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High-Resolution Separation of DNA Restriction Fragments Using Capillary Electrophoresis with Near-IR, Diode-Based, Laser-Induced Fluorescence Detection

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The near-IR dye thiazole green (TAG) was used as a monomeric nuclear staining dye for the low-level detection of DNA restriction fragments separated via high-performance capillary electrophoresis with near-IR laser-induced fluorescence detection. TAG possessed an absorption maximum at 735 nm and an emission maximum at approximately 765 nm and, in the presence of dsDNAs, showed a fluorescence enhancement ratio of approximately 102, with a binding constant to dsDNAs determined to be 6.1 × 10^6 M^{-1}. The high-resolution separation of the *Hae*III restriction digest of ΦX174 was carried out using capillary electrophoresis on the native, ethidium bromide-stained, and TAG-stained DNA fragments. The TAG-stained DNA fragments resulted in higher plate numbers compared to the native and EtBr-stained restriction fragments as well as enhanced resolution; however, the 271/281 fragments could not be resolved using these CE conditions. To investigate the detection sensitivity of the TAG-stained DNA in capillary electrophoresis, an all-solid-state diode-based, laser-induced fluorescence detector was constructed, which consisted of a GaAlAs diode laser, with a principal lasing line at 750 nm and an avalanche photodiode. Using a running buffer composed of an entangled polymer (HPMC) and 1 μM TAG with no prestaining of the dsDNA prior to the electrophoresis, the limit of detection was found to be 20 fg (SNR = 3) of DNA per electrophoretic band. In addition, using the LIF system, the 271/281 bp fragments were nearly baseline resolved, with plate numbers exceeding 1 × 10^6 plates/m.

Capillary electrophoresis (CE) in conjunction with entangled polymer solutions as a sieving matrix has been shown to be an attractive fractionating method for dsDNAs due to the high efficiencies and resolution that are attainable and the speed associated with the separation technique.[1−7] However, due to the low mass-loading levels associated with capillary electrophoresis, stringent requirements associated with the detection protocol are necessary in order to detect modest concentration levels of dsDNAs. While native UV detection at 254 nm can be implemented, it suffers from poor sensitivity due to the short optical path length associated with capillary electrophoresis. Therefore, laser-induced fluorescence (LIF) with intercalating dyes has proven to be the method of choice in critical applications requiring low levels of detection.[8−13]

LIF detection has typically been undertaken using intercalating dyes which possess absorption and emission maxima in the visible region of the electromagnetic spectrum (450−630 nm). The detection is affected by monitoring the perturbation in the spectroscopic property of the dye when in the bound state. Cationic dyes which have planar aromatic or heteroaromatic rings and exhibit enhancements in their fluorescence emission upon complexation with dsDNA include the monointercalating dyes such as ethidium bromide (EtBr)[14] thiazole orange (TO),[15] and oxazole yellow (YO).[15] EtBr shows a fluorescence enhancement of 20 when it interacts with dsDNA, and the dye has been found to show a binding constant of 1.1 × 10^6 M^{-1} to dsDNAs.[16] In addition, the dimeric forms of these monointercalating dyes have been used for the analysis of dsDNAs.[17−19] The dimeric staining dyes consist of two chromophores covalently linked through a polycationic chain and show larger binding constants to dsDNA when compared to their monomeric counterparts. Because the fluorescence quantum yields of the dimeric form of the dyes are significantly improved in the bound state compared to those in free solution, the background fluorescence from free dye is very low, which makes these dyes excellent probes for the low-level quantification of dsDNA.

(1) Heiger, D. N.; Cohen, A. S.; Karger, B. L. *J. Chromatogr.* **1990**, *516*, 33−48.
(2) Schwartz, H. E.; Ulfelder, K.; Sunzeri, F. J.; Busch, M. P.; Brownlee, R. G. *J. Chromatogr.* **1991**, *559*, 267−283.
(3) Strege, M.; Lagu, A. *Anal. Chem.* **1991**, *63*, 1233−1236.
(4) Grossman, P. D.; Soane, D. S. *J. Chromatogr.* **1991**, *559*, 257−266.
(5) Pariat, Y. F.; Berka, J.; Heiger, D. N.; Schmitt, T.; Vilenchik, M.; Cohen, A. S.; Foret, F.; Karger, B. L. *J. Chromatogr.* **1993**, *652*, 57−66.
(6) Barron, A. E.; Soane, D. S.; Blanch, H. W. *J. Chromatogr.* **1993**, *652*, 3−16.
(7) Grossman, P. D. *J. Chromatogr.* **1994**, *663*, 219−227.
(8) Schwartz, H. E.; Ulfelder, K. J. *Anal. Chem.* **1992**, *64*, 1737−1740.
(9) McCord, B. R.; McClure, D. L.; Jung, J. M. *J. Chromatogr.* **1993**, *652*, 75−82.
(10) Srinivasan, K.; Girard, J. E.; Williams, P.; Roby, R. K.; Weed, V. W.; Morris, S. C.; Kline, M. C.; Reeder, D. J. *J. Chromatogr.* **1993**, *652*, 83−91.
(11) Kim, Y.; Morris, M. D. *Anal. Chem.* **1994**, *66*, 1168−1174.
(12) Zhu, H.; Clark, S. M.; Benson, S. C.; Rye, H. S.; Glazer, A. N.; Mathies, R. A. *Anal. Chem.* **1994**, *66*, 1941−1948.
(13) Figeys, D.; Arriaga, E.; Renborg, A.; Dovichi, N. J. *J. Chromatogr.* **1994**, *669*, 205−216.
(14) Le Pecq, J. B.; Paoletti, C. *Anal. Biochem,* **1966**, *17*, 100−107.
(15) Rye, S.; Yue, S.; Wemmer, D.; Quesada, M.; Haugland, R.; Mathies, R.; Glazer, A. *Nucleic Acids Res.* **1992**, *20*, 2803−2812.
(16) Gaugain, B.; Barbet, J.; Capelle, N.; Roques, B.; Le Pecq *J. Biochem.* **1978**, *17*, 5078−5088.
(17) Benson, S.; Singh, P.; Glazer, A. *Nucleic Acids Res.* **1993**, *21*, 5727−5737.
(18) Rye, S.; Quesada, M.; Peck, K.; Mathies, R.; Glazer, A. *Nucleic Acids Res.* **1990**, *19*, 327−332.
(19) Glazer, A.; Peck, K.; Mathies, R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3851−3855.

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In capillary electrophoresis, the monomeric dyes are typically added to the running buffers due to their smaller binding constants, and the dsDNA need not be prestained prior to the electrophoresis. Because the binding constants to dsDNA for the dimeric forms of the dyes are much higher, the DNA is typically prestained with the bis-intercalating dye, and it is unnecessary to add the dye to the running buffer during the electrophoretic separation. The limit of detection using the dimers is typically improved compared to that obtained using the monomers, but it suffers from poor resolution due to the multiple binding modes of the dye.[11,13]

The instrumental difficulty associated with utilizing visible LIF detection is that the excitation source is a gas-ion laser, for example an Ar ion or He−Ne laser, which has a limited operational lifetime and, in some cases, can be expensive and difficult to operate. In addition, visible excitation can encounter interferences in the form of scattering and fluorescence impurities, which can degrade the limit of detection, especially when using bis-intercalating dyes.

An attractive alternative to visible fluorescence is the use of near-IR fluorescence detection. Near-IR fluorescence has recently been demonstrated to be a viable detection strategy in liquid chromatography,[20−22] free solution capillary electrophoresis,[23−27] and capillary gel electrophoresis for DNA sequencing applications.[28] One of the principal advantages of using near-IR fluorescence is that simple diode lasers with lasing lines >700 nm can be used as the excitation source for the LIF detector. These diode lasers are compact, employ simple turn-key operation, have relatively high powers (20 mW), provide long operational lifetimes (>40 000 h), and reduce the cost of setting up such a detection system. In addition, near-IR fluorescence typically displays fewer interferences from the background matrix resulting from impurity fluorescence and/or a smaller scattering contribution as a result of the $1/\lambda^4$ dependence on the scattering cross section, improving the limit of detection.[28] This potentially lower background will be particularly attractive when using dimeric staining dyes in conjunction with CE, when the dsDNA is prestained and the dye not required to be present in the running buffer.

In this work, we report on the first demonstration of near-IR, diode-based LIF detection of restriction fragments separated by CE with an entangled polymer solution and staining using a monomeric intercalating dye which shows absorption and emission properties in the near-IR (>700 nm). The staining dye, thiazole green (TAG), was prepared by extending the methine chain in the common monointercalator TO-PRO 3, resulting in a shift in the absorption maxima into the near-IR.[29] The LIF system consisted of a GaAlAs diode laser operating at 750 nm and a single-photon avalanche diode (SPAD). Both of these devices are solid-state and easily operated, producing an LIF detection system that has a potentially longer operational lifetime and is very inexpensive but displays detection sensitivities for dsDNAs comparable to those of the visible systems that use gas ion lasers.

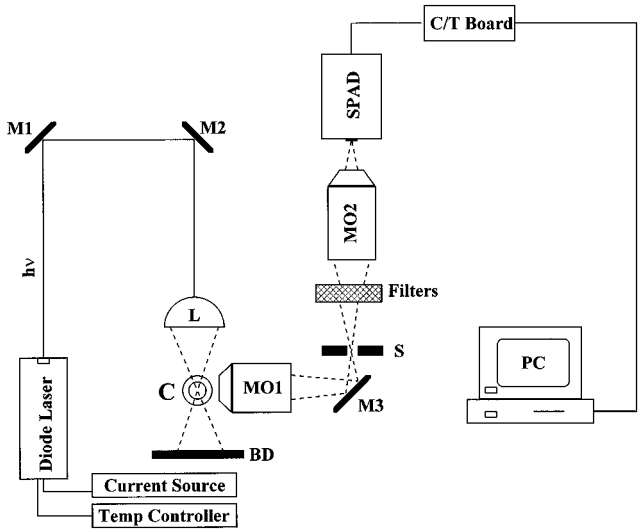


Figure 1. Near-IR LIF system for CE analysis of DNA restriction fragments. M1, M2, and M3, mirrors; L, laser focusing lens; C, capillary tube; BD, beam dump; MO1 and MO2, microscope objectives (see text for details); S, spatial filter; F, optical filters (see text); SPAD, single-photon avalanche diode; C/T, counter/timer; PC, computer.

EXPERIMENTAL SECTION

Instrumentation. Electrophoretic separations of native, ethidium bromide-stained, and TAG-stained ΦX174 *Hae*III-digested DNA were performed on a Beckman electrophoresis apparatus with UV detection at 254 nm (Foster City, CA). The electrophoresis system was operated in the reverse polarity mode (negative potential at the injection end of the capillary), and the data were acquired using the chromatographic software on a personal computer for displaying and analyzing the data. The near-IR LIF detection system was constructed in-house and is schematically shown in Figure 1. It consisted of a 7 mW, 750 nm diode laser (GaAlAs, Melles Griot, Irvine, CA) and a diode laser driver (Model 06 DLD 201, Melles Griot). The laser head contained an anamorphic prism pair to produce a circular beam that was single mode and also a thermoelectric cooler to maintain the diode temperature to prevent mode-hopping due to temperature fluctuations. Incorporation of external cavity optics improves the quality of the laser beam, allowing tighter focusing for use in electrophoresis devices with small inner diameter columns. The laser beam was focused onto the capillary detection window with a planoconvex lens (25 mm diameter, 25.0 mm focal length, Edmund Scientific, Barrington, NJ) and produced a beam waist ($1/e^2$) of 7 μm. The capillary was affixed horizontally (parallel to the optical bench) onto a home-made Plexiglas capillary holder, which was mounted on top of an $X-Y$ micropositioner for positioning the capillary with respect to the laser beam and collection optics. The fluorescence was collected at right angles with respect to the laser beam using a 40× epifluorescence microscope objective (Melles Griot) with a numerical aperture of 0.65 and was imaged onto a slit serving as a spatial filter to reduce the amount of scattered photons generated at the air/glass and glass/liquid interfaces of the capillary from reaching the detector. The fluorescence was further isolated from the scattering photons

(20) Sauda, K.; Imasaka, T.; Ishibashi, N. *Anal. Chim. Acta* **1986,** *187*, 353−356.
(21) Sauda, K.; Imasaka, T.; Ishibashi, N. *Anal. Chem.* **1986**, *58*, 2649−2653.
(22) Williams, R. J.; Lipowska, M.; Patonay, G.; Strekowski, L. *Anal. Chem.* **1993**, *65*, 601−605.
(23) Higashijima, T.; Fuchigami, T.; Imasaka, T.; Ishibashi, N. *Anal. Chem.* **1992**, *64*, 711−714.
(24) Fuchigami, T.; Imasaka, T. *Anal. Chim. Acta* **1993**, *282*, 209−213.
(25) Bachteler, G.; Drexhage, K.-H.; Jacob, J.; Han, K.-T.; Kollner, M.; Muller, R.; Sauer, M.; Seeger, S.; Wolfrum, J. *J. Lumin.* **1994**, *62*, 101−108.
(26) Fuchigami, T.; Imasaka, T. *Anal. Chim. Acta* **1994**, *291*, 183−188.
(27) Flanagan, J. H.; Legendre, B. L.; Hammer, R. P.; Soper, S. A. *Anal. Chem.* **1995**, *67*, 341−347.
(28) Williams, D. C.; Soper, S. A. *Anal. Chem.* **1995**, *67*, 3427−3432.
(29) Glazer, A.; Zeng, Z.; Benson, S. *Anal. Biochem.* **1995**, *231*, 247−255.

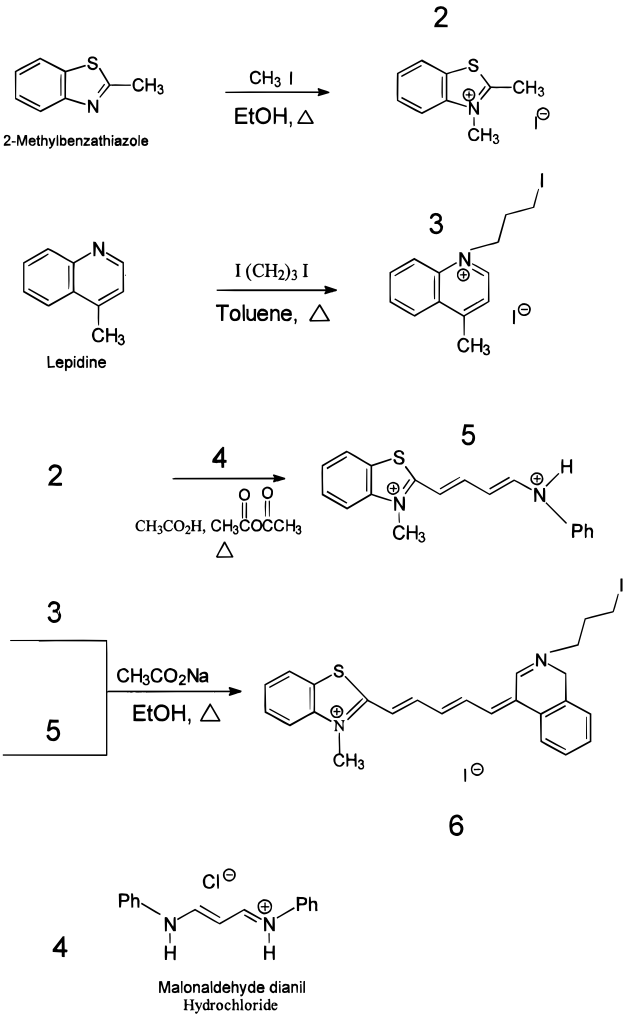
by a 780 nm band-pass filter (Oriel, Stratford, CT) and a 780 nm long-pass filter (Edmund Scientific, Barrington, NJ). The fluorescence was then focused onto the photoactive area of the detector with a 20× microscope objective (Melles Griot). The detector was a SPAD (Model SPCM-AQ-141, EG&G Optroelectronics Canada, Vaudreuil, Canada) with a 200 μm diameter photoactive area and possessed a dark and maximum light count rate, before saturation, of approximately 30 and 16 × 10^6 counts/s, respectively. To increase the linear dynamic range, the SPAD was operated in an actively quenched mode. The LIF signals were acquired on a personal computer (Gateway 2000, Model P5-120) using a 16-bit counter/timer board (Model CYRCTM 05, Cyber-Research Inc., Brandford, CT). The CE data were not subjected to any type of filtering algorithm prior to presentation. The high-voltage power supply was obtained from Spellman (CZ1000R, Plainview, NY).

Reagents. Tris-HCl, γ-[(methoxyacryl)oxy]propyltrimethoxysilane (MAPS), and ethidium bromide were obtained from Amresco (Solon, OH) and used as received. Hydroxypropylmethylcellulose (MW = 86 000), anhydrous ethylenediaminetetraacetic acid (EDTA), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Sigma Chemical Co. (St. Louis, MO). The ΦX174/*Hae*III digest was received from Life Technologies (Gaithersburg, MD) and was stored in a freezer at −20 °C until used for the electrophoresis. Calf thymus DNA, used for studies of the binding of TAG to dsDNA, was obtained from United States Biochemical (St. Louis, MO) and was sonicated in an ultrasonicator prior to use. During sonication, the DNA was placed on ice, and following sonication, it was stored in ultrapure water at −20 °C. The nucleotide base concentration was determined by measuring the absorbance at 260 nm and assuming an extinction coefficient of 6000 cm^{-1} M^{-1} per nucleotide base.

Sample and Sieving Buffer Preparation for Electrophoresis. *Hae*III restriction digest samples of ΦX174 were diluted (1:4; total [DNA] = 100 μg/mL) using 18 MΩ Milli-Q water prior to electrophoresis and injected directly onto the CE capillary using electrokinetic injection. The sieving matrix consisted of a 0.25% HPMC solution, which was made by adding the appropriate amount of polymer to the TAE running buffer (40 mM TRIS, 20 mM acetate, 2 mM EDTA, pH = 7.61). The sieving buffer was placed in a water bath and stirred with heating to completely dissolve the polymer. Prior to electrophoresis, the sieving buffer was filtered twice with 0.45 μm filter paper to remove particulates and undissolved polymer.

Capillary Preparation. Fused silica capillaries (75 μm i.d., 375 o.d.) were purchased from Polymicro Technologies, Inc. (Phoenix, AZ) and were coated with a linear acrylamide (1%T) according to published procedures.[30] Briefly, the capillary was rinsed first with 1.0 M NaOH and then 1.0 M HCl, each followed with copious rinsing with doubly distilled H_2O. The capillary was rinsed overnight with a 50:50 MAPS/MeOH solution, after which the column was dried in an oven for several hours at 80 °C. A 1%T linear acrylamide solution was made in 1× TBE (TRIS, borate, EDTA) from a 40%T/0%C stock solution. The solution was thoroughly degassed by water aspiration for approximately 2.5 h. Twenty-five microliters of fresh 10% ammonium persulfate (APS) in H_2O was added to the degassed acrylamide solution, followed by 5 μL of TEMED. The capillary was quickly filled with the acrylamide solution and allowed to rest horizontally until complete polymerization occurred, after which high pressure was used to remove excess linear acrylamide. The total capillary length used for both conventional and LIF CE was 39 cm (31 cm from injection to detection).

Scheme 1



The capillary and cathodic and anodic reservoirs were filled with the running buffer solution, which contained the TAE buffer, 0.25% (w/v) hydroxypropylmethylcellulose, and the appropriate concentration of the staining dye. Before injection of sample, the column was preconditioned by applying a field strength of 170 V/cm for 15 min. Nonstained dsDNA samples were injected into the capillary electrokinetically using an applied voltage of 2 kV for 5 s for the LIF system (30 s for conventional CE).

Synthesis of Near-IR Cyanine Dye. The near-IR nuclear staining dye TAG was prepared following modifications of previously described procedures.[29] The synthetic steps are outlined in Scheme 1. 2-Methylbenzathiazole (0.067 mol) was added to 3 equiv of methyl iodide and refluxed for 18 h in ethanol at 72 °C to form **2**. The iodoalkyl derivative **3** was prepared by adding 0.034 mL of lepidine to 5 equiv of 1,3-diiodopropane (0.176 mol) and refluxed in toluene at 110 °C for 18 h. The yellow-brown precipitate was filtered with extensive washing using ethyl ether and collected. Malonaldehyde dianil hydrochloride was prepared from malonaldehyde bis(dimethyl acetal) and aniline. The intermediate was produced by refluxing **2** (0.0121 mol) and **4** (0.100 mol) in a 1:1 mixture of acetic acid and acetic anhydride. The reaction progress was monitored via UV absorption and judged

(30) Hjerten, S. J. *J. Chromatogr.* **1985**, *347*, 191−198.

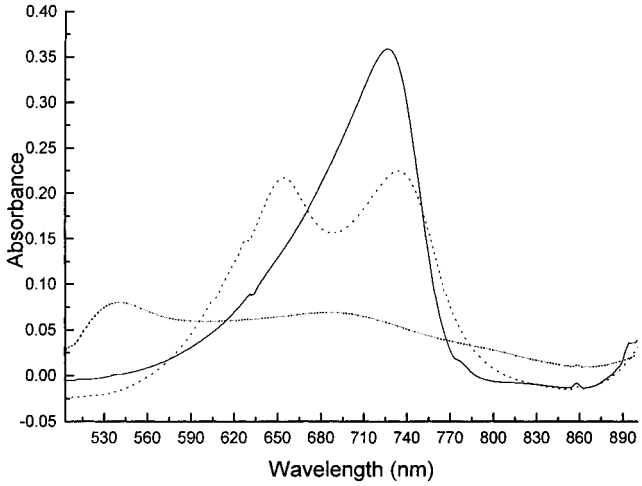


Figure 2. Absorption spectra of TAG in methanol (solid line), TRIS/acetate buffer with no DNA (dot/dashed line), and a 100-fold molar excess, in nucleotide bases, of calf thymus DNA (dotted line). In all cases, the dye concentration was 2×10^{-5} M.

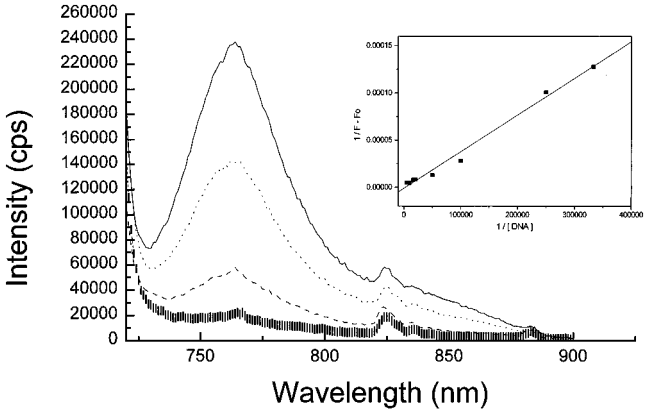


Figure 3. Spectrofluorometric titration of TAG with calf thymus DNA. In all cases, the DNA concentration was measured in terms of the nucleotide bases and was varied from 5×10^{-6} to 1×10^{-4} M. The fluorescence was excited at 710 nm and the emission monochromator scanned from 720 to 900 nm. The dye concentration used was 1.0×10^{-6} M.

complete when the 286 nm band nearly vanished. Reaction of **3** (0.64 mol) and **5** (0.0059 mol) in ethanol with sodium acetate after 30 min of refluxing produced **6** (thiazole green, TAG). This reaction was also monitored by UV/vis absorption, which indicated the presence of the symmetrical (665 nm) and unsymmetrical (735 nm) dye. TAG was purified by flash chromatography using a chloroform/methanol solvent (9:1 v/v). The NMR and mass spectral analysis revealed the identity and purity of **6**.

Absorbance and Fluorescence Measurements. The absorption spectra were acquired on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. The fluorescence spectra were obtained on a SPEX Fluorolog spectrofluorometer (SPEX, Edison, NJ) equipped with a 75 W xenon lamp. The emission gratings were blazed for 750 nm, and the photomultiplier tube was a Hammamatsu R636 red-sensitive photomultiplier tube.

RESULTS AND DISCUSSION

Absorption and Emission Properties of TAG. In Figure 2 are shown the absorption spectra of TAG in methanol, TAE buffer, and TAE with a 100-fold molar excess (in nucleotide bases) of calf thymus DNA. In methanol, the dye gave a single band with an absorption maximum at 735 nm. This band could be assigned to the monomer, since it is expected that the dye shows little tendency to aggregate in this solvent.[31] In TAE buffer, the spectrum shows two broad bands at approximately 705 and 533 nm, superimposed on a diffuse background. These spectral properties are indicative of extensive dye aggregation, forming dimers and other higher order aggregates, which is a typical phenomenon for the extended cyanine dyes.[32] In the presence of double-stranded calf thymus DNA, the monomer band is partially restored ($\lambda_{max} = 740$ nm), and another prominent band appears at 650 nm, which could arise from a dimeric form of the dye.

In Figure 3 is shown a series of fluorescence spectra which were obtained at various concentrations of calf thymus DNA (dye concentration 1 μM). As can be seen, drastic enhancements in the fluorescence were observed upon addition of calf thymus DNA. Construction of a modified Benesi−Hildebrand plot[33] for these data (see insert to Figure 3) indicated that the binding constant of TAG to dsDNA was approximately 6.1×10^6 M^{-1}, nearly 50× greater than the binding constant of EtBr to dsDNA, which has been determined to be 1.5×10^5 M^{-1}.[16] Integration of the spectra in the buffer only and in a 100-fold molar excess (in nucleotide bases) of dsDNA indicated that the fluorescence enhancement ratio of bound to free dye was 102.

CE Analysis of Native and Stained DNA. In order to make a comparison of the separation efficiency of native, EtBr-stained, and TAG-stained DNA, the *Hae*III restriction fragments of ΦX174 were analyzed using a conventional CE system with UV detection at 254 nm. In Table 1 are shown the plate numbers (N, m^{-1}) and the apparent electrophoretic mobilities (μ_{app}) of these restriction fragments under different staining conditions. Generally, the TAG-stained restriction fragments produced higher plate numbers than the EtBr-stained DNA or the nonstained DNA. Also seen in this data is that the apparent electrophoretic mobilities of the TAG-stained DNA fragments are smaller than those associated with the EtBr-stained fragments. This result is supported by the binding constant measured for the TAG/DNA complex, which indicated a value larger than that associated with EtBr to dsDNA. Therefore, the increased stability of the dye/DNA complex would result in a lower mobility due to reductions in the negative charge of the biopolymer and increases in the frictional coefficient resulting from elongation of the double helix produced from intercalation and the increased molecular mass associated with the complex. It should be noted that, in all cases, we were unable to resolve the 271/281 bp fragments in this restriction digest under these CE conditions.

Near-IR LIF Detection. Figure 4 shows the CE separation of the *Hae*III digest of ϕX174 in a polyacrylamide-coated capillary using near-IR LIF detection. From the apparent mobility and electrokinetic injection conditions (2 kV for 5 s), it was estimated that approximately 3.1 pg of the 603 bp fragment was inserted onto the capillary. The SNR for this peak, which was determined by integrating the area under the peak and dividing by the square root of the average background integrated over the same time

(31) Soper, S. A.; Mattingly, Q. L. *J. Am. Chem. Soc.* **1994**, *116*, 3744−3752.
(32) West, W.; Pearce, S. *J. Phys. Chem.* **1965**, *69*, 1894−1903.
(33) Green, M. D.; Potanay, G.; Ndou, T.; Warner, I. M. *Appl. Spectrosc.* **1992**, *46*, 1724−1728.

Table 1. CE Analysis of the *Hae*III Restriction Digest of the Native ΦX174, EtBr, and TAG-Stained Fragments[a]

	native		EtBr		TAG		
fragment size	N (m^{-1})[b] ($\times 10^5$)	μ_{app} ($cm^2\ V^{-1}\ s^{-1}$) ($\times 10^{-4}$)	N (m^{-1}) ($\times 10^5$)	μ_{app} ($cm^2\ V^{-1}\ s^{-1}$) ($\times 10^{-4}$)	N (m^{-1}) ($\times 10^5$)	μ_{app} ($cm^2\ V^{-1}\ s^{-1}$) ($\times 10^{-4}$)	N (m^{-1})[b] ($\times 10^5$)[c]
72	4.77 (±0.08)	4.26 (±0.04)	5.63 (±0.07)	4.04 (±0.05)	5.11 (±0.08)	3.99 (±0.04)	22.2 (±0.3)
118	3.84 (±0.05)	4.16 (±0.03)	3.88 (±0.05)	3.97 (±0.04)	5.01 (±0.09)	3.89 (±0.03	23.5 (±0.4)
194	3.16 (±0.09)	3.98 (±0.03)	3.98 (±0.05)	3.82 (±0.03)	5.17 (±0.07)	3.72 (±0.07)	26.2 (±0.5)
234	4.60 (±0.06)	3.88 (±0.06)	4.19 (±0.04)	3.74 (±0.05)	5.07 (±0.08)	3.62 (±0.05)	27.6 (±0.3)
271	*d*	*d*	*d*	*d*	*d*	*d*	29.3 (±0.4)
281	*d*	*d*	*d*	*d*	*d*	*d*	29.6 (±0.3)
310	6.00 (±0.08)	3.70 (±0.05)	4.50 (±0.04)	3.59 (±0.04)	6.29 (±0.10)	3.44 (±0.03)	7.68 (±0.01)
603	4.44 (±0.08)	3.22 (±0.05)	4.47 (±0.05)	3.18 (±0.03)	7.13 (±0.08)	2.99 (±0.02)	10.6 (±0.2)
872	4.49 (±0.10)	2.95 (±0.04)	4.11 (±0.06)	2.94 (±0.03)	8.28 (±0.06)	2.73 (±0.03)	12.5 (±0.3)
1,078	5.27 (±0.11)	2.82 (±0.03)	4.42 (±0.04)	2.81 (±0.04)	9.45 (±0.08)	2.60 (±0.03)	13.7 (±0.4)
1,353	5.43 (±0.11)	2.70 (±0.06)	4.77 (±0.03)	2.69 (±0.04)	9.87 (±0.09)	2.48 (±0.03)	14.8(±0.2)

[a] Detection was accomplished using UV absorption at 254 nm. The restriction fragments were injected electrokinetically onto the CE column for 30 s at −5 kV. The standard deviations, which were determined from three replicate measurements, are given in parentheses. [b] Plate numbers were calculated, assuming a Gaussian peak, from the fwhm of the electrophoretic peak and the migration time. [c] Plate numbers determined for the LIF system. [d] The 281 fragment comigrated with the 271 fragment.

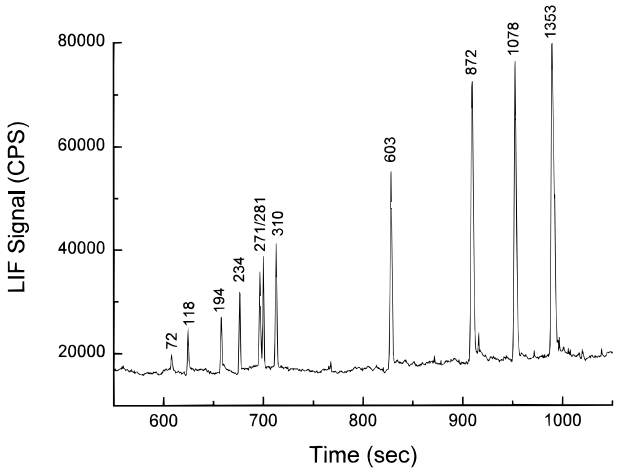


Figure 4. Electropherogram of ΦX174 DNA restriction fragments with LIF detection. The dye concentration added to the running buffer was 1.0 μM, and the running buffer was composed of 40 mM TRIS, 20 mM sodium acetate, 2.0 mM EDTA (pH 7.6), and 0.25% HPMC. The sample was electrokinetically injected for 6 s at −2.0 kV onto the column. The electric field strength used for the separation was 175 V/cm. The laser power was set at approximately 8.5 mW.

interval, was 465. The on-column mass limit of detection for this fragment under these electrophoresis conditions was estimated to be 20 fg (SNR = 3), comparable to the visible LIF detection of this same digest stained with TO.[13]

The magnitude of the background signal, which in part determines the limit of detection, depends on several experimental parameters, including the concentration of the dye in the running buffer, the applied electric field strength, the magnitude of Raman and Rayleigh scattering, and the intensity of the laser beam. Since the running buffer consisted of the staining dye, one of the major contributions to the background is fluorescence from free dye and scattering in the form of Raman and/or Rayleigh scattered photons. Using a 1 μM dye solution added to the running buffer, we observed an increase in the background signal of approximately 10−15% with respect to the HPMC containing TAE solution only. In addition, we noticed that significant decreases in the background signal in the presence of free dye was observed at lower electric field strengths. Using a dye concentration of 1 μM, the background signal decreased by 9.3% when the electric field was decreased from −270 to −135 V/cm. We attributed this reduction in the fluorescence intensity resulting from free dye due to photobleaching of the staining dye, since many near-IR dyes have been shown to exhibit poor photochemical stability as compared to the visible fluorescent dyes.[31] Since the dye travels from the anodic to cathodic reservoirs due to its cationic nature and the DNA travels in the opposite direction, the bleached dye can compete with unbleached dye for the limited number of binding sites associated with the DNA in each electrophoretic band. Therefore, lower field strengths can degrade the limit of detection in situations where the staining dye is added to the running buffer. In the present case, we observed a SNR for the 603 bp fragment of 191 when the electrophoresis was run using a field strength of −135 V/cm; when the field strength was increased to −270 V/cm, the SNR increased to 231 when the DNA sample was injected onto the column using identical conditions and similar DNA concentrations for both cases.

From the electropherogram depicted in Figure 4, it is seen that the 271/281 bp fragments are nearly baseline resolved, with a resolution determined to be 1.3, significantly better than that observed for the conventional CE results where the native, EtBr-stained, and TAG-stained 271/281 bp fragments comigrated (see Table 1). Since the capillary length and conditions were similar in both cases and the sieving buffer was identical, the enhanced resolution in the LIF system most likely results from improvements in the efficiency of the separation and not differences in selectivity. This supposition is supported by our data, which showed that the plate numbers for these restriction fragments when stained with TAG were found to be significantly better for the LIF system compared to the conventional CE case. For the conventional system, the plate numbers for the 603 fragment were 4.13×10^5 plates/m, while for the LIF system, the plate numbers for this same fragment was 1.06×10^6 plates/m. The significant improvement in efficiency most likely results from the fact that the zone variances arising from the finite injection volume and detection volume are smaller in the LIF case compared to the conventional case since, in the LIF system, the beam length was significantly smaller and the amount of sample inserted onto the column was reduced. The need for extended injection conditions in the case of UV detection resulted from the need to obtain sufficient signal strengths for analysis by inserting more material onto the column.

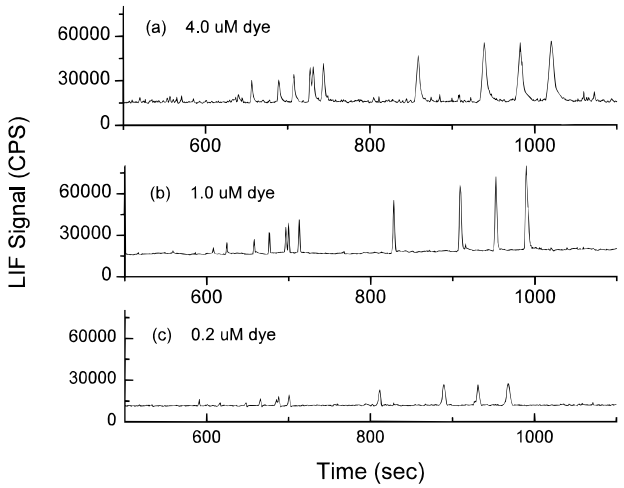


Figure 5. Electropherograms of ΦX 174 DNA restriction fragments obtained using (a) 4.0, (b) 1.0, and (c) 0.2 μM dye in the running buffer. See Figure 3 for experimental details.

We next investigated the effect of dye concentration placed in the running buffer on CE detectability and efficiency for TAG-stained DNA restriction fragments. The results of this investigation for three different dye concentrations are shown in Figure 5 (0.2, 1.0, and 4.0 μM). The fluorescence intensity of the DNA fragments decreased significantly when the dye concentration in the buffer was decreased to 0.2 μM due to less dye incorporated into the dsDNA fragment, which is based on thermodynamic considerations (see Figure 5c). In addition, it was found that the peaks broadened as well when the dye concentration was reduced to 0.2 μM as compared to the case when 1 μM dye was used ($N = 1.38 \times 10^6$ plates/m for 0.2 μM dye and $N = 1.06 \times 10^6$ plates/m for 1.0 μM dye for the 603 bp fragment). Increasing the dye concentration in the running buffer to 4.0 μM deteriorated the peak shapes of all DNA fragments, causing significant peak broadening and tailing (see Figure 5a, $N = 6.80 \times 10^5$ plates/m for the 603 bp fragment). In comparison to the data obtained with 1.0 μM dye, the peak areas were found to be approximately 4 times larger for the 4.0 μM dye solution. However, the peak heights were nearly equivalent (slightly higher and lower for 72−310 and 603−1353 bp fragments, respectively), indicating that the peaks were broadened under these conditions, as apparent from the reduced plate numbers for the 4 μM dye case. Schwartz et al.[2] postulated that the excess dye possibly binds to ssDNAs as well. If this binding process becomes thermodynamically preferable, the presence of excess dye could destabilize the DNA double helix. As a result, it is likely to result in broad peaks with longer migration times, consistent with our data.

CONCLUSIONS

We have prepared a near-IR, monomeric nuclear staining dye (TAG) which forms stable complexes to dsDNA and exhibits a fluorescence enhancement ratio of bound to free dye of approximately 102, making it an ideal probe for the detection of DNA restriction fragments or other dsDNAs, such as PCR products. In addition, this dye has an absorption maximum near the principal lasing line of a GaAlAs diode laser, allowing the construction of an LIF detector for capillary electrophoresis which consists of all-solid-state components, reducing cost and simplifying operation. The detection limit of the dsDNA fragments was determined to be 20 fg of DNA per electrophoretic band, comparable to those obtained with visible LIF systems using Ar ion laser excitation. Using capillary electrophoresis with an entangled polymer sieving matrix, TAG-stained dsDNA fragments were found to have improved efficiency compared to EtBr-stained fragments, most likely resulting from the higher affinity of TAG to dsDNA compared to EtBr.

The attractiveness of near-IR LIF detection also arises from the fact that the inexpensive diode laser and avalanche diode can easily be miniaturized and integrated directly onto a microfabricated electrophoresis system for the analysis of dsDNAs, producing a fully miniaturized apparatus. Work in our laboratory is currently underway toward developing miniaturized, high-sensitivity LIF detectors for microelectrophoresis systems. In addition, we are synthetically preparing nuclear staining homodimers which will also have absorption and emission properties associated with the near-IR region in order to improve detection limits.

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