-lactoglobulin A with SQ-3 in the boric acid and Tris–HCl buffers, since both proteins and dye would be negatively charged under these solution conditions as well. Although β-lactoglobulin A showed limited interaction with SQ-3 in these buffers (as indicated by poor fluorescence enhancements and poor correlation between $1/F$ and $1/[P]$), HSA (and BSA) showed strong interaction with SQ-3 in all buffers studied. However, the greatest enhancement in fluorescence for HSA with SQ-3 was observed in citric acid buffer. Although the pK_a values of the dyes are not known, it is likely under these solution conditions that SQ-3 would be either neutral or positively charged (depending upon the protonation of the nitrogen atom off of the phenyl ring adjacent to the squarylium ring—see Fig. 2), and the protein would be positively charged (buffer $pH < pI$ of HSA, BSA). This clearly indicates that forces other than electrostatic interactions must play some significant role in albumin–SQ-3 complex formation. Most likely, electrostatic interactions (or repulsions) between the protein and one site on the dye molecule can occur simultaneously with hydrophobic or intercalation effects at another site.

Similar arguments can be made regarding electrostatic versus hydrophobic interactions between NN127 and the various proteins studied herein. NN127 likely demonstrates no net charge in the Tris and boric acid buffers, and no net charge or a positive charge in citric buffer, depending upon the extent of protonation of the heterocyclic nitrogen atom bonded to "R_2" and the originally deprotonated oxygen atom on the squarylium ring (Fig. 1). Interestingly, NN127 demonstrated strong interactions with each protein regardless of electrostatic considerations (albeit the worst correlation between protein concentration and fluorescence enhancement was observed for HSA in citric and boric acid buffers). Previous work by Patonay and co-workers concluded that hydrophobic forces between NN127 and BSA were the primary mode of dye–protein interaction [23], and that only one strong binding site on BSA exists for NN127 [24]. If parallels can be drawn between BSA and HSA binding, then the current work supports the idea of hydrophobic rather than electrostatic forces predominating for this dye–protein couple, although the current work indicates that more than one dye molecule can be bound per HSA molecule under acidic and basic pH conditions.

3.6. Capillary electrophoresis of squarylium dye–protein mixtures

In order to better assess the utility of SQ-3 and NN-127 as possible fluorescent labels for protein analytes, we attempted CE–LIF separations of various protein–dye mixtures. A single peak was observed in electropherograms of SQ-3/HSA mixtures (data not shown), and although the size of this peak increased as protein concentration increased, there was no change in its migration time as one might expect, since the protein–dye complex should migrate more slowly than the dye alone. We were also unable to resolve excess, unbound SQ-3 dye from the SQ-3–HSA complex.

Fig. 5 shows a series of electropherograms recorded for NN127 with various concentrations of HSA. Because of the very low fluorescence of NN127 in aqueous media, virtually no signal is detectable in the electropherogram for dye alone. However, a single peak near 8 min is seen for NN127–HSA mixtures using a boric acid separation buffer. This buffer was chosen in an attempt to minimize interactions between the protein and capillary wall, although the low peak efficiencies in Fig. 5 indicate that some interaction is still occurring. Analogous to the results obtained for SQ-3, the size of the NN127–protein peak increases as the concentration of HSA is increased. However, unlike SQ-3, it is not necessary to resolve excess, unbound NN127 from the NN127–HSA complex, since the

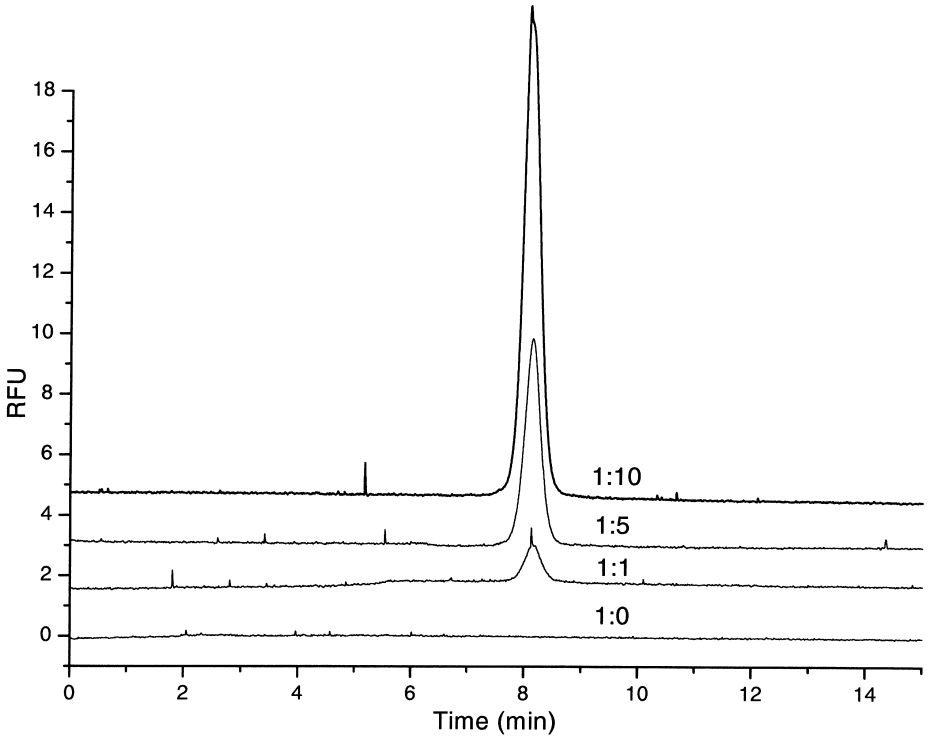

Fig. 5. Electropherograms of 1.5×10^{-7} *M* NN127 with varying amounts of HSA added. Dye/protein ratios are specified in the figure. CE–LIF conditions were as follows: 10 p.s.i. s injection; 15.0 kV separation voltage; 50 cm×50 μm I.D. uncoated fused-silica capillary (45.4 cm effective length); 25 m*M* boric acid running buffer (pH 11.5); 25 °C; excitation with 650-nm diode laser. Electropherograms are offset vertically for clarity of presentation.

former is not fluorescent and so will not interfere with the desired signal from the protein–dye complex.

4. Conclusions

Both symmetric and asymmetric squarylium dyes NN127 and SQ-3 show enhancement of fluorescence upon noncovalent interaction with protein. Although NN127 interacts strongly with HSA, β-lactoglobulin A and trypsinogen, SQ-3 shows markedly greater interactions with HSA and BSA. The very low fluorescence of free NN127 makes it an ideal candidate for on-column labeling of proteins in CE–LIF, and this work is being continued in our laboratory. However, the use of SQ-3 as an on-column labeling reagent is likely only feasible under acidic conditions, when the fluorescence of the free dye is somewhat quenched relative to its intensity under neutral or basic pH conditions. Further refinement of CE separation conditions to allow the resolution of excess, unbound dye from dye–protein complex is also warranted. This work, too, with SQ-3 and NN127 is being continued in our laboratory.

5. Nomenclature

BSA	bovine serum albumin
CE	capillary electrophoresis
HSA	human serum albumin
LIF	laser-induced fluorescence

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