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# Non-covalent labeling of human serum albumin with indocyanine green: a study by capillary electrophoresis with diode laser-induced fluorescence detection☆

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**Abstract**

Indocyanine green (ICG) is a negatively charged, water-soluble, tricarbocyanine dye used primarily for medical imaging. ICG is only weakly fluorescent in the near-infrared region in its free (unbound) state in dilute aqueous solution. However, when non-covalently bound to protein, its fluorescence is greatly enhanced, making it a candidate for diode laser-induced fluorescence (diode-LIF) detection of proteins in capillary electrophoresis (CE). This paper investigates the suitability of ICG as a fluorescent label for the separation and detection of human serum albumin (HSA) by CE with diode-LIF detection. Specifically, we have considered the separation conditions necessary to resolve free ICG from ICG–HSA complexes; the limits of detection for free and HSA-bound ICG; the stability of aqueous ICG and ICG–HSA solutions over time; and the stoichiometry of the ICG–HSA complex. 

*Keywords:* Indocyanine green; Human serum albumin

## 1. Introduction

Capillary electrophoresis (CE) has become a primary technique in the separation and quantitation of proteins. Typically, CE analysis of proteins is performed utilizing absorbance detection methods. However, laser induced fluorescence (LIF) detection has become increasingly more attractive due to its advantages of lower background, increased sensitivity and increased selectivity relative to UV absorbance detection. Inexpensive diode lasers are particularly attractive in this role, since proteins and other biological molecules are not natively fluorescent at the wavelengths associated with diode lasers and, so, background is significantly reduced. However, labeling of the analyte molecules with a near-infrared fluorescent dye is required for their detection.

Fluorescent labeling of analytes offers many advantages. Specifically, such labeling permits natively non-fluorescent analytes to be detected by LIF schemes, thus enhancing their limits of detection relative to absorbance detection by at least several orders of magnitude [1]. Fluorescent labels may be chosen that react selectively with an analyte(s) of interest, thus reducing possible interference from

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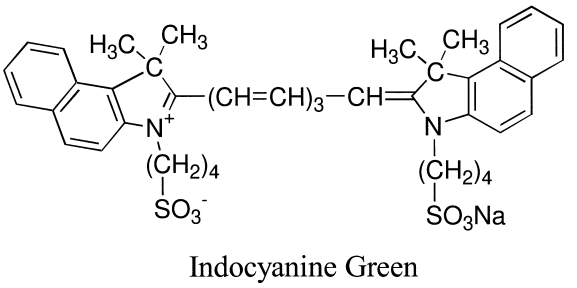
unwanted signals in the electropherogram [1]. Additionally, very careful control of labeling conditions may result in the generation of a single, fully tagged protein analyte, resulting in much sharper peaks with greatly improved efficiencies (and limits of detection) relative to the native analyte [2].

Fluorescent labeling is not without some disadvantages. Such derivatization necessitates increased sample preparation, and may be difficult to achieve with very dilute samples. Often, covalent labeling procedures require very specific control of solution pH, and so may not be readily achieved at the physiological pH of many biological samples. As well, it has been observed that fluorescent derivatization of an analyte prior to separation may result in peak broadening and loss of separation efficiency. In a study involving green fluorescent protein, Craig and Dovichi [3] recently presented compelling evidence that such band broadening is due to the formation and partial resolution of multiple, differently labeled species. Finally, the cost of LIF detection systems with visible or UV gas lasers may be prohibitive for their routine inclusion in an analytical lab. For all of these reasons, we propose to use a near-infrared dye, indocyanine green (ICG), which can be excited by an inexpensive diode laser, and which will non-covalently bind to proteins, requiring no special sample preparation and, thus, lends itself to post-separation labeling schemes if desired.

The applicability of diode lasers to LIF detection in CE was first demonstrated in 1992 by Higashijima et al. [4], who used a diode laser emitting at 670 nm to detect as few as 10 pmol of amino acids derivatized with a newly synthesized thiazine chromophore with a succinimidyl ester group for covalent linkage. Mank and Yeung [5] used a similar diode laser to detect covalently labeled amino acids and peptides in a biological sample. Chen et al. [6] were the first to describe the use of Cy5, one of the few commercially available long wavelength dyes, for labeling an oligonucleotide $M_{13}$ primer for CE with diode-LIF detection. Many studies with respect to the spectroscopic properties and separation of near-infrared, tricarbocyanine dyes have been published by Soper and coworkers [7–10], suggesting the possible utility of such non-covalent dyes in CE with diode-LIF detection.

One such dye ICG is a negatively charged, polymethine dye with a molar absorptivity of 180 000 [11]. The molecular structure of this dye is shown in Fig. 1. ICG has been employed primarily in the medical imaging field as a diagnostic for blood-volume determinations, ophthalmological examination, and cardiac and hepatic function tests [12–17]. It is non-toxic to the body and, therefore, can be administered in relatively large doses to patients without any harm. ICG has an absorption maximum at 780 nm, but when non-covalently bound to a protein, the maximum shifts slightly to longer wavelengths [18]. In its unbound state, ICG is only weakly fluorescent (with a fluorescence quantum yield of only 0.01 in aqueous solution [7]), but upon binding to protein, its fluorescence intensity is enhanced [11].




Indocyanine Green

Fig. 1. Structural formula of indocyanine green (MW, 775 g $mol^{-1}$).

There are some reports of assays for ICG in serum or plasma in the literature. Rappaport and Thiessen [19] developed a high-performance liquid chromatographic (HPLC) assay for ICG. They found that both ICG and its degradation products absorbed light near 780 nm and, so, previously employed spectrophotometric assays [20] may have overestimated ICG concentrations due to their inability to distinguish between ICG and its degradation products. However, Svensson et al. [21] found that both HPLC and spectrophotometric assays of ICG yielded essentially identical estimates of ICG concentration in plasma. Later, Hollins et al. [22] reported on a spectrofluorometric assay for ICG in plasma that could accurately report ICG concentrations since the degradation products are not fluorescent at the wavelength of maximum emission of native ICG. Sauda et al. [11] attempted to determine protein in human serum by labeling with ICG followed by HPLC separation.

Unfortunately, dissociation of the dye–protein complex was found to take place during sample separation in the column, due to the greater affinity of the dye for the column relative to the protein analyte.

In this paper, we will investigate the suitability of ICG as a fluorescent label for the separation and detection of human serum albumin (HSA) protein by CE with diode-LIF detection. We have considered the separation conditions necessary to resolve free ICG from ICG–albumin complexes, and have determined the limits of detection for free ICG, free HSA and ICG–HSA complexes by this method. The stability of aqueous ICG versus ICG–HSA solutions as a function of solution age was studied. Finally, studies of the stoichiometry of the ICG–HSA complex were performed in order to determine the stoichiometry of the most predominant form of ICG–HSA complex present in aqueous solution.

## 2. Experimental

### 2.1. Reagents

Human serum albumin (≥96%), was used as received from Sigma (St. Louis, MO, USA). It was dissolved in 25 m*M* borate buffer, pH 8.9. The fluorescent dye, ICG (~95%), was purchased from Sigma and was used as received. It was dissolved in 25 m*M* borate buffer. Stock dye solutions were prepared daily and diluted to $5.26\times10^{-6}$ *M* concentration unless otherwise noted. They were stored in the dark at 10°C until used. The borate buffer was made by dissolving boric acid (99.9%) from J.T. Baker (Phillipsburg, NJ, USA) in Milli-Q distilled and deionized water. The pH was adjusted with 0.1 *M* NaOH, made by dissolving NaOH (98.3%) from Fisher Scientific (Norcross, GA, USA) in Milli-Q water. Before its use, the buffer was filtered using a 0.20-μm filter.

### 2.2. Instrument

The experiments were carried out using a Bio-Rad (Hercules, CA, USA) BioFocus 3000 CE system with LIF detection. Samples were pressure injected at 10 p.s.i.·s (1 p.s.i.=6894.76 Pa) unless otherwise noted. They were excited with the 780 nm line from a red diode laser from Oz Optics (Carp, Canada) and the emission was detected at 820 nm. An 820DF20 emission filter from Omega Optical (Brattleboro, VT, USA) was used. Unless otherwise stated, 50 cm×50 μm I.D. uncoated fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) were used. The effective length from the inlet to the detector was 45.4 cm. Before each run, the capillary was sequentially rinsed with 0.1 *M* NaOH, water, and buffer. The capillary was kept at 15–20°C during the run.

### 2.3. Procedure

The non-covalent labeling was carried out by taking appropriate portions of the dye and protein and adding them together with buffer in a 500-μl microcentrifuge tube. Samples were vortex-mixed for 20 s, followed by 1 min of degassing by centrifugation. Before use, they were further diluted with buffer, as necessary.

## 3. Results and discussion

### 3.1. Stability of ICG in aqueous solution

The use of non-covalent, tricarbocyanine dyes for protein labeling in CE with diode-LIF detection offers several advantages, as discussed previously in this article. However, the stability of these dyes in aqueous solution is often poor, and so, we wanted to investigate the specific case of the effect of solution age on ICG fluorescence in aqueous solution, both in the presence and absence of HSA. Barbier and De Weerdt [18] studied ICG by paper and thin-layer chromatography and by infrared spectroscopy, and found that an additional ‘fast running’ fraction was observed as few as 5 min after preparation of an aqueous solution of ICG. These authors attributed this new fraction to the formation of a new ring structure via the $SO_3^-$ group of ICG, accompanied by the saturation of the unstable C=N group, the loss of the $SO_3^-$ function, and the creation of C=O character. Rappaport and Thiessen [19] argued that discrepancies between HPLC and spectrophotometric determinations of aqueous ICG were due to the presence of

ICG degradation products that absorbed light near 780 nm.

Our stability studies of ICG by CE with diode-LIF detection did not reveal the presence of such degradation products. These results do not necessarily indicate that degradation products are absent, rather, they imply that if such products are present, they are not fluorescent at the wavelength at which we are monitoring ICG fluorescence (820 nm). Indeed, studies of the intensity of fluorescence emission from solutions of ICG and ICG–HSA prepared in 25 m*M* borate buffer (pH 8.9) clearly show that the solutions degrade fairly rapidly with time (see Fig. 2). Just 24 h after preparation of solutions, areas decreased by 74 and 41% for free ICG and ICG–HSA complex, respectively. Hence, the presence of protein in a 1:5 molar ratio with ICG did somewhat enhance the stability of the dye in aqueous solution, although significant degradation still occurred. Several other reports [11,14,18] have stated that ICG is greatly stabilized in the presence of protein, albeit at much greater levels of protein relative to the dye. Based on our results, we were careful to prepare diluted ICG and ICG–HSA solutions just prior to analysis by CE with diode-LIF detection. Additionally, we conducted all separations with the capillary and sample carousel thermostatted at 20°C, to minimize the effects of sample degradation.

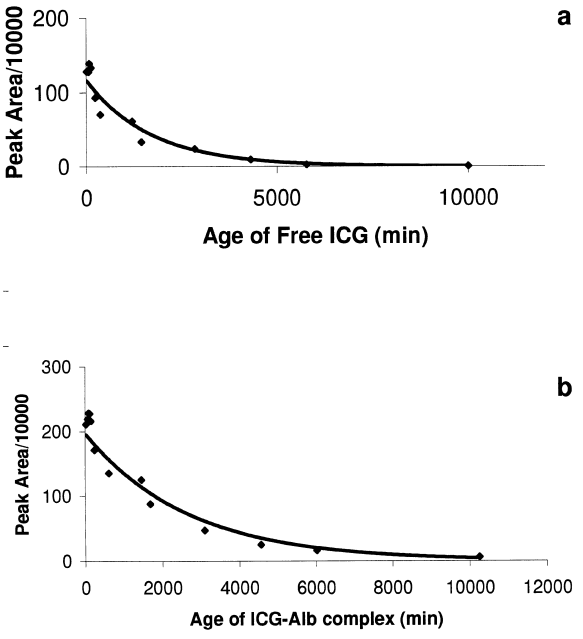



Fig. 2. Effect of solution aging on the peak area of (a) free ICG and (b) an ICG–HSA complex. The ICG concentration was $2.10\times10^{-6}$ *M* in both cases. The HSA concentration in (b) was $4.2\times10^{-7}$ *M*. Circles represent experimental points, and solid lines represent exponential fits of the data with $R^2$ factors of 0.98 and 0.97 for free ICG and ICG–HSA, respectively.

### *3.2. Optimization of ICG–HSA separation conditions*

Protein separations are most commonly performed using coated capillaries due to adsorption effects between the untreated silica capillary wall and the protein [8]. However, from an experimental point of view, it is often more convenient to work with standard, uncoated fused-silica capillaries. This obviates the need for carrying out sometimes labor- and time-intensive capillary coating procedures. By adjusting the pH of the running buffer to a pH greater than the p*I* of the proteins being determined, the charge on the capillary wall is altered so that protein–capillary wall interactions are greatly reduced. Thus, it is necessary to choose a buffer system that can provide a relatively high pH, such as borate or phosphate, to render both the capillary wall and the protein negatively charged.

Various buffer systems were employed in these studies in order to determine the one that was best suited to free solution separations of ICG and ICG–HSA complexes in uncoated capillaries. It was found that the sharpest peaks for ICG could be obtained using a 25-m*M* boric acid buffer. Fig. 3 shows electropherograms of a mixture of ICG with HSA in 25 m*M* boric acid buffer in a pH range of 8.2 to 12.0. At a pH of 8.9, two peaks are most clearly evident, but they are not yet completely resolved. The first of these represents uncomplexed ICG, while the second is the ICG–HSA complex, as confirmed by solution-spiking experiments.

It was our goal to devise separation conditions that would permit complete resolution of free ICG from ICG complexed with HSA. Such a separation would permit excess quantities of the dye to be used (if necessary) without the need for sample clean-up by way of solid phase extraction to remove excess dye prior to CE analysis. Such clean-up was carried out in other similar work [23,24] and necessarily added

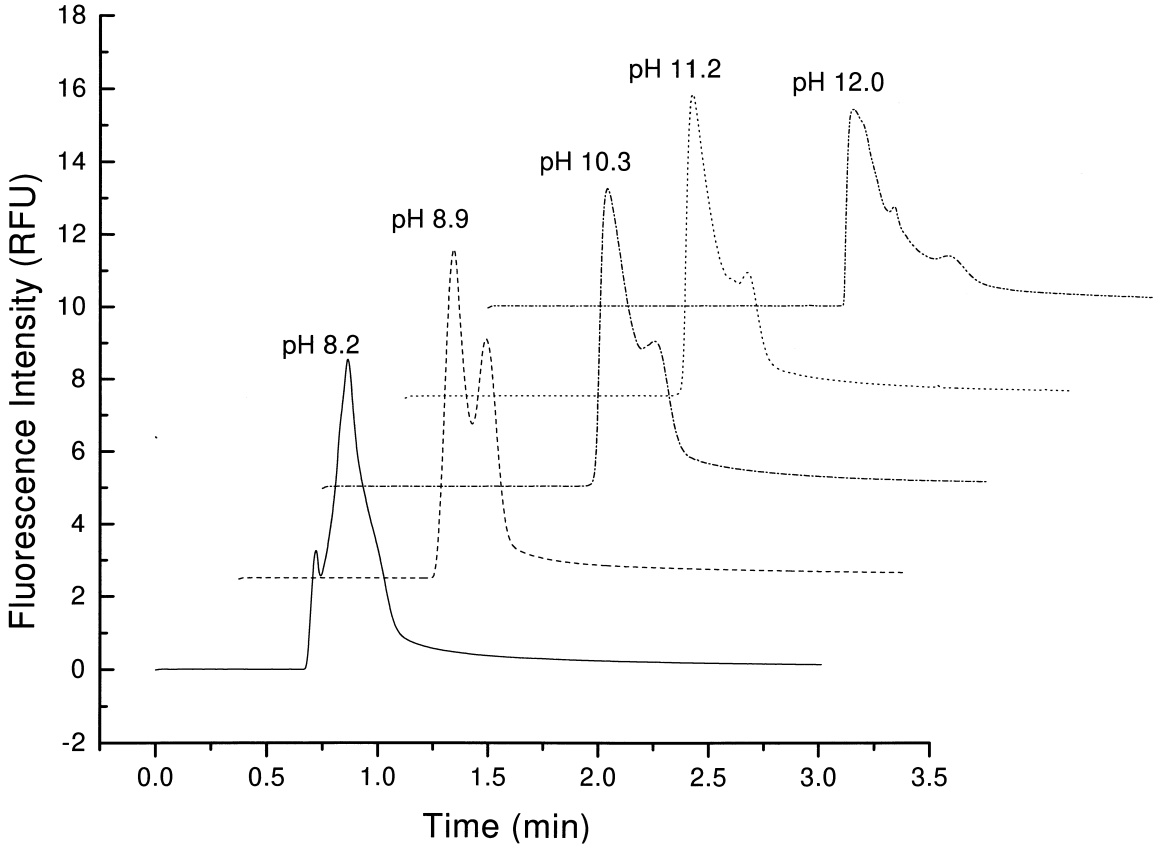



Fig. 3. pH dependence of the separation of a mixture of $5.26\times10^{-6}$ *M* ICG and $1.06\times10^{-6}$ *M* HSA in 25 m*M* boric acid buffer. Other separation conditions were as follows: 30.0 and 25.4 cm total and effective capillary lengths, respectively; 50 μm I.D.; 20 kV separation voltage; 10 p.s.i.·s pressure injection; 15 and 10°C capillary and sample carousel temperatures, respectively. Electropherograms are offset for clarity.

to total analysis time, the risk of contamination and the risk of sample loss. To this end, we increased the length of the separation capillary and decreased the separation voltage to obtain the near baseline-resolution shown in Fig. 4. These separation conditions were used in all subsequent work.

### *3.3. Detection limit studies of free and bound ICG*

To determine the free ICG detection limit by way of CE with diode-LIF detection, a stock solution of $1.94\times10^{-4}$ *M* ICG was made by dissolving 1.5 mg of ICG in 10 ml of 25 m*M* boric acid buffer, pH 8.9. From this stock solution, serial dilutions in the range of $3.88\times10^{-6}$ to $1.55\times10^{-7}$ *M* were prepared and separated according to the conditions described in Section 3.2, until peaks were no longer discernible above the baseline noise. The same technique was applied to ascertain a detection limit for free ICG in absorbance mode at 220 nm; however, samples were diluted in distilled, deionized water instead of buffer. The limits of detection for free ICG were found to be $3.88\times10^{-7}$ and $8.26\times10^{-7}$ *M* by way of diode-LIF and absorbance detection, respectively. This relatively insignificant difference in the observed limits of detection is presumably due to the relatively low fluorescence quantum yield of ICG in aqueous solution [7] compared to its relatively high molar absorptivity [11].

A stock solution of a 1:1 molar mixture of ICG–HSA ($1.00\times10^{-6}$ *M* in each) was prepared and the method described above for serial dilution followed

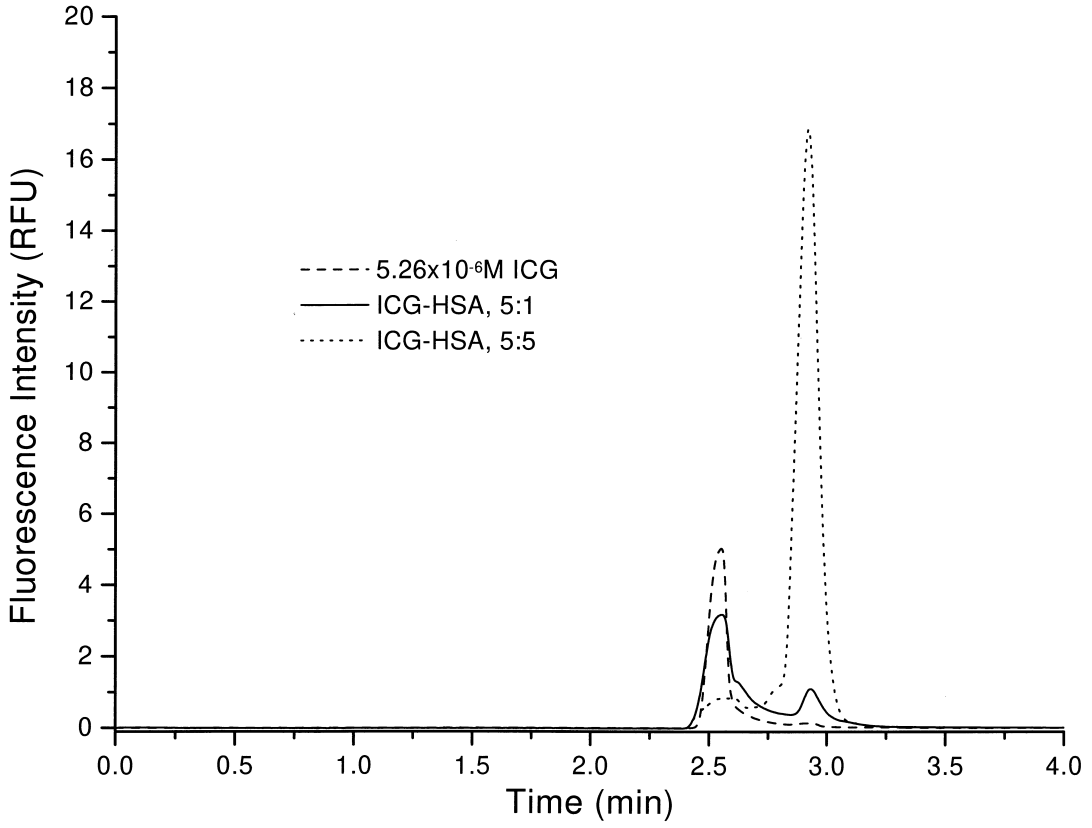



Fig. 4. Electropherograms of free ICG and ICG–HSA mixtures. Sample concentrations are as indicated in the figure. Run buffer was 25 m*M* boric acid, pH 8.9. The capillary was 50 μm I.D., 50.0 cm total length and 45.4 cm effective length. The temperature of the capillary was 25°C. Sample temperature was maintained at 20°C. Injection was at 10 p.s.i.·s. The separation voltage was 20 kV.

by CE analysis was used to obtain a detection limit of $1.4\times10^{-9}$ *M* for ICG (and for HSA) in the dye–protein mixture. Fig. 5 shows electropherograms of the ICG–HSA complex peak at, above, and below the estimated limit of detection. This dramatic increase in the fluorescence of ICG when bound to protein, yielding a limit of detection two orders of magnitude greater than that for uncomplexed ICG, confirms similar reports of enhanced fluorescence upon complexation found in the literature [8,11]. Such enhanced fluorescence indicates that ICG may, indeed, be a suitable non-covalent label for protein detection in CE with diode-LIF detection.

### *3.4. Stoichiometry and binding*

It has been suggested that tricarbocyanine dyes associate with the hydrophobic cavities of protein molecules [11], or that they may interact with charged regions on the protein surfaces [8]. However, it is difficult to determine the stoichiometry of a dye–protein complex, since it is likely that many different complexes are, in fact, formed in a simple mixture of the dye with protein. It is this very formation (and subsequent partial resolution) of multiple, differently labeled products that is suspected of being responsible for the observation of rather broad, ill-defined complex peaks, as seen in the current work.

In order to derive some information about the composition of the ICG–HSA complex(es) occurring in the present work, we recorded electropherograms for ICG–HSA mixtures prepared at a variety of different molar ratios. A series of such electropherograms is presented in Fig. 6. In these experiments, all solutions contained a fixed con-

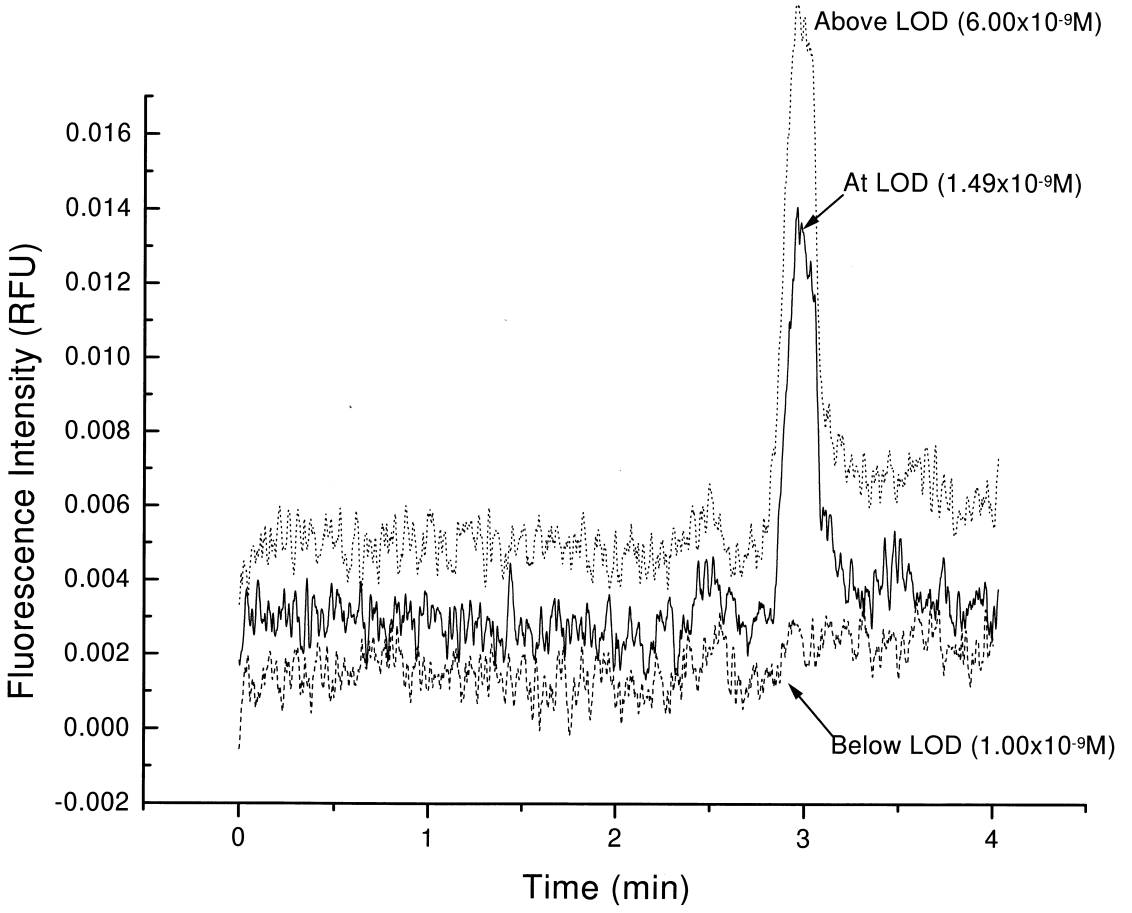



Fig. 5. Limit of detection of a 1:1 molar mixture of ICG–HSA by CE with diode-LIF detection. Separation conditions were as described in Fig. 4, except that injection was at 30 p.s.i.·s. Electropherograms are offset for clarity.

centration of ICG ($1.55\times10^{-5}$ *M*). However, the HSA content was increased from an initial concentration of $3.16\times10^{-6}$ *M* (representing essentially a 1:5 protein-to-dye molar ratio) to a final concentration of $3.16\times10^{-5}$ *M* (representing essentially a 2:1 protein-to-dye molar ratio). Initially, with a five-fold excess of dye present, both a free (uncomplexed) ICG peak and an ICG–HSA complex peak were visible in the electropherogram. However, as the concentration of protein relative to dye increased, the free ICG peak essentially disappeared from the electropherogram. A concomitant increase in the size of the ICG–HSA peak was observed.

A saturable binding curve was thus prepared for ICG–HSA, as seen in Fig. 7. Here, the change in fluorescence observed upon increasing the concentration of HSA in a solution mixture (from 0 to $2.6\times10^{-5}$ *M*) relative to a fixed concentration of ICG ($5.26\times10^{-6}$ *M*) is plotted, and shows a distinct plateau above an added HSA concentration of about $9\times10^{-6}$ *M*. Beyond this concentration, no significant increase in fluorescence was observed, indicating that all ICG present had been complexed with protein, and so, the addition of still more HSA had no effect on the product(s) formed.

Using the data obtained in Fig. 7, we first attempted to determine the stoichiometry of the complex by way of the method described by Beltran-Porter et al. [25]. This method, however, necessarily assumes that a single complex is formed. Since we were unable to determine a fixed stoichiometry by this method, we confirmed that, indeed, multiple products must be formed with different stoichiometries, and it is this formation of multiple products that undoubtedly

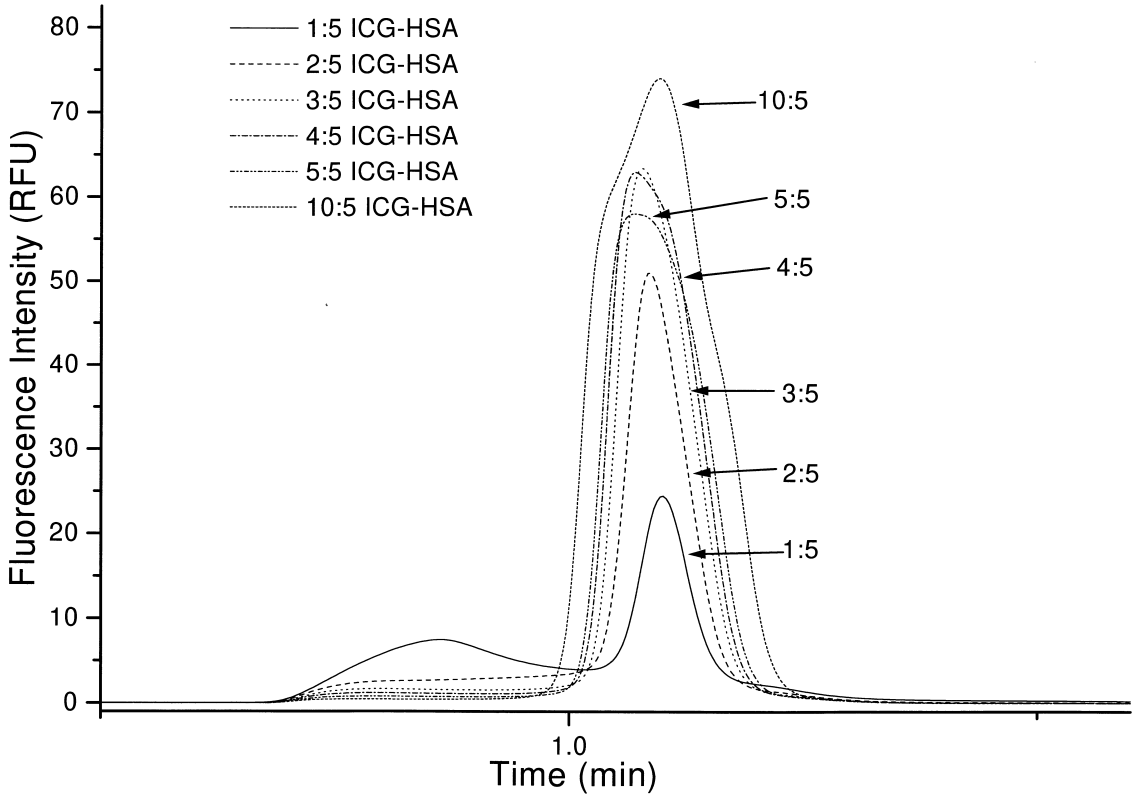



Fig. 6. Electropherograms obtained by increasing the concentration of HSA added to a fixed concentration of ICG ($1.55\times10^{-5}$ *M*). Initial and final albumin concentrations were $3.16\times10^{-6}$ and $3.16\times10^{-5}$ *M*, respectively. Separation conditions were as described in Fig. 4.

reduces the efficiency of our separations of free ICG from ICG–HSA complex.

Instead, we constructed a Job's plot (see Fig. 8) to determine the predominant form of the equilibrium complex formed according to the equation: $x$ ICG + $y$ HSA $\Leftrightarrow$ $(ICG)_x(HSA)_y$. In these experiments, we maintained a fixed total solution concentration of $5.26\times10^{-4}$ *M* (combined HSA and ICG concentration), but varied the mole fraction of ICG present. The peak of the Job's plot corresponds to the composition of the most predominant form of the complex present in a mixture [26]. In our case, this corresponded to an ICG mole fraction of 0.6, or a stoichiometry of $(ICG)_{1.5}(HSA)_1$. This stoichiometry differs from a previously published stoichiometry for ICG–HSA, which was three molecules of ICG per 100 molecules of HSA [11]. It is clear from our work, however, that the proposed 1.5: 1 stoichiometry is simply an 'average' or most probable composition and that, in fact, multiple ICG–HSA complexes are formed.

## 4. Conclusions

Indocyanine green (ICG) is a suitable non-covalent label for fluorescently tagging HSA to allow for its separation and detection by CE with diode-LIF detection. In order to be able to resolve excess free dye from the dye–protein complex, we determined that a simple 25 m*M* boric acid buffer (pH 8.9) was most suitable, along with a separation voltage of 20 kV, and total and effective capillary lengths of 50.0 and 45.4 cm, respectively. It is believed that higher efficiency separations with greater resolution could be achieved by the judicious use of surfactants, and this work is presently underway in our laboratory.

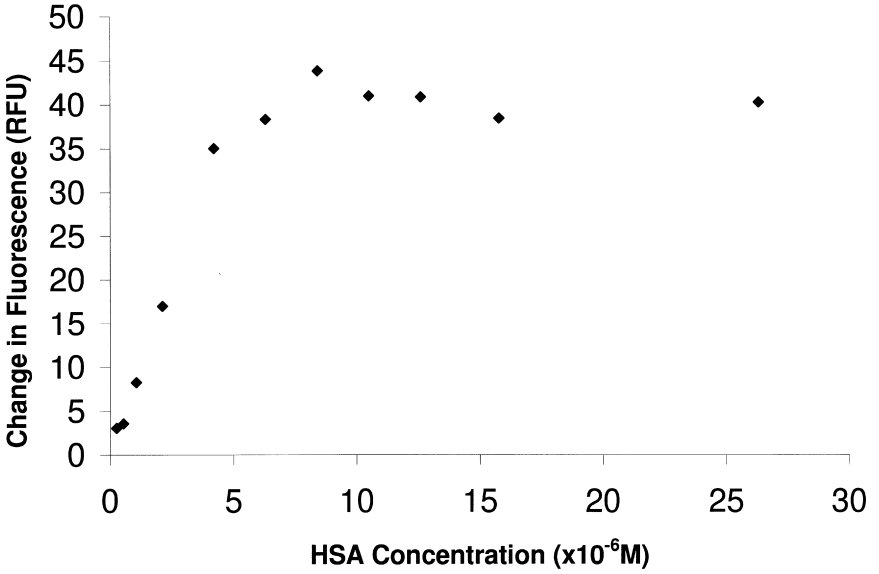



Fig. 7. Saturable binding curve: change in fluorescence (corrected for free ICG fluorescence) of the ICG–HSA complex as a function of added HSA concentration. The concentration of ICG was $5.26\times10^{-6}$ *M* in all cases.

Although ICG is quite soluble in water, its aqueous solutions are not very stable, and a 74% decrease in fluorescence intensity for a $2.1\times10^{-6}$-*M* solution was found to occur over a period of approximately 24 h. The presence of $4.2\times10^{-7}$ *M* HSA had some stabilizing effect on the ICG solution. Limits of detection for freshly prepared ICG solutions were found to be $3.88\times10^{-7}$ and $1.49\times10^{-9}$ *M* in the

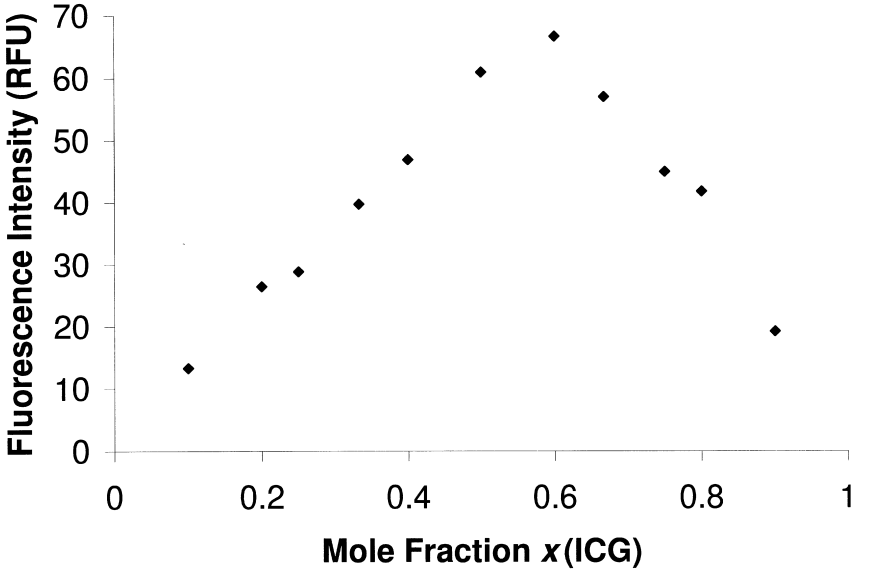



Fig. 8. Job's plot for the determination of ICG–HSA stoichiometry. A fixed, total solution concentration of $5.26\times10^{-4}$ *M* was used, with varying relative amounts of ICG and HSA.

absence and presence, respectively, of $4.2\times10^{-7}$ *M* HSA. This two-orders-of-magnitude increase in the detectability of ICG upon complexation with HSA indicates the potential for this dye to be used as a non-covalent fluorescent label in the determination of other proteins by CE with diode-LIF detection. Indeed, there are many references in the literature to ICG's preferential association with globulin rather than albumin-type proteins [13,27–29] and so, it seems reasonable to assume that this method could be successfully applied to mixtures of proteins.

From our binding and stoichiometry studies, it is clear that a fixed amount of ICG may be 'saturated' upon the addition of HSA beyond roughly a 1:1 molar ratio. Further addition of protein beyond this saturated molar ratio leads to no significant increase in fluorescence signal. Hence, it appears that ICG should, indeed, be useful for labeling and determining low levels of proteins. Furthermore, the speed and ease with which the ICG–HSA complex is formed corroborates the potential of this dye for on-column or postcolumn labeling schemes. Although multiple products are believed to be formed when ICG is mixed with HSA, the stoichiometry of the most predominant form of the complex, as determined from a Job's plot analysis, was found to be $(ICG)_{1.5}(HSA)_1$.

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