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DNA and protein applications of near-infrared dyes

John Sowell
Lucjan Strekowski
Gabor Patonay
Georgia State University
Department of Chemistry
University Plaza
Atlanta, Georgia 30303

Abstract. The near-infrared region of the spectrum (650–1100 nm) offers distinct advantages over the traditional UV/vis region for spectroscopic measurements. In the past, the lack of commercially available equipment capable of working in the near infrared limited the utility of near-infrared techniques. However, since the advent of photodiodes and semiconductor lasers, much progress has been made in the development of near-infrared techniques. This paper discusses the use of near-infrared dyes used in DNA and protein applications.
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1 Introduction

One of the most common detection schemes used in bioanalytical applications is laser-induced fluorescence. Fluorescence detection schemes offer high sensitivity and selectivity, since the fluorophore is usually attached to the species of interest and therefore functions as a reporter molecule. The majority of fluorescence detection schemes use visible fluorophores, with the fluorescein–argon ion laser couple being one of the most popular. However, a problem often encountered with visible laser-induced fluorescence is autofluorescence from the sample matrix. Autofluorescence arises from the intrinsic fluorescence that many biological compounds have in the visible region of the spectrum (Figure 1). The presence of matrix effects degrades both sensitivity and selectivity.

Very few biological molecules posses intrinsic fluorescence in the near-infrared (NIR) region of the spectrum (650–1100 nm). Consequently, near-infrared laser induced fluorescence detection can significantly improve sensitivity and selectivity. The improvements in detection limits available with near-infrared laser-induced fluorescence detection arise from the lack of intrinsic fluorescence of matrix compounds in this region of the spectrum. The near-infrared region is therefore well suited for bioanalytical applications where autofluorescence of biological compounds is a concern. Additional improvements in sensitivity arise from decreased light scatter. Light scatter is dependent on the wavelength of detection by $1/\lambda^4$. As a result, detection at 820 nm offers a sixfold reduction in scatter over detection at 500 nm.

Advances in solid state technology during the past decade have allowed for the development of near-infrared fluorescence applications. The most common excitation source for fluorescence detection schemes is the laser, due to its high intensity and narrow bandwidth. Visible lasers, such as the argon ion, are often expensive, bulky, and have limited operational lifetimes. Diode lasers are the sources most often used with near-infrared laser-induced fluorescence detection and do not have any of the previously mentioned disadvantages. They are rugged, inexpensive, compact, and have long operational lifetimes.

Address all correspondence to Gabor Patonay. Tel: 404-651-3856; E-mail: cheggp@panther.gsu.edu

The typical signal transducer for visible fluorescence detection is the photomultiplier tube (PMT). While PMTs can be used as detectors in the near-infrared region, avalanche photodiodes (APD) make a much more attractive choice, offering higher quantum yields in the near-infrared region where commercial dyes are currently available. Additionally APDs are inexpensive, rugged, compact, and have long operational lifetimes. One advantage that PMTs currently have over APDs is that the photoactive range of PMTs can extend out to 1200 nm. However, this advantage is somewhat minimal at this point in time due to the fact that no commercially available dyes exist for this region of the spectrum. Additionally, the photoactive area of PMTs is larger than that of APDs. The difference in photoactive area can be an advantage or disadvantage, depending on the application.

2 DNA and Near-Infrared Dyes

The human genome project was initiated to determine the three billion base pair sequence of the human genome. Due to the complexity of this task, a multitude of advancements in DNA sequencing technology emerged. Near-infrared laser-induced fluorescence sequencing technology began to appear during this time period. Soper and co-workers have done much work with respect to applying near-infrared dye technology to DNA applications. Williams and Soper demonstrated the superior detection limits that are attainable using near-infrared laser-induced fluorescence.[1] The 5 ft end of a M13 sequencing primer was labeled with a tricarbocyanine dye. Molar concentrations of the bases were 4:2:1:0, thus allowing for sequence determination based on peak height while only using a single lane. The same procedure was carried out with a fluorescein tagged primer, allowing for sensitivity comparison between the two. The quantum yields of the two dyes were 0.9 for fluorescein and 0.07 for the tricarbocyanine dye. Despite the lower quantum yield of the tricarbocyanine dye, detection limits for this dye were nearly two orders of magnitude lower, 34 zmol for the NIR dye versus 1.5 amol for fluorescein. The superior detection limit of the NIR dye is attributed to the absence of matrix autofluorescence.

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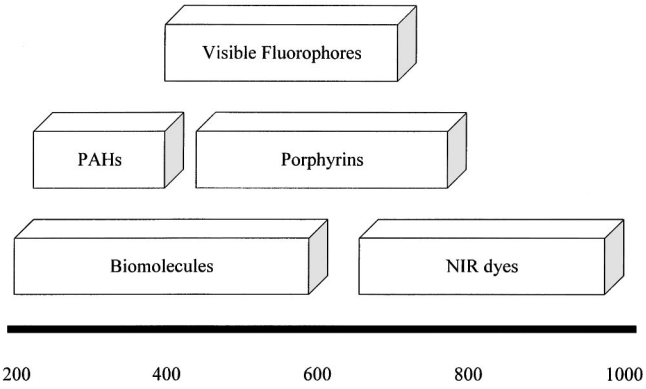

Fig. 1 Spectral regions of autofluorescence of various compounds.

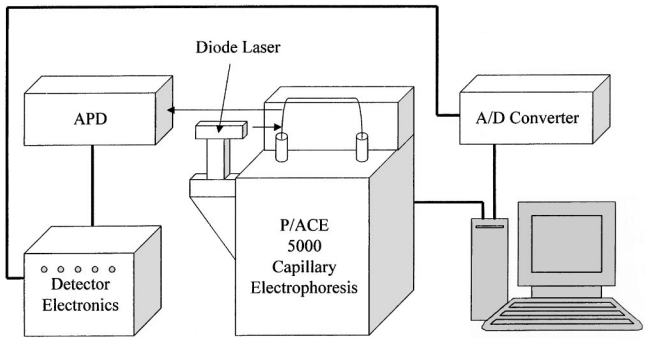

Fig. 2 Schematic of modified capillary electrophoresis.

Generally, spectral discrimination is the most common method of base calling in DNA sequencing, however, temporal discrimination does offer distinct advantages. The lifetime of the fluorophore is not dependent on concentration. Fluorescence lifetimes may be determined with greater accuracy than fluorescence intensity. Additionally, the problem of broad emission profiles is negated and a single detection channel may be used. Soper and co-workers have developed a temporal method for DNA sequencing using near-infrared dyes.[2] The near-infrared dyes used have been modified through the incorporation of heavy atoms in the dye structure, thereby giving a set of dyes with unique decay times.[3–5] Incorporation of the heavy atom does not alter the absorption and emission profiles of the dyes, allowing for a single excitation source. Due to the lack of interfering photocounts, much higher precision is obtainable using the method. Additionally, the electrophoretic mobilities of the strands remained unchanged and therefore postrun corrections were not necessary.

In order to investigate the advantages of temporal versus spectral discrimination, a comparison was done by Soper and co-workers.[6] A standard four-dye, one-lane sequencing approach was used for spectral discrimination. A two-dye, two-lifetime approach was used for the temporal base calling. The apparatus used a scanner that was translated across a slab gel. The scanner consisted of a single avalanche photodiode and a pulsed diode laser, with an output of 5 mW at 680 nm. The two-dye, two-lane method not only gave higher read accuracy but eliminated insertions and deletions. Furthermore, spectral information may be obtained simultaneously with temporal discrimination, if necessary. An application of this would be the ability to simultaneously sequence in the forward and reverse directions, minimizing the chance for miscalls.

A further development of near-infrared temporal discrimination evaluated by Soper involves its incorporation into a microarray format.[7] DNA microarrays provide a means of screening for a specific target sequence in the presence of large numbers of interfering compounds. DNA microarrays may be used as DNA probes, expression profiling, and sequencing.[8–10] Temporal detection methods offer several advantages over the traditional spectral detection methods commonly used in the microarray format. Temporal detection methods have the ability to monitor multiple probes while using a single channel. The use of near-infrared dyes with temporal discrimination can improve sensitivity due to the absence of interfering photocounts. Additionally, the instrumentation for temporal detection in the near infrared is much less complex than the corresponding apparatus for visible time-resolved fluorescence. Detection limits of 0.38 molecules/μm^2 were obtained for the microarray developed by Soper. The detection limits were further improved by an order of magnitude through the use of time gated detection.

3 Protein Applications of Near-Infrared Dyes

3.1 Noncovalent Labeling

A covalent or noncovalent labeling scheme may be utilized when tagging a species with a fluorescent dye. The capability of fluorescent dyes to reversibly label proteins such as human serum albumin has long been known.[11] Soper and co-workers used the near-infrared dye indocyanine green to noncovalently label β-casein, β-lactoglobulin, and chicken egg albumin.[12] Also, Colyer has used capillary electrophoresis to characterize the binding between ICG and human serum albumin.[13] Noncovalent labeling schemes potentially offer distinct advantages over traditional covalent labeling methods. Covalent labeling schemes can be time consuming and require purification steps. Often, elevated pH levels are required for the derivitization reaction to proceed. Biological compounds are not always stable at high pH levels. Furthermore, it has been demonstrated that covalent labeling schemes may lead to band broadening in chromatographic applications, due to analyte:dye heterogeneity.[14] Noncovalent labeling schemes can overcome many of these problems. Noncovalent labeling is characterized by fast labeling reactions, little or no pH dependence, and the potential for no postreaction purification provided sufficiently high binding constants and known reaction stochiometry.

Sowell and co-workers developed a capillary electrophoresis based dye displacement approach for drug-albumin binding constant determination that utilized a noncovalent labeling scheme.[15,16] A modified Beckman P/ACE capillary electrophoresis was used for all experiments (Figure 2). Pharmacological properties such as toxicity, activity, excretion, distribution, and solubility are affected by drug-albumin binding constants. It is therefore necessary to develop methods that allow for drug binding constant determination.

The dye displacement method for drug-albumin binding constant determination developed by Sowell and co-workers has several advantages over traditional chromatographic binding constant determination methods. Binding constants are

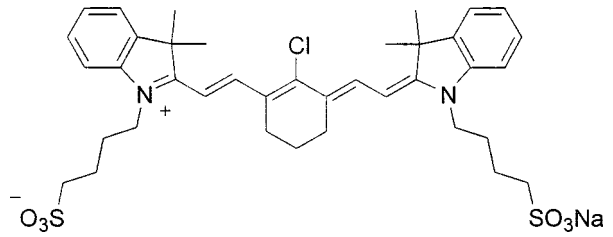

Fig. 3 Structure of heptamethine cyanine dye (sodium 4-[2-[4-chloro-7-[3,3-dimethyl-1-(4-sulfonatobutyl) indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-3H-indolio] butane sulfonate) used for noncovalent labeling.

obtained in a single run and consequently a minimal amount of reagent is used. The dye displacement method utilizes a near-infrared dye (Figure 3) synthesized in house, to noncovalently label human serum albumin.[8] Aliquots of dye and albumin were vortexed and injected onto the modified capillary electrophoresis in order to determine if the dye was suitable as a noncovalent label (Figure 4). It can be seen from Figure 4 that as the protein concentration increases, relative to a fixed concentration of dye, the peak corresponding to free dye decreases while the peak associated with the dye labeled protein increases. Consequently, it was concluded that the dye was suitable for noncovalent labeling of human serum albumin.

If a drug that is known to bind to human serum albumin is introduced to the dye labeled protein, two types of interactions may occur, competitive or noncompetitive, with respect to dye. Noncompetitive interactions will occur if the dye and drug have separate and distinct binding locations on the protein. Competitive interactions will occur if the dye and drug

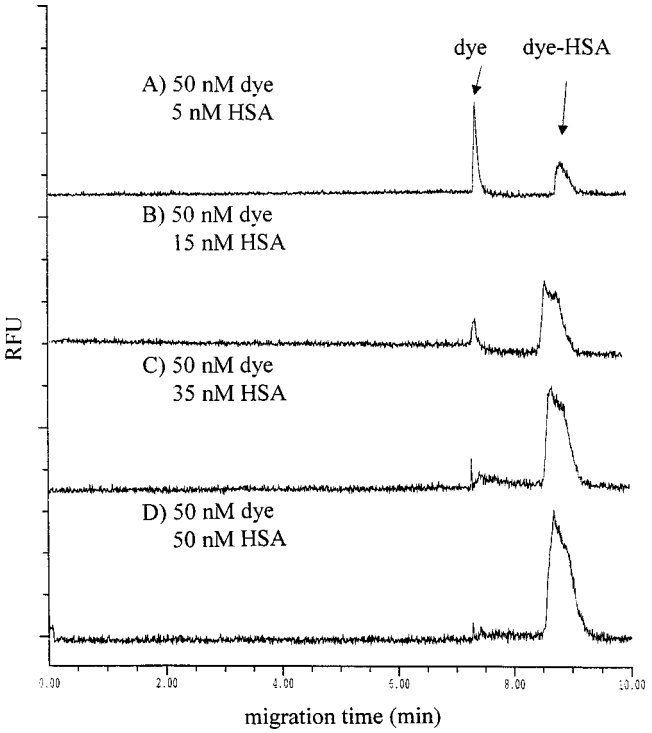

Fig. 4 Electropherogram illustrating noncovalent labeling.

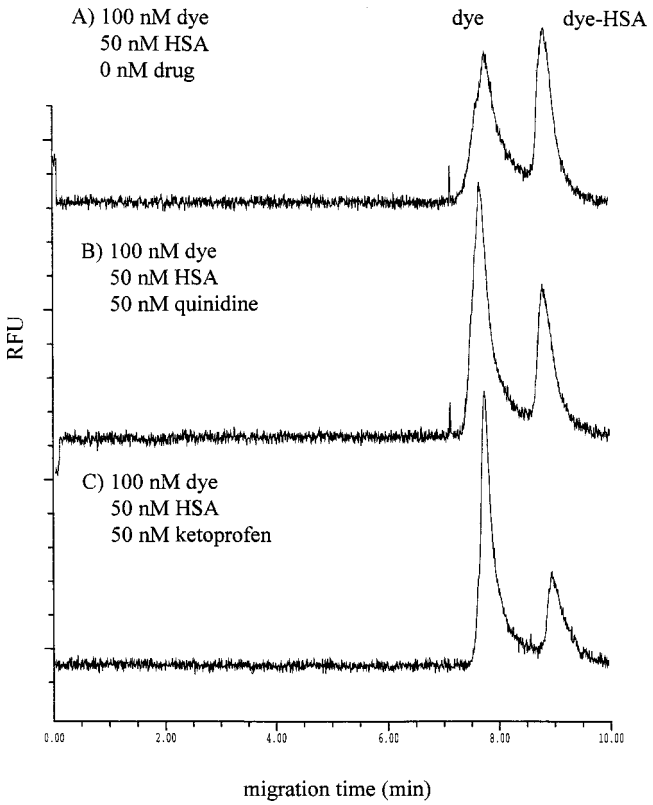

Fig. 5 Electropherogram illustrating competitive binding interactions.

share a common binding site on the protein. While binding constant determinations may not be determined with noncompetitive interactions, these types of interactions do provide some insight into the binding location of the dye. Figure 5 illustrates competitive interactions. As a drug is introduced to fixed concentration of dye labeled protein, the peak corresponding to dye labeled protein decreases and the free dye peak increases. This suggests that the dye and the drug are competing for the same binding location on the protein. The degree of dye displacement induced by introduction of drug is related to the binding constant of the drug. Therefore, it is possible to develop a calibration curve that would allow for binding constant determination in a single run. The dye displacement method of binding constant determination is advantageous in that a binding constant is calculated in a single run. Other chromatographic techniques for binding constant determination, such as affinity capillary electrophoresis, frontal analysis, and vacancy peak, require multiple runs to obtain a binding constant. Furthermore, these methods consume relatively large amounts of reagent.

3.2 Covalent Labeling

While noncovalent labeling does have advantages, there are certain instances where covalent labeling schemes must be used. Covalently labeled compounds are much more stable. Furthermore, the opportunity for nonspecific interactions is greatly minimized. Favorable characteristics of a fluorescent covalent label include photostability, water solubility, and good photophysical properties. The dye should not interfere with the measurement being made, i.e., no nonspecific inter-

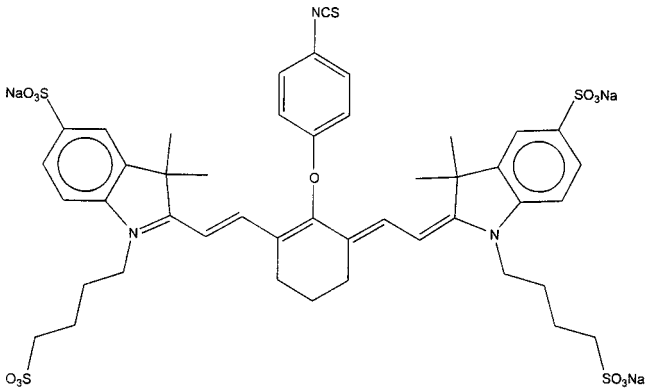

Fig. 6 Structure of NN382.

actions. Additionally, the labeling reaction should be fast and efficient. Dye analyte specificity is usually accomplished through the attachment of functional groups to the dye. For example, isothiocyanate groups may be attached to the dye in order to make the dye reactive towards amino groups. Applications developed that utilize covalent attachment of near-infrared dyes include capillary electrophoresis methods as well as immunoassays.

Immunoassays are bioanalytical techniques that take advantage of the highly specific interaction between an antigen and antibody. The most popular format of immunoassay presently in use is enzyme linked immunosorbent assay (ELISA). With the ELISA format, an enzyme is used as a tracer, as opposed to a fluorescent tag. The enzyme produces a substrate whose presence is monitored. Due to the fact that one enzyme molecule can produce large numbers of substrate molecules, ELISA is a highly sensitive technique. However, it is not without disadvantages. ELISA is sensitive to factors such as temperature and *p*H. Additionally, nonspecific binding and antibody-antigen recognition are problems. Consequently, the use of fluorescent tags in immunoassays is growing rapidly.

As mentioned previously, the use of visible fluorophores in bioanalytical applications often results in a high degree of matrix autofluorescence. For this reason, Patonay and co-workers developed a near-infrared solid phase immunoassay.[17] Detection limits of 500 pM concentrations were obtained. A problem encountered with the assay was excessive light scatter. Consequently, new instrumentation, consisting of a LI-COR 4200 fluorescence microscope coupled to an orthogonal scanner, was developed to overcome the light scatter problem. The heptamethine cyanine dye NN382 was used for the experiments (Figure 6). The dye, which contains an isothiocyanate group, was reacted with human IgG. Defecation limits of 20 pM were obtained, giving roughly an order of magnitude improvement over those obtainable with ELISA. Additionally, the NIR fluorescence immunoassay was not nearly as time intensive as the ELISA format.

Another area where covalent labeling schemes see use is in capillary electrophoresis. There have been a few groups to explore capillary electrophoresis with red and near-infrared laser induced fluorescence. Higashijima and co-workers reported detection limits of 800 pM using the far red dye Azure B to label amino acids.[18] Baars and Patonay used the dye NN382 to label and separate six variants of the peptide angiotensin.[19] Micellar electrokinetic chromatography was used to separate the six variants. Detection limits in the low zeptomole range were obtained.

4 Conclusion

It can be seen that much potential exists for the use of near-infrared fluorescence in bioanalyitical analysis. Very few biological molecules possess intrinsic fluorescence in the near-infrared region of the spectrum. As a result, matrix autofluorescence is almost nonexistent. Consequently, sensitivity of measurements made using near-infrared laser-induced fluorescence are greatly improved. Also, the complementary nature of diode lasers and avalanche photodiodes increases the attractiveness of the technique. The combination of advances in the synthetic chemistry of near-infrared dyes, in conjunction with the gradual development of commercially available equipment, have resulted in more analytical techniques using near-infrared dyes, due to the unique advantages they offer.

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