Near infrared dyes in biotechnology and bioanalysis: overview and future trends

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Introduction

Fluorescence detection is one of the most popular detection methods used in bioanalytical analysis. Fluorescence detection methods offer the advantages of high sensitivity combined with selectivity. Generally, the chromophore is attached to the species of interest and acts as a reporter molecule. Most fluorescence detection schemes involve the use of visible fluorophores, fluorescein being one of the most common. A problem often encountered with the used of fluorescence detection in the visible region of the spectrum is sample autofluorescence. Many compounds have intrinsic fluorescence in this region. Background fluorescence is present throughout the visible and UV region of the spectrum (fig 1). Consequently, background noise can be very high and, as a result, the overall sensitivity of the measurement is decreased.

Very few molecules exhibit intrinsic fluorescence in the near infrared region of the spectrum (650-1100 nm). As a result, the background noise associated with fluorescence detection in the visible region is nearly eliminated. Consequently, the near infrared region offers the potential for significant improvements in sensitivity, especially in situations where matrix autofluorescence is a concern. Near infrared fluorescence detection methods are therefor well suited to bioanalytical applications. Furthermore, light scatter, another source of interference, is decreased in the near infrared region due to its $1/\lambda^4$ dependence. Detection at 820 nm offers a

Biomedical Nanotechnology Architectures and Applications, Darryl J. Bornhop, et al., Editors,
Proceedings of SPIE Vol. 4626 (2002) © 2002 SPIE · 1605-7422/02/$15.00

six-fold reduction in light scatter over detection at 500 nm.

Instrumentation associated with the use of near infrared dyes also offers some advantages over traditional instrumentation. Laser induced fluorescence is one of the most commonly used fluorescence detection methods, due to the high intensity associated with laser light. Lasers that operate in the visible region of the spectrum are often bulky, expensive, and have limited operational lifetimes. The advent of solid state diode lasers do not have any of the aforementioned disadvantages. They are rugged, inexpensive, compact, and have long operational lifetimes. The typical signal transducer for fluorescence measurements is the photomultiplier tube (PMT). However, PMT's make poor choices for signal transducers in the near infrared region due to their low quantum yields at these wavelengths. Consequently, Avalanche photodiodes (APD) are used. APD's offer high quantum efficiencies in the near infrared region. Additionally, they are rugged, cheap, and have long operational lifetimes.

Bioanalytical Applications of Near Infrared Dyes Using Noncovalent Labeling

Near infrared dyes may be attached to the species of interest either by covalent or noncovalent labeling schemes. The labeling of serum proteins with indocyanine green was first demonstrated in 1974 [1]. ICG was also used by Soper and coworkers to noncovalently label the proteins β-casein, β-lactoglobulin, and chicken egg albumin [2]. Coyler recently used capillary electrophoresis to study the noncovalent interactions between ICG and albumin [3]. Depending on the application, noncovalent labeling schemes offer many advantages over traditional covalent labeling schemes. Some covalent labeling reactions require elevated pH levels in order to proceed. Some proteins are not stable at these elevated pH levels. Additionally, the labeling reactions are often time consuming and may require purification steps. A further disadvantage of covalent labeling schemes is dye:analyte heterogeneity, which leads to band broadening in

chromatographic / electrophoretic applications [4]. Noncovalent labeling schemes offer fast labeling reactions, decreased pH dependence, and can potentially eliminate the need for purification steps.

For these reasons, Sowell and coworkers developed a capillary electrophoresis based dye displacement method for the determination of drug binding constants to human serum albumin that utilizes a noncovalent labeling scheme [5,6]. All experiments were done on a modified Beckman P/ACE 5000 capillary electrophoresis instrument (figs 2,3). Albumin is the most abundant serum protein and is know for its ability to reversibly bind a wide array of compounds [7]. Drug albumin binding constants affect several important pharmoclogical properties such as activity, distribution, toxicity, excretion, and solubility. Consequently, it is of interest to develop methodologies that allow for the determination of drug albumin binding constants.

The dye displacement method developed utilizes a NIR dye, synthesized in house [8] to noncovalently label serum albumin (fig 4). The dye displacement method has advantages over traditional electrophoretic binding constant determination techniques in that a it uses a minimal amount of reagent and a binding constant is calculated in a single run. In order to determine if the near infrared dye was suitable as a noncovalent label, aliquots of dye and albumin were vortexed and then injected on the CE (fig 5). As the concentration of protein increases relative to a fixed concentration of dye, the free dye peak decreases and the dye-HSA peak increases, suggesting that the dye is indeed suitable for noncovalent labeling of albumin. If a drug that is known to bind to albumin is introduced to the dye labeled protein, two types of interactions may occur. Noncompetitive interactions occur when the drug and dye bind at different locations on the protein. Competitive interactions occur when the drug and dye compete for the same binding location on the dye. Noncompetitive interactions do not allow for the determination of a binding

constant, however, they do provide some insight into the binding location of the dye. Figure 6 illustrates the competitive binding of drugs toward serum albumin. As drug is introduced into the system the free dye peak increases and the dye albumin peak decreases (relative to the dye protein system with no drug present), suggesting that the drug introduced is competing with the dye for the same binding location on albumin. Furthermore, the degree of dye displaced from protein is directly correlated to the binding constant of the drug introduced. Consequently this information may be used to construct a curve that allows for the determination of drug albumin binding constants in a single run. This method of binding constant determination has the advantage that a binding constant is calculated in a single run, unlike traditional CE based binding constant determination methods such as affinity capillary electrophoresis (ACE), frontal analysis (FA), and vacancy peak (VP), where multiple runs are required.

Bioanalytical Applications of Near Infrared Dyes Using Covalent Lableling

While noncovalent labeling schemes do have their advantages, it is often necessary to covalently tag the species of interest. Covalent labeling schemes offer some advantages that noncovalent labeling cannot. Covalently tagged analytes are much more stable. Additionally, covalent labeling minimizes the opportunity for non specific interactions and consequently increases selectivity. Criteria that contribute to a dye's potential as a covalent label include favorable photophysical properties, stability, and water solubility. The dye should not interfere in the measurement or application in which it is being used, i.e., no nonspecific interactions. Additionally, the labeling method should be specific and efficient. Specificity is generally achieved through the attachment of various functional groups to the near infrared dye. Examples include isothiocyanate groups, specific for amines, and iodoacetamides, specific for thiols. Some applications developed using covalent labeling schemes with near infrared dyes include

immunoassays and ultrasensitive CE based peptide detection.

Immunoassays are analytical techniques that rely on the highly specific interaction between an antigen and antiobody. Enzyme linked immunosorbent assay (ELISA) is probably the most popular format in use today. ELISA uses an enzyme as a tracer. The enzyme produces a substrate whose presence is typically monitored by some type of spectroscopic technique. The high turnover number of the enzyme results in a highly sensitive technique. Disadvantages associated with ELISA include sensitivity to pH and temperature, nonspecific enzyme binding, and interference with respect to antigen - antibody recogntition. For these reasons, fluorescence detection techniques are becoming increasingly more popular. As mentioned previously, the use of visible absorbing fluorescent molecules is the presence of matrix autoflourescence. Consequently, near infrared fluorescent tags make attractive alternatives in immunoassays.

Williams and coworkers have developed instrumentation for solid phase immunoassays [9]. Detection limits of 500 pM concentrations were obtained, comparable to what was achieved using ELISA. However, the assay suffered from excessive scatter. For these reasons a NIR fluorescence immunoassay that overcame this problem was developed. The instrumentation consisted of a LI-COR 4200 fluorescence microscope was coupled with an orthogonal scanner (fig 7). The heptamethine cyanine dye NN382 was used for the experiments (fig 8). The dye contains an isothiocyanate group that is reactive toward primary amines. The sulfonates present on the dye increase its water solubility. The developed assay was capable of 20 pM detection limits if human IgG, roughly an order of magnitude improvement in detection limits relative to traditional methods. Additionally, the NIR LIF immunoassay was not nearly as time intensive as the ELISA method.

Covalent labeling schemes have also been used in capillary electrophoresis applications. Imasaka used the far red dye Azure B to label amino acids with reported detection limits of 800 zmol [10]. Yeung and Mank developed a micellar electrokinetic chromatography method for the separation of amino acids tagged with a near infrared dye [11]. Williams and coworkers used the dye NN382 to label six variants of the angiotensin peptide [12]. The variants were separated in a micellar electrokinetic format. Detection limits were in the low zeptamol range, illustrating the superior detection limits achievable through the use of NIR LIF detection.

Conclusions

The advantages of near infrared fluorescence detection in conjunction with covalent and noncovalent labeling schemes has been illustrated. Due to the fact that very few molecules exhibit intrinsic fluorescence in the near infrared region, near infrared absorbing chromophores are well suited for bioanalytical applications. The lack of background fluorescence translates into increased sensitivity. The complimentary nature of solid stated diode lasers and avalnache photodiodes makes for instrumentation that is compact, inexpensive, and robust. As the availability of commercial instrumentation capable of near infrared fluorescence increases, combined with an increasing knowledge base in near infrared synthetic dye chemistry, the implementation of this technology will continue.

References

1) K. Kamisaka, I. Listowski, J. J. Betheil, and I.. M. Arias, "Competitive binding of Bilirubin, sulfobromophthalein, indocyanine green, and other organic anions to human and bovine serum albumin," *Biochim. Biophys. Acta.* **365**, pp. 169-180, 1974.

2) B. L. Legendre Jr. and S. A. Soper, *Appl. Spectrosc.* **50**, pp. 1196-1202,1996.

3) E. D. Moody, P. J. Viskari, and C. L. Colyer, "Non-covalent labeling of human serum albumin with indocyanine green: a study by capillary electrophoresis with diode laser-induced fluorescence detection," *J. Chromatogr. B.,* **729**, pp. 55-64, 1999.

4) D. B. Craig, N. J. Dovichi, Anal. Chem. 70 (1998) 2493.

5) J. Sowell, K. A. Agnew-Heard, J. C. Mason, C. Mania, L. Strekowski, and G. Patonay, "Use of non-covalent labeling in illustrating ligand binding to human serm albumin via affinity capillary electrophoresis with near-infrared laser induced fluorescence detection," *J. Chromatogr. B.*, **755**, pp. 91-99, 2001.

6) J. Sowell, J. C. Mason, L. Strekowski and G. Patonay, "Binding constant determination of drugs toward subdomain IIIA of human serum albumin by near-infrared dye displacement capillary electrophoresis," *Electrophoresis*, **12**, pp. 2512-1517, 2001.

7) T. Peters, *All About Albumin: Biochemistry, Genetics and Medical Applications*, Academic Press, San Diego, CA 1996, pp. 76-131.

8) M. Lipowska, G. Patonay, and L. Strekowski, "New near-infrared cyaninie dyes for labeling proteins," *Synth. Commun.*, **23**, pp. 3087-3094, 1993.

9) R. J. Williams, N. Narayanan, G. A. Casey, M. Lipowska, L. Strekowski, J. M. Peralta, V. C. W. Tsang, G. Patonay, "Instrument to Detect Near-Infrared Fluorescence in Solid Phase Immunoassay", *Anal. Chem.*, **66**, pp. 3102-3107, 1994.

10) T. Higashijima, T. Fuchigami, T. Imasaka, N. Ishibashi, Anal. Chem., 64 (1992) 711.

11) A. J. Mank, E. S. Yeung, J. Chromatogr. A., 708 (1995) 309.

12) M. J. Baars and G. Patonay, "Ultra sensitive detectio of closely related peptides using CE with NIR LIF detection," *Anal. Chem.* **71**, 667-671, 1998.

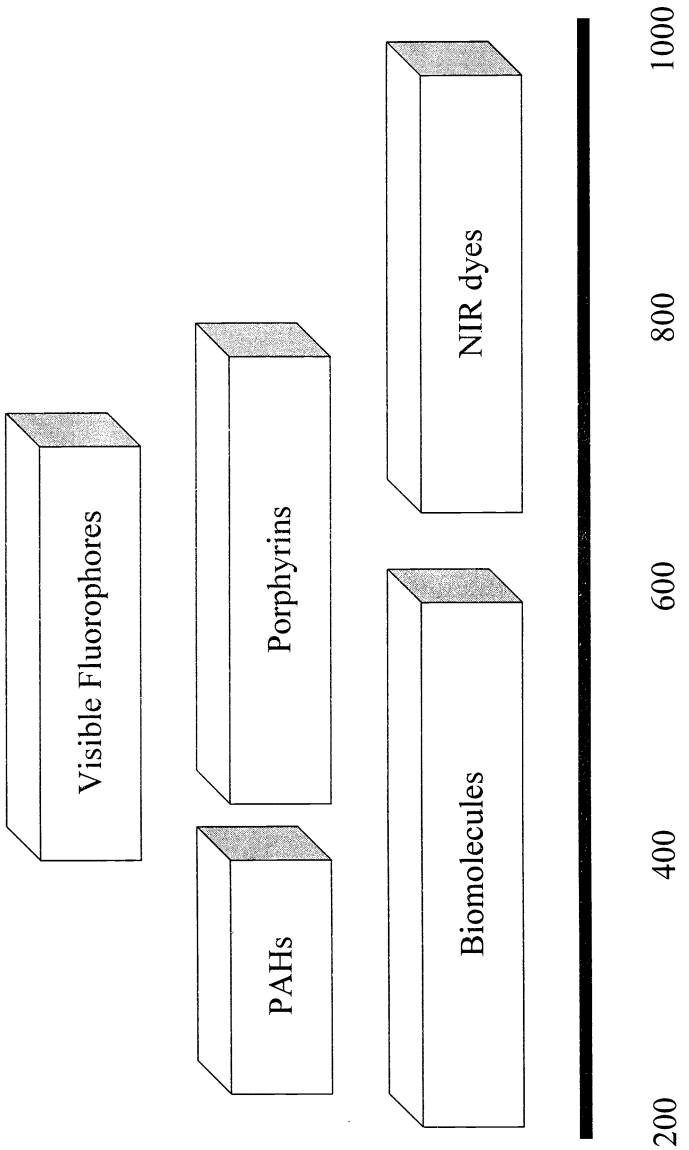

Figure 1 Interference regions from intrinsic fluorescence

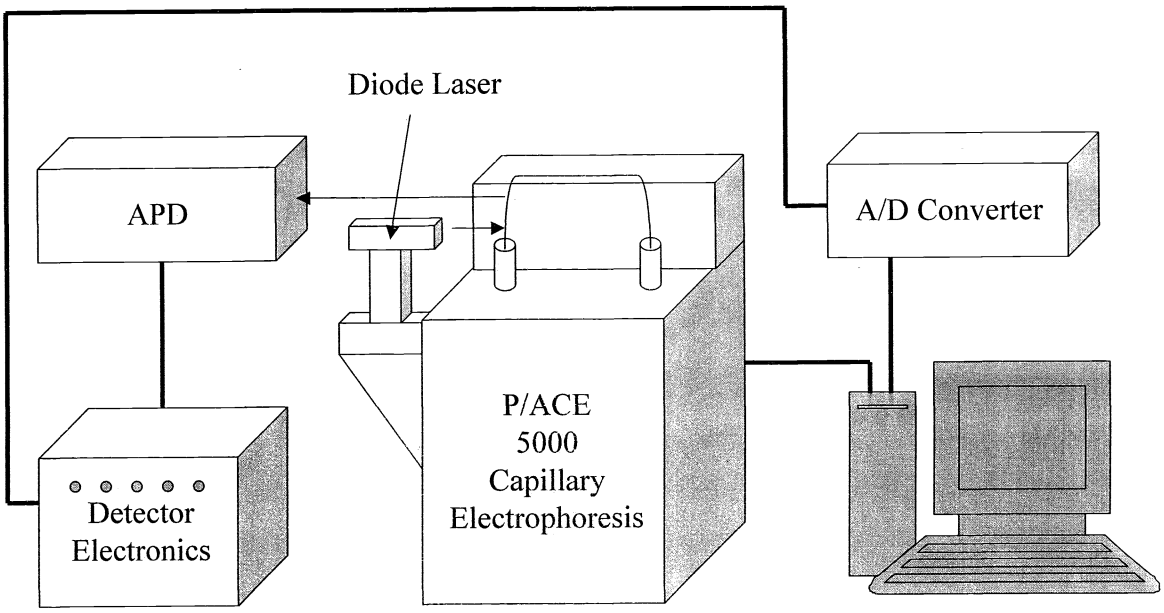

Figure 2 Diagram of modified capillary electrophoresis

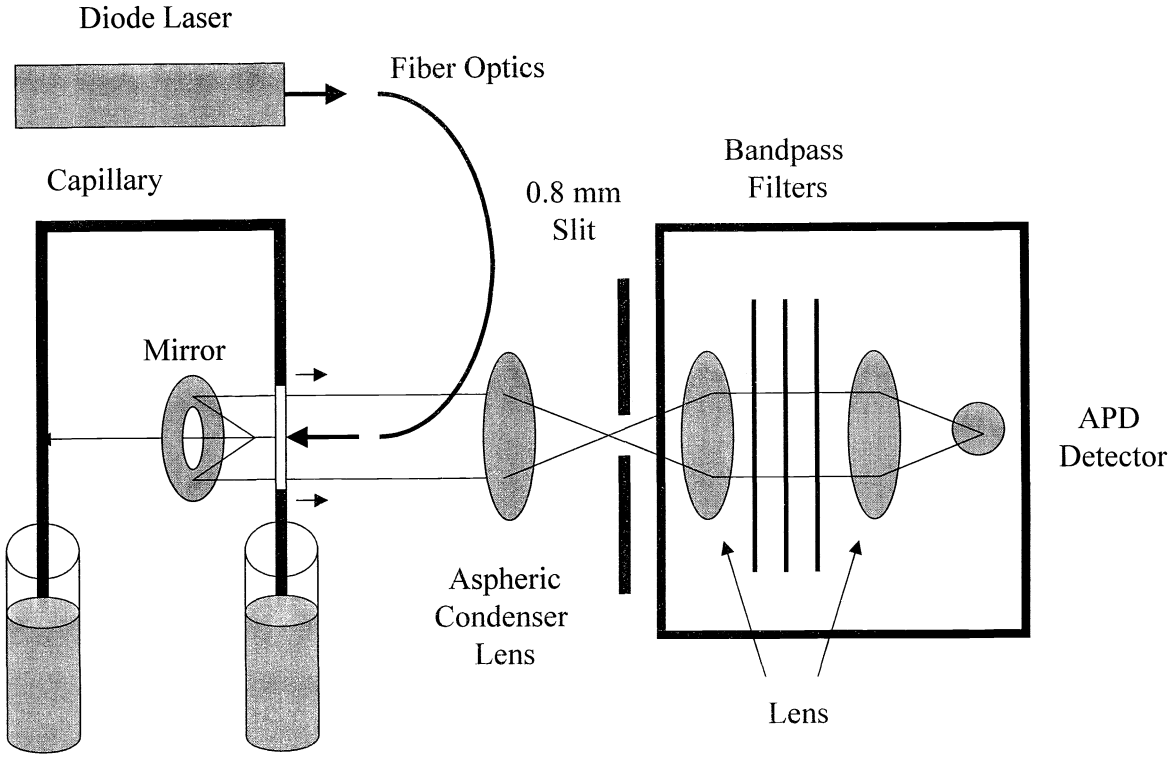

Figure 3 Optical path of capillary electrophoresis

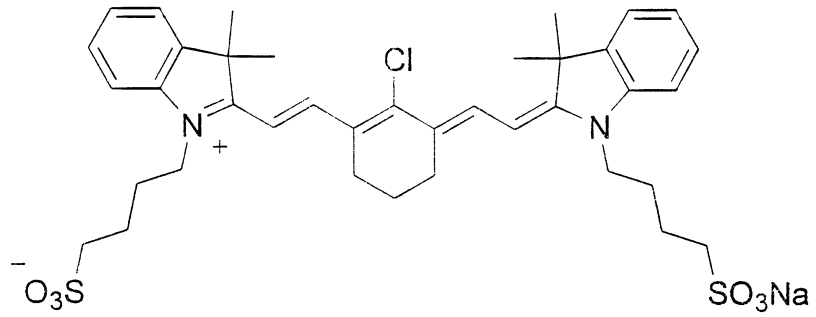

Figure 4 Structure of near infrared dye used for noncovalent labeling

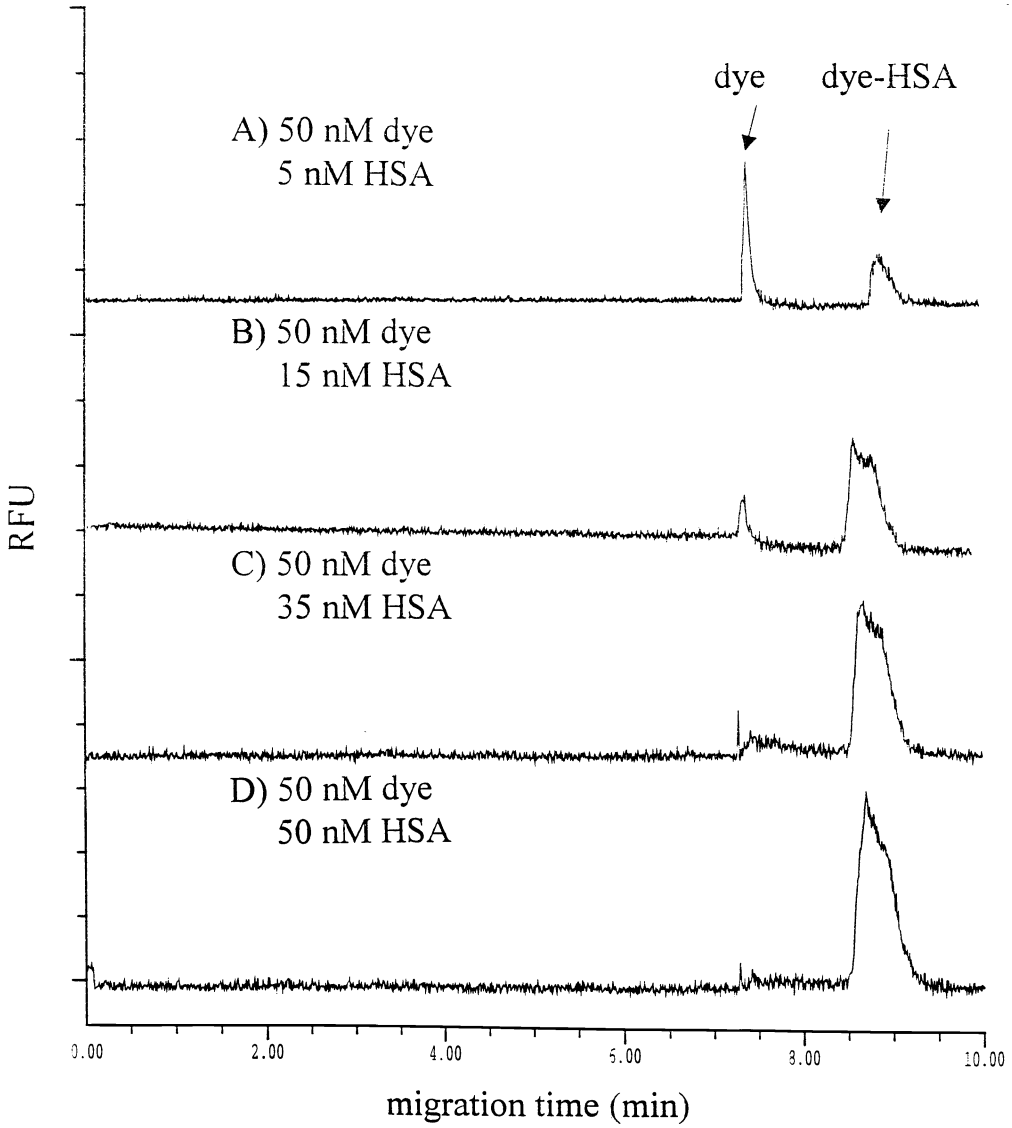

Figure 5 Electropherogram illustrating noncovalent labeling of human serum albumin

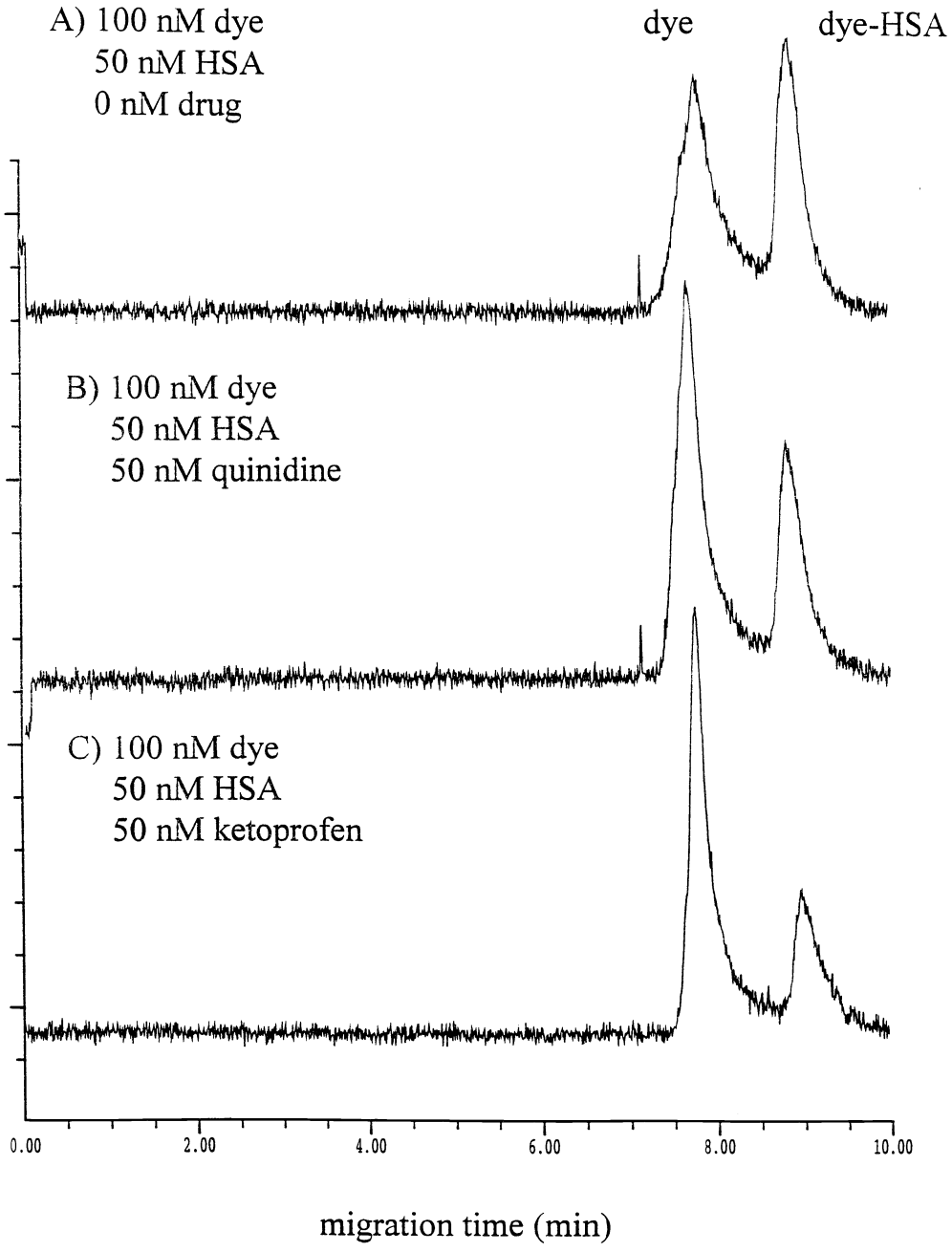

Figure 6 Electropherogram illustrating competitive interaction between drug and dye

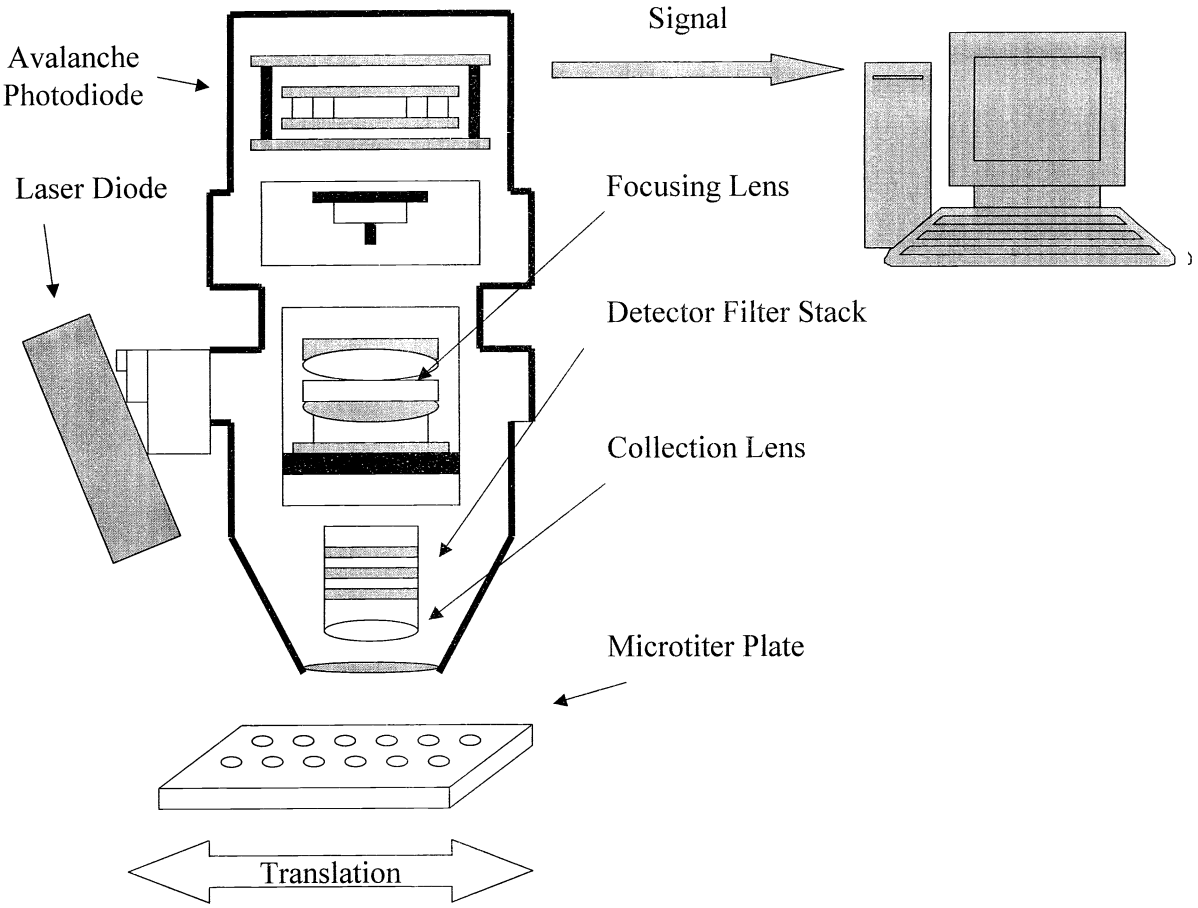

Figure 7 Diagram of near infrared fluorescence immunoassay instrument

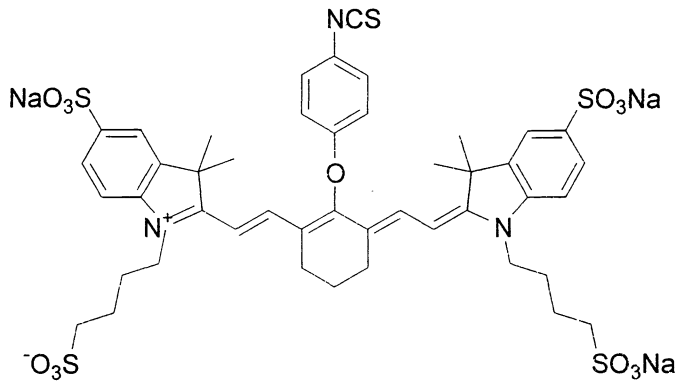

Figure 8 Structure of NN382