Asymmetric Mono- and Bis-Squarylium Dyes as Pre-Column and On-Column Labels for Protein Analysis by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Abstract

Labeling proteins with fluorescent dyes offers a powerful tool for monitoring protein interactions in vitro and in vivo. In order for this tool to be effective, the nature of the dyes - absorbance and emission properties, solution stability, pH range, and mechanisms for protein interaction - must first be considered. Two new asymmetric, squarylium dyes, bis-SQHN-4d and SQHN-3c, were shown to be only weakly fluorescent in aqueous buffers in the absence of proteins. However, their spectra showed a dramatic increase in fluorescence intensity upon the addition of Human Serum Albumin (HSA) or Bovine Serum Albumin (BSA) as model proteins. The enhanced fluorescence properties, attributed to noncovalent binding, allowed the use of the new squarylium dyes as probes for the low-level detection of proteins in a mixture (including myoglobin (pI=7.16), transferrin (pI=5.9), and HSA (pI=4.8)), separated by Capillary Electrophoresis with Laser-Induced Fluorescence detection (CE-LIF). Because of the low background fluorescence of these probes, on-column labeling was feasible and led to simple and rapid protein detection. This labeling protocol offered greater sensitivity than the more conventional pre-column labeling protocol (with a 10-fold lower limit of detection for HSA with bis-SQHN-4d). A limit of detection for HSA (by CE-LIF with on-column labeling with bis-SQHN-4d) of 3.42 x 10^{-8} M indicates that this dye is well suited to the development of other protein assays.

Introduction

The implication of proteins in various biological functions and as markers of various states of disease provides the impetus for developing improved protein assays. Various techniques, including two-dimensional Polyacrylamide Gel Electrophoresis (PAGE) [1], High-Performance Liquid Chromatography (HPLC) [2,3], and mass spectrometry [4], have been successfully employed for protein analysis. Alternatively, fluorescence-based methods have been shown to be some of the most sensitive methods for protein analysis [5]. By combining the sensitivity and selectivity of Laser-Induced Fluorescence (LIF) detection with the high efficiency and speed of Capillary Electrophoresis (CE) separations of proteins, many have shown the utility of this system for protein analysis [6-13]. However, since just a small number of proteins possess natural fluorescence in a suitable spectral range (beyond UV), CE-LIF requires protein analytes to be labeled with a fluorescent tag or dye. Covalent labeling (e.g. via the ε-amino group of lysine, the α-amino group of the N-terminus, or the thiol group of a cysteine residue) often necessitates increased sample preparation, and may be difficult to achieve with very dilute samples. Instead, non covalent dye–protein interactions can be exploited to facilitate protein determination by CE-LIF. Non covalent labeling can occur by 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 (wavelength and/or intensity) of the fluorophore–protein complex relative to that of the free, uncomplexed fluorophore, which can be easily monitored by fluorimetric studies [8].

Squarylium dye molecules are polymethine dyes with a cyclobutene core in the middle of the pi-conjugation systems. These dyes and their derivatives are gaining significant interest in many fields such as solar-energy conversion [9], biochemical labeling [10], chromo/fluorogenic probes [11], and neutral molecule recognition [12]. The properties of squarylium dye molecules have been extensively studied by Liang et al. [13]. Typically, they exhibit a characteristically intense absorption band in the range of 640–850 nm [14]. Nakazumi et al. [15], showed that squarylium dyes experience enhanced fluorescence emission upon binding to BSA and HSA. By modifying the structure of the molecule, it is possible to design different kinds of squarylium dyes that will function in specific media or exhibit enhanced selectivity and sensitivity towards specific analytes. Two squarylium dyes designed with these goals in mind are shown in Figure 1. They exhibit different selectivity and sensitivity for protein labeling because of their different molecular structures.

The aim of the current work is to determine the effects of solution conditions on the nature of protein-dye interactions for bis-SQHN-4d and SQHN-3c dyes; the emission properties of the two novel squarylium dyes in their bound and Free states; and the efficiency of the separation of dye-labeled proteins. To this end, the absorbance and emission properties of the new dyes were determined in a variety of solvents and aqueous buffers at different pHs prior to titration of fixed quantities of the dyes with increasing concentrations of model proteins.

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Received April 07, 2012; Accepted May 26, 2012; Published June 01, 2012


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Human Serum Albumin (HSA) or Bovine Serum Albumin (BSA). An investigation of aggregation effects along with protein-dye incubation time and solution age was conducted. Finally, the utility of the new dyes bis-SQHN-4d and SQHN-3c as noncovalent protein probes was explored by designing and quantifying pre-column and on-column labeling protocols for CE-LIF.

Materials and Methods

Reagents, buffers, and sample solutions

3-((Z)-4-((2’-((E)-(1-Butyl-3,3-dimethylindolin-2-ylidene) methyl)-3,3,4,4’-tetraoxo-[1,1’-bi(cyclobutane)]-1,1’-dien-2-yl) methylene)quinolin-1(4H)-yl)propanoic acid (bis-SQHN-4d) and (Z)-4-((5-carboxy-1-ethyl-3,3-dimethyl-3H-indol-1-ium-2-yl) methylene)-2-(4-(diethylamino)-2-hydroxyphenyl)-3-oxy-cyclobut-1-enolate (SQHN-3c) dyes (Figure 1). were synthesized by modifying previously published procedures by Nakazumi and co-workers [16], [17]. The solid dyes are stable in the dark at room temperature for several months or longer. A 1.70 x 10⁻⁴ M stock solution of bis-SQHN-4d was prepared in dimethylformamide, DMF (Fisher), and subsequently diluted in HPLC-grade methanol (G.J. Chemical Company, Newark, NJ, USA) to the desired concentration (1.00 or 5.00 µM) just prior to use. A 1.30 x 10⁻⁵ M stock solution of SQHN-3c was prepared in dimethyl sulfoxide, DMSO (Fisher) and subsequently diluted to a working solution of 5.00 x 10⁻⁶ M in DMSO just prior to use. Dye stock solutions were stored in the dark at 4°C when not in use. Pre-column mixtures of protein with dye were stored overnight, when not in use, filled with water; and a separation voltage of 20 kV with 4 psi/sec injection was also employed. Between each run, the capillary was rinsed with purified water for two minutes, then running buffer for 2 min.

A BioRad Biofocus 3000 CE system (Hercules, CA, USA) with an LIF detector equipped with a 650 nm external diode laser (OZ Optics, Carp, Canada) and a 664 nm DF20 emission filter (Omega Optical, Brattleboro, VT, USA) was used for the electrophoretic separations of protein mixtures labeled on-column and pre-column with SQHN-3c. Unless otherwise noted, uncoated, fused silica capillaries (25 μm ID x 30.0 cm total length x 25.4 cm effective length) were used and were stored overnight, when not in use, filled with water; and a separation voltage of 20 kV with 4 psi/sec injection was also employed. Between each run, the capillary was rinsed with purified water for two minutes, then running buffer for 2 min.

Results and Discussion

Spectral properties of bis-SQHN-4d and SQHN-3c alone and with added protein

Spectral properties of the novel squarylium dyes (7.00 x 10⁻⁴ M bis-SQHN-4d or 7.50 x 10⁻⁴ M SQHN-3c) were determined under various solution conditions in order to assess their suitability as fluorescent probes for proteins. Absorbance maxima for bis-SQHN-4d were observed at 220, 414, 607, and 657 nm in methanol, and red shifts of all but the shortest wavelength absorbance band were observed for dye samples prepared in aqueous buffers. However, the absorbance maximum for SQHN-3c was slightly blue shifted relative to methanol (640 nm) in all aqueous buffers except under acidic conditions (citric acid buffer), for which a slight red shift was observed. A summary of the wavelengths of maximum absorbance and emission of the dyes, both
with and without added protein, is presented in Table 1. Significant differences between absorbance and emission wavelengths for both dyes in the presence of protein (for example, 52 nm in the case of bis-SQHN-4d with BSA in Tris) indicate good analytical discrimination between excitation and emission signals, thus allowing for more sensitive detection. The wavelengths of maximum absorbance and emission for SQHN-3c exceeded those for bis-SQHN-4d. These longer wavelengths can be beneficial because they allow the optical detection to be shifted away from the region of native protein absorbance bands and possible autofluorescence of biological matrices, therefore reducing interference and improving detection limits.

As noted above, there are four absorbance bands in methanol and in aqueous buffered solutions for the bis-SQHN-4d dye. The band around 420 nm was the strongest and is reported in Table 1. In Na2HPO4 buffer (pH 11.3, 25.0 mM), the fluorescence emission of bis-SQHN-4d alone was very weak with a maximum at 475 nm (and the dye color in Na2HPO4 buffer at pH 11.3 is light purple to the eye, but it is blue in other buffers). When an equimolar quantity of BSA was added, the fluorescence emission was very strong with a maximum at 620 nm. It should be noted that there was a substantial increase (98%) in the fluorescence emission for bis-SQHN-4d with BSA in Na2HPO4 buffer (pH 11.3, 25.0 mM) relative to the emission of the dye alone with no added protein in this same buffer system. No other increases of this magnitude were observed for other buffer systems, which indicated that the interaction of bis-SQHN-4d with BSA is significantly affected by the pH of the solution. At pH 11.3, both the dye and protein are predicted to be negatively charged, and so electrostatic repulsion would suggest an unfavorable condition for interaction at this pH. However, if the dye forms H-type (parallel) aggregates at low solution concentrations, as documented recently by Saito et al. [18], for another squarylium-based protein label, then it is possible that the higher solution pH instigates disaggregation, availing the dye for protein labeling. Even though electrostatic interactions are not likely favored under these solution conditions, other interactions (i.e. hydrophobic interactions, hydrogen bonds and Van der Waals forces) between dye-proteins will exist and could subsequently affect the noncovalent interactions between dye and proteins as reflected in changes in fluorescence, causing a more significant enhancement in fluorescence to be observed.

Unlike bis-SQHN-4d, there was no fluorescence enhancement observed for SQHN-3c upon addition of BSA in the phosphate buffer (pH 11.3, 25.0 mM); however, there was a fluorescence enhancement upon the addition of BSA in all other buffers, with the enhancement in the citric acid buffer (pH 3.1, 25.0 mM) being the most significant. In the citric acid buffer the fluorescence emission of the unbound SQHN-3c was the weakest; however, upon the addition of BSA the fluorescence emission was strong with a substantial 96% increase in signal relative to the emission of SQHN-3c alone. In the borate and Tris buffers there were 89% and 58% increases in signal, respectively. While these increases are not as significant as in the citric acid buffer, this does demonstrate that the binding of SQHN-3c with protein is not as pH dependent as the binding of bis-SQHN-4d with protein.

To further explore the nature of bis-SQHN-4d–BSA interactions, a fixed concentration (7.00 x 10^-7 M) of dye was titrated with increasing concentrations of BSA to achieve dye: protein ratios ranging from 1:0 to 1:30 in the Na2HPO4 buffer (pH 11.3). The enhancement of fluorescence increased with increasing concentration of added protein up to 2.10 x 10^-5 M added protein (a 30-fold molar excess of protein relative to dye). A correlation coefficient of r^2 = 0.991 affirmed a linear correlation between emission intensity and protein concentration over the range of 0–1.00 x 10^-5 M BSA, with a regression equation as follows: [fluorescence emission, RFU] = 4.02 x 10^5[concentration BSA, mol/L]. However, the addition of BSA to a fixed concentration of bis-SQHN-4d beyond a molar ratio of 1:14 (dye: protein) did not result in proportional increases in fluorescence. This indicates a saturation, whereby all available dye molecules are bound by protein and so the addition of still more protein could not significantly alter the emission properties of the dye.

### Stability of dye and dye–protein mixtures

Since the stability of most dyes in aqueous solution is poor [19], we investigated aging effects for both the dye solution and the dye-protein mixture in Na2HPO4 buffer on fluorescence for bis-SQHN-4d. The stability of bis-SQHN-4d (1.00 x 10^-6 M) and a bis-SQHN-4d (1.00 x 10^-6 M) – BSA (1.00 x 10^-5 M) mixture was analyzed by CE-LIF. The fluorescence of the CE-LIF peak observed for bis-SQHN-4d samples alone remained the same over a period of 48 hours, indicating a stable dye solution in Na2HPO4 buffer. However, the fluorescence of the peak attributable to the dye–BSA complex increased during the 48 hour period, but stabilized thereafter. There was a substantial peak area increase (63%) for bis-SQHN-4d with BSA in Na2HPO4 buffer (pH 11.3, 25.0 mM) after 24 hours relative to the total fluorescence response of the dye with BSA within one hour of mixing in this same buffer system. This might imply a slow rate of association for the bis squarylium dye with protein, whereas previous research confirmed a fast reaction rate [20]. Even though our own subsequent studies employing bis-SQHN-4d for on-column labeling reactions confirm a fast enough reaction to yield sufficient sensitivity for protein detection (see Section 3.4), it is possible that greater sensitivities could be achieved by allowing longer reaction times.

The effect of age of the dye–protein mixture on fluorescence in citrate buffer (pH 3.1, 25 mM) for SQHN-3c was also investigated. The stability of a SQHN-3c (7.00 x 10^-7 M) – BSA (7.00 x 10^-5 M) mixture was analyzed by fluorimetry. The fluorescence of the peak attributable to the complex in the resulting spectra rapidly degraded during the first 4 hours after mixing, after which time the signal remained very weak. There was a substantial decrease (30%) in fluorescence signal from time 0 to 10 minutes, and another substantial decrease (38%) from 10 minutes to 15 minutes. The most stable fluorescence was observed between 1-2 hours after mixing; however the strongest fluorescence signal was observed immediately after mixing. As such, SQHN-3c may

<table>
<thead>
<tr>
<th>Solvent or Buffer</th>
<th>7.00 x 10^-7 M Bis-SQHN-4d</th>
<th>1.00 x 10^-6 M BSA</th>
<th>1.00 x 10^-5 M BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ_max (nm)</td>
<td>λ_em (nm)</td>
<td>λ_max (nm)</td>
</tr>
<tr>
<td>methanol</td>
<td>414</td>
<td>460</td>
<td>432</td>
</tr>
<tr>
<td>citric acid</td>
<td>421</td>
<td>470</td>
<td>432</td>
</tr>
<tr>
<td>Tris (25.0 mM, pH 7.4)</td>
<td>418</td>
<td>470</td>
<td>432</td>
</tr>
<tr>
<td>boric acid</td>
<td>424</td>
<td>470</td>
<td>432</td>
</tr>
<tr>
<td>Na2HPO4 buffer</td>
<td>420</td>
<td>475</td>
<td>432</td>
</tr>
</tbody>
</table>

Table 1: Absorbance and fluorescence properties for bis-SQHN-4d and SQHN-3c dyes in various solvent systems.
be considered suitable for on-column or pre-column labeling of protein samples in cases when the subsequent separation can be completed in a very short period of time, although quantitation of protein based on CE-LIF peak height in such instances may not necessarily be attained through a simple linear calibration.

**CE-LIF analysis of protein-dye mixtures**

CE-LIF can be used analogously to fluorimetry to monitor changes in fluorescence emission of a dye upon titration with increasing concentrations of protein. In Figure 2, a linear increase in peak height for BSA–SQHN-3c complex (regression data reported in Table 2) is seen for increasing BSA concentration via on-column labeling. In these experiments, the citric acid running buffer contained a fixed concentration of SQHN-3c dye (5.00 x 10^{-6} M), while injected protein samples (prepared in water) ranged in concentration from 0.625 x 10^{-6} M to 10.0 x 10^{-6} M. Peak shape degraded as protein concentration samples (prepared in water) ranged in concentration from 0.625 x 10^{-6} M to 10.0 x 10^{-6} M. Peak shape degraded as protein concentration samples ranged in concentration from 0.625 x 10^{-6} M to 10.0 x 10^{-6} M. As can be seen, when the injected HSA concentration was held constant at 1.00 x 10^{-5} M, the peak area of the complex increased when the bis-SQHN-4d dye concentration increased from 1.0 x 10^{-5} M (Figure 3a) to 5.0 x 10^{-5} M (Figure 3c), but the peak area of the complex did not show a further increase (and, in fact, decreased) when the dye concentration was further increased to 1.0 x 10^{-4} M (Figure 3b). Thus, 5.0 x 10^{-5} M dye in running buffer was chosen for further on-column labeling studies to maximize dye–protein signal. Similarly, a study of optimal SQHN-3c dye concentration for on-column protein labeling revealed that 1.0 x 10^{-5} M SQHN-3c provided the best compromise between sensitivity towards added protein and minimal dye background signal (data not shown).

**On-column vs. pre-column labeling protocols**

In the previous section, on-column, noncovalent protein labeling was employed to establish optimal dye concentrations for analytical method development. For noncovalent protein labeling in CE-LIF, the commonly used modes are on-column and pre-column labeling [21-23]. The mode of pre-column labeling involves mixing the protein and fluorophore together prior to introduction of a discrete plug of the mixture at the inlet of a buffer-filled capillary. On-column labeling, however, involves the injection of a discrete plug of protein alone into a capillary filled with buffer that contains the dye. In this case, the dye concentration is essentially constant in the buffer throughout the capillary, and the equilibrium between the free dye and the protein-bound dye is readily established and maintained as the protein migrates through the capillary. On-column labeling provides a simpler protocol with no sample pretreatment required, no risk of sample contamination, and no sample dilution, and it typically offers an enhancement in sensitivity as demonstrated in previous work with related squarylium dyes [24].

A comparative study was undertaken with bis-SQHN-4d, to...
the dye is present everywhere in the buffer, but again, the quantum yield of the unbound dye is lower than that of the bound dye and so background fluorescence in the case of on-column labeling with bis-SQHN-4d is not likely to reduce the overall sensitivity of the method. It can be seen that the HSA–bis-SQHN-4d peak resulting from on-column labeling (Figure 4(c)) is larger (by 51% in terms of peak area) than the comparable peak from pre-column labeling (Figure 4(c)), which confirms the increased sensitivity achieved for on-column labeling in other work [24]. In addition, on-column labeling offers the advantage of reduced sample handling. The increase in migration time observed for the protein–dye complex peak in the on-column labeling experiment arises due to the presence of dye in the running buffer, which increases the ionic strength of the buffer, thus decreasing the overall electroosmotic mobility and concomitantly increasing the migration time.

A similar comparative study of noncovalent, pre-column vs. on-column labeling of BSA with SQHN-3c was conducted by CE-LIF, and the resulting electropherograms are shown in Figure 5. In this instance, 5.00 x 10^{-6} M SQHN-3c dye was used for on-column labeling of two different injected concentrations of BSA sample (Figure 5(a) 5.00 x 10^{-6} M and (b) 1.25 x 10^{-6} M), and pre-column labeling of 50.0 x 10^{-6} M BSA (Figure 5(c)) in a 25.0 mM citric acid buffer system (pH 3.4). Analogous to the studies with bis-SQHN-4d, on-column labeling with SQHN-3c resulted in a 54-fold increase in peak area (or an approximately 10-fold increase in peak height) relative to pre-column labeling, which confirms the increased sensitivity achieved for on-column labeling in other work [24]. In Figure 5 (c) it can be seen that a single peak for the protein–dye complex was observed. No peak was observed for free dye, presumably because an excess of protein in the sample, as described in other work [24]. In addition, on-column labeling offers the advantage of reduced sample handling. The increase in migration time observed for the protein–dye complex peak in the on-column labeling experiment with SQHN-3c likely arises due to the presence of dye in the running buffer, which increases the ionic strength of the buffer, thus decreasing the overall electroosmotic mobility and concomitantly increasing the migration time.

A quantitative comparison of sensitivity differences between on-column and pre-column labeling methods were conducted. In this work, the Limit of Detection (LOD) was calculated as 3s/m, where ‘s’ is the standard deviation of the baseline signal, found from 1/5 of the peak-to-peak noise of a blank signal, and m is the slope or sensitivity of a calibration curve constructed from peak height versus injected protein concentration. Protein concentration ranges were from 0.78 – 3.5 μM HSA (pre-column labeled with bis-SQHN-4d), 0.70 – 7.0 μM HSA (on-column labeled with bis-SQHN-4d), and 0.625 - 10 μM BSA (on-column SQHN-3c). The LODs for pre-column and on-column labeling of HSA with bis-SQHN-4d and on-column labeling of BSA with SQHN-3c are reported in Table 2. As can be seen, the LOD for pre-column labeling with bis-SQHN-4d is more than an order of magnitude lower than the comparable peak from pre-column labeling (Figure 4(c)), than the LOD for BSA obtained with SQHN-3c, indicating that the former provides an advantage of reduced sample handling. The increase in migration time observed for the protein–dye complex peak in the on-column labeling experiment arises due to the presence of dye in the running buffer, which increases the ionic strength of the buffer, thus decreasing the overall electroosmotic mobility and concomitantly increasing the migration time.
interacts more strongly with proteins, possibly through the kind of 'clamshell' interactions described in work by Patonay et al. [20].

**Separations of protein mixtures**

A mixture containing three model proteins with different pIs including myoglobin (pI=7.16), transferrin (pI=5.9), and HSA (pI=4.8) was labeled on-column with bis-SQHN-4d and fully resolved by CE-LIF, as shown in Figure 6. Trypsinogen was also labeled and resolved in subsequent experiments, although separation efficiency was significantly lower for this protein (data not shown). A thorough study of separation parameters was undertaken, including capillary dimensions, injection times, cartridge temperatures, separation voltage, buffer composition and ionic strength. Larger separation efficiencies were achieved for on-column labeling of a protein mixture employing a 25.0 mM phosphate separation buffer (pH 11.3) with 5.00 x 10^{-6} M bis-SQHN-4d (a) or 6.70 x 10^{-5} M (b) myoglobin (pI=7.16); II, 5.00 x 10^{-4} M (a) or 3.30 x 10^{-4} M (b) transferrin (pI=5.9); III, 5.00 x 10^{-5} M (a) or 4.00 x 10^{-6} M (b) HSA (pI=4.8).

Unfortunately, similar CE-LIF conditions did not yield successful protein separations with SQHN-3c as the labeling probe. The lower sensitivities achieved with this dye with on-column labeling protocols also bodes well for its promise as a protein probe. On-column labeling methods have the advantage of reduced sample handling (and thus lower risk of contamination or loss). Sample derivatization prior to injection typically necessitates larger sample sizes (to ensure proper mixing of reagents and a complete reaction) than does any given CE-LIF injection, and sample derivatization prior to injection often results in sample dilution, thus impinging on detection limits. However, on-column, noncovalent protein labeling as described herein obviates the need for sample derivatization prior to injection, which holds much promise for analytical method development for clinical, forensic, and environmental samples. By combining the high resolving power of CE with the sensitivity of LIF detection and the convenience of on-column labeling, applications for novel, asymmetric squarylium dyes as protein probes are expected to grow.

**Conclusions**

The spectral properties of two novel squarylium dyes, bis-SQHN-4d and SQHN-3c, were explored under a variety of solution conditions in order to determine the best conditions for noncovalent protein labeling and analysis by CE-LIF. Although these dyes are not water soluble, stable stock solutions can be prepared in organic solvents and subsequently diluted with aqueous buffers to the appropriate working concentration just prior to use. In particular, bis-SQHN-4d shows great potential as a noncovalent label for a wide range of proteins, and the enhanced detection sensitivity observed for this dye with on-column labeling protocols also bodes well for its promise as a protein probe. On-column labeling methods have the advantage of reduced sample handling (and thus lower risk of contamination or loss). Sample derivatization prior to injection typically necessitates larger sample sizes (to ensure proper mixing of reagents and a complete reaction) than does any given CE-LIF injection, and sample derivatization prior to injection often results in sample dilution, thus impinging on detection limits. However, on-column, noncovalent protein labeling as described herein obviates the need for sample derivatization prior to injection, which holds much promise for analytical method development for clinical, forensic, and environmental samples. By combining the high resolving power of CE with the sensitivity of LIF detection and the convenience of on-column labeling, applications for novel, asymmetric squarylium dyes as protein probes are expected to grow.

**Acknowledgements**

This work was supported, in part, by Wake Forest University and the National Science Foundation under grant no. 0809756.

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