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Characterization of TO-PRO-3 as an intercalator for double-stranded DNA analysis with red diode laser-induced fluorescence detection

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Abstract

We report the analysis of double-stranded DNA (dsDNA) with a blue intercalating dye, TO-PRO-3 (TP3) using a visible diode laser-induced fluorescence (LIF) detection. In the presence of single-stranded DNA (ssDNA), such as $pd(A)_{40-60}$ or $pd(T)_{20-40}$, TP3 absorption maximum shifts slightly from 631 to 633 nm and, when intercalated to dsDNA, the absorption maxima shifts to 643 nm. TP3 itself does not fluoresce in the presence of either $d(A)_{18}$ or $d(T)_{18}$ alone, but the combination of both oligonucleotide species yielded intense fluorescence, about two orders of magnitude higher. The LIF detection was based on the use of a 2.5-mW diode laser emitting light at 635 nm and the fluorescence of the resulting dsDNA-bound TP3 was collected at 670 nm. The capillary electrophoretic (CE) separation of fragments from 72 to 1353 basepairs (bp) of ΦX174/*Hae*III digest were well-resolved within 10 min in the gel–buffer system with TP3. The application of TP3 as an intercalating dye for the analysis of dsDNA fragments produced by polymerase chain reaction (PCR) was examined. Excellent correlations between CE-LIF area and fragment size in basepairs were obtained with TP3 and ethidium bromide as the intercalating dyes. TP3-based chemistry along with the diode laser-induced fluorescence detection system is well-suited for rapid, high-sensitivity and automated DNA analysis of the PCR-amplified dsDNA products and DNA restriction fragments. © 1997 Elsevier Science B.V.

Keywords: Detection, electrophoresis; DNA; TO-PRO-3

1. Introduction

The use of capillary electrophoresis (CE) for the separation of DNA fragments is well-established [1–6]. Analysis of DNA restriction fragments or polymerase chain reaction (PCR)-amplified DNA with 100–1500 basepairs (bp) can usually be completed within 10–20 min by a CE procedure with a high separation efficiency that cannot be readily achieved by the traditional gel electrophoresis method. The sensitivity of the CE procedure by the direct UV detection method (260 nm for DNA) is relatively poor with a detection limit of a few mg/ml of dsDNA sample [6], similar to that of the conventional gel-based DNA analysis using the ethidium bromide staining method [20].

To increase the detection sensitivity for the analysis of dsDNA by CE, Schwartz and Ulfelder [7] initiated the use of thiazole orange (TO) as an intercalator for laser-induced fluorescence (LIF; excitation, 488 nm) detection for CE–LIF analysis of DNA. The dsDNA-bound TO fluoresces (emission, 520 nm) at a substantially higher intensity than that of the free TO (emission at 580 nm). Excellent

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detection sensitivity using TO for DNA analysis by CE–LIF at 0.1 ng/ml was reported recently by Zhu et al. [8]. Similarly, YO-PRO-1, {1-(4-[3-methyl-2,3-dihydro- (benzo-1,3-oxazole) -2-methylidene-]-quinolinium)-3-trimethylammonium propane diiodide} (YP1) was studied extensively by McCord et al. [9] as an intercalator for CE–LIF analysis of dsDNA with a detection sensitivity of a few ng/ml dsDNA using an argon-ion laser (488 nm).

Ethidium bromide is a popular intercalating agent for the analysis of dsDNA and can be used in LIF detection with a green He–Ne laser emitting at 543 nm [10,11] or an argon-ion laser emitting at 488 nm [9]. The fluorescent emission maximum of dsDNA-bound ethidium bromide is at 610 nm [9–11], while that of the free ethidium bromide is 635 nm.

Diode lasers are relatively inexpensive and are a reliable illumination source for many analytical applications [12,13]. The output power requirement for the laser in LIF detection is usually a few mW, and the commercially available diode lasers emitting at the near infrared region between 635 and 830 nm are an ideal light source with a typical output of 3–20 mW. The use of a diode laser for LIF detection in CE has been reported [14–16].

The objective of this study is to investigate blue intercalating fluorescent dyes suitable for dsDNA analysis using a red diode laser for excitation.

A blue fluorescent dye, TO-PRO-3 {1-(4-[3-methyl-2,3-dihydro- (benzo-1,3-thiazole) -2-propylidene]-quinolinium)-3-trimethylammonium propane diiodide} (TP3) was proposed as an intercalating agent, with a reported excitation maximum at 642 nm and emission at 661 nm [17].

TP3 was reported to be one of the fluors for measuring relative DNA content in a fixed-cell system by flow cytometry using a red He/Ne laser emitting at 632.8 nm [18]. The following report is a summary of the characterization of TP3 and its interaction with dsDNA.

2. Experimental

2.1. Materials

TO-PRO-3 (TP3) was purchased from Molecular Probes (Eugene, OR, USA) as a 1.0 m*M* solution in dimethylsulfoxide. Boric acid and other buffer components and ΦX174/*Hae*III restriction fragments were obtained from Sigma (St. Louis, MO, USA). Single-stranded oligomers, $pd(T)_{20-40}$ and $pd(A)_{40-60}$ were purchased from Pharmacia Biotech (Piscataway, NJ, USA). Pure oligonucleotides, $d(A)_{18}$ and $d(T)_{18}$ were supplied by Dr. Jang Rampal of Beckman Instruments.

2.2. Measurement of UV–Vis and fluorescence spectra

UV–Vis spectra of TP3 and TP3-dsDNA in TBE buffer, pH 8.3, were recorded on a Beckman 7500 diode-array spectrophotometer (Fullerton, CA, USA). Fluorescence spectra were recorded on a Perkin-Elmer LS50 Luminescence Spectrometer (Beaconsfield, Buckinghamshire, UK).

The fluorescence intensity of the TP3 in dsDNA, or single-stranded (ssDNA) such as $pd(T)_{20-40}$ or $pd(A)_{40-60}$, or a combination of both in TBE buffer was measured at 670 nm with an excitation of 635 nm.

2.3. Capillary electrophoresis procedures

CE separations were performed on a P/ACE 2200™ equipped with a laser-induced fluorescence detection system and a 2.5-mW, 635-nm diode laser (Beckman Instruments). The fluorescence emission filter of 670±9 nm was purchased from Omega Optical (Brattleboro, VT, USA).

For capillary zone electrophoresis, samples were introduced by pressure injection (0.5 p.s.i.; 1 p.s.i.= 6894.76 Pa) for 20 s into an untreated fused-silica capillary of 27 cm length (20 cm to detector window)×20 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA), and the running buffer was a sodium salt of 100 m*M* borate containing 1 μ*M* TP3, pH 10.2. Between runs, the capillary was washed sequentially with 1.0 *M* sodium hydroxide and water (12 s of high pressure rinsing at 15 p.s.i.), followed by reconditioning with the running buffer for 1 min.

For gel electrophoresis, a coated capillary (27 cm in length (20 cm to detector window)×75 μm I.D. (dsDNA-1000 kit from Beckman Instruments) was used for DNA size analysis. The gel consists of 3%

linear polyacrylamide in 89 m*M* Tris, 89 m*M* boric acid and 2 m*M* EDTA at pH 8.3 (dsDNA-1000 kit from Beckman Instruments). TP3 at final concentration of 0.05–1.0 μ*M* was added to the above gel solution.

Samples were introduced to the capillary by either pressure injection (0.5 p.s.i./5 s) or voltage injection (typically 5 kV/5 s) and the separation was performed at 22°C with the applied voltage shown in each of the electropherograms. Between runs, the capillary was rinsed with the running buffer for 5 min (high pressure rinsing at 15 p.s.i.) to ensure reproducibility.

3. Results and discussion

3.1. Spectral characteristics of TP3

The structure of TP3 and its visible spectra are shown in Fig. 1. The absorption λ_{max} of TP3 is 631 nm in aqueous solution with significant absorbance at 543 nm. Molar absorptivity of TP3 was reported to be 110 000 M^{-1} cm^{-1} at 631 nm [17]. In the presence of dsDNA, the absorption λ_{max} of TP3 shows a significant shift to 643 nm.

This spectral shift can be reproduced by measuring TP3 in ethanol, indicating that the intercalation of TP3 in dsDNA is similar to its solvation in ethanolic medium. In the presence of single-stranded DNA (ssDNA), the absorption maximum shifted slightly to 633 nm (data not shown).

Excitation of TP3 with either 635 or 543 nm wavelength results in a relatively weak fluorescence emission λ_{max} at 650 nm in an aqueous solution. Addition of the dsDNA to TP3 in an aqueous solution, however, results in a drastic enhancement in fluorescence intensity with an emission λ_{max} at 665 nm.

In contrast, the intercalation of ethidium bromide [11] or thiazole orange [12] with dsDNA, results in blue shift of the fluorescence emission maximum.

In the presence of a single-stranded DNA, such as $pd(A)_{40-60}$ or $pd(T)_{20-40}$, the fluorescent intensity of

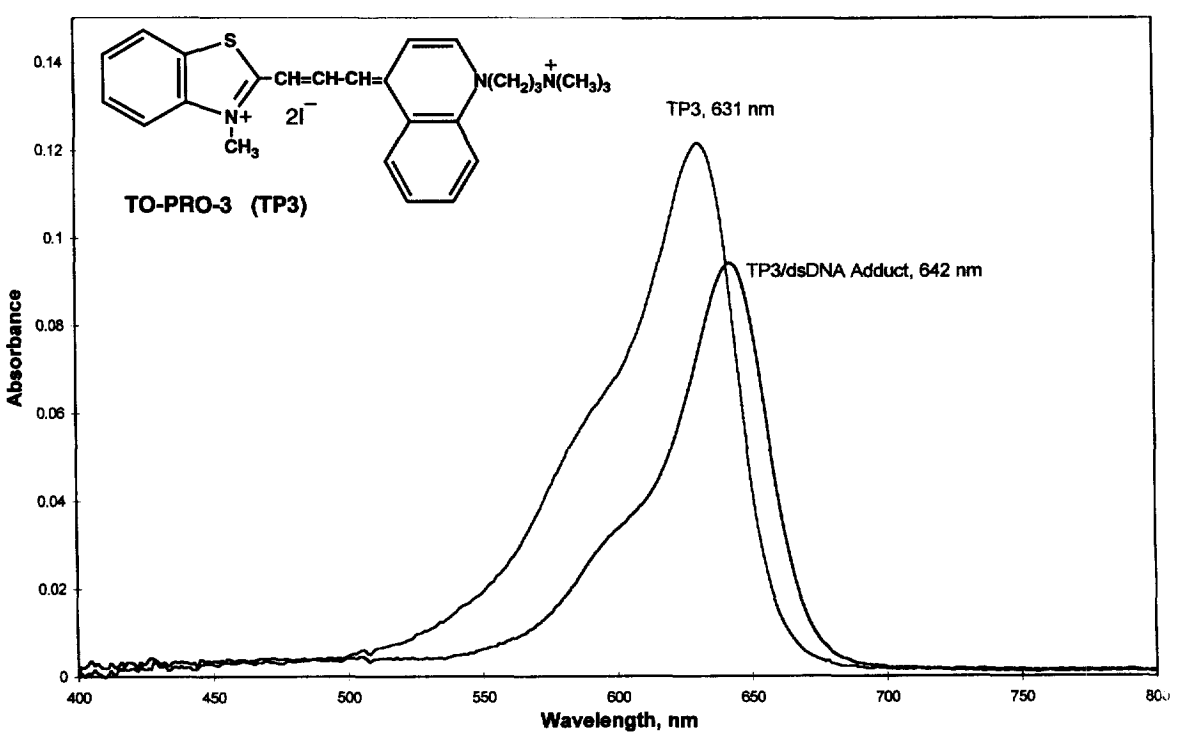


Fig. 1. Structure and visible spectra of TP3 and its adduct with dsDNA.

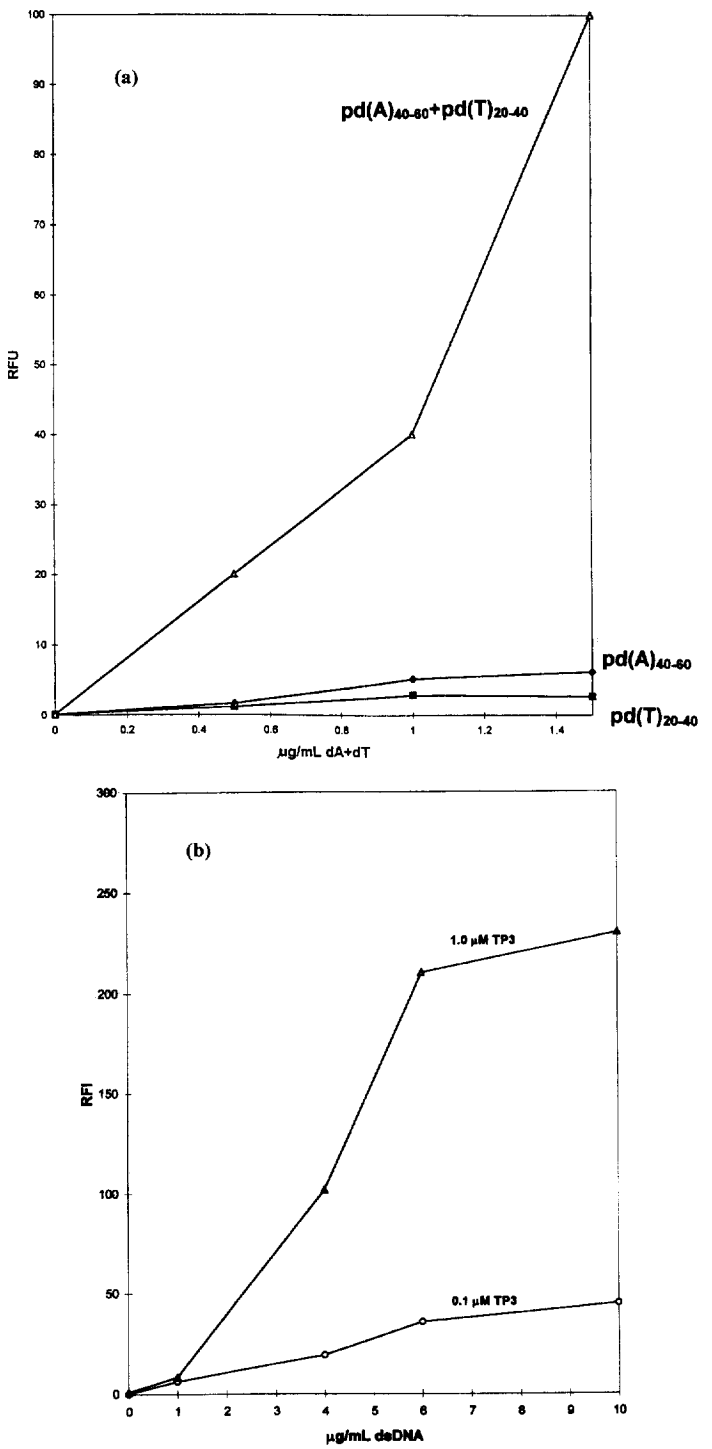
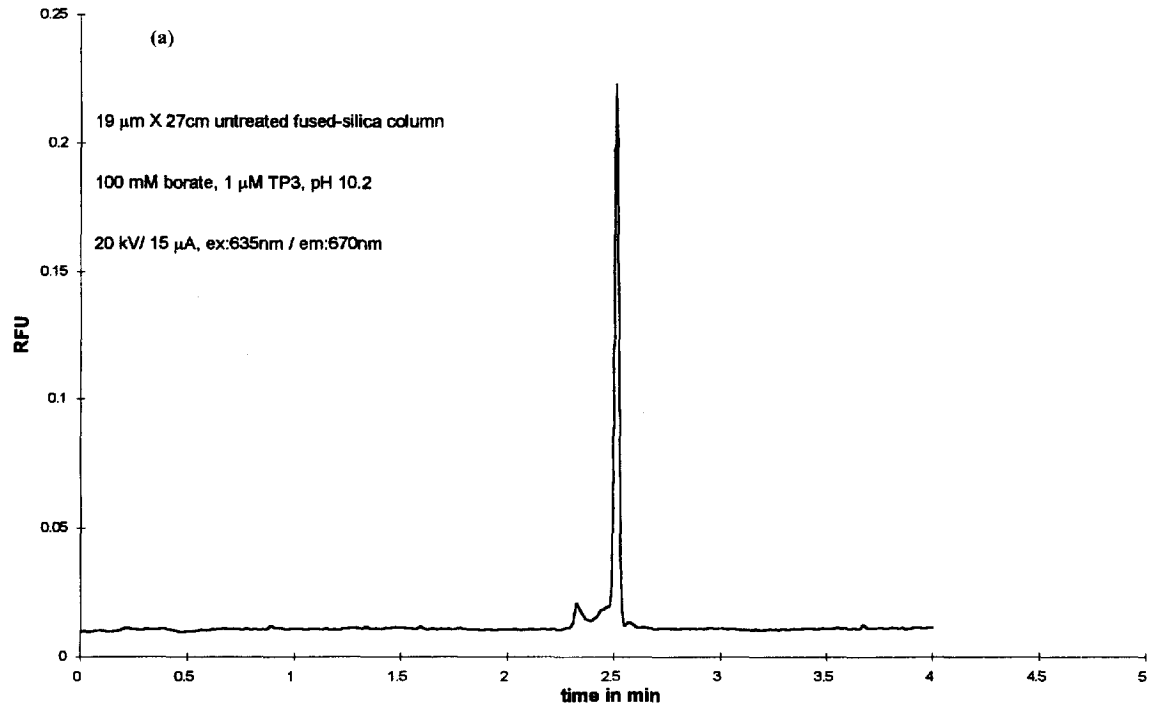


Fig. 2. (a) Fluroescent intensity of TP3 in the single-stranded $pd(A)_{40-60}$ and $pd(T)_{20-40}$ and combination of both $pd(A)_{40-60}$ and $pd(T)_{20-40}$. (b) Relative fluorescence intensity (RFI) of TP3 with dsDNA (ΦX174/*Hae*III) at 0.1 and 1.0 µ*M*.



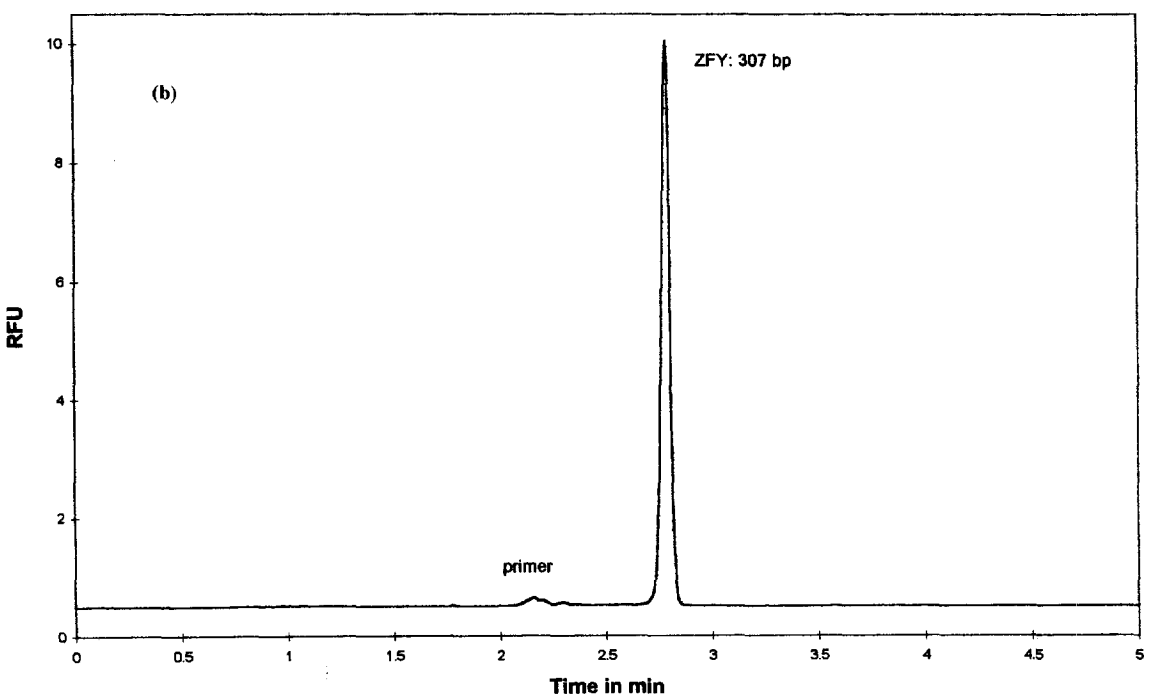


Fig. 3. (a) Electropherogram of $d(A)_{18}$ and $d(T)_{18}$ and TP3 complex. Conditions: 27 cm×20 μm untreated fused-silica capillary; running buffer, 100 m*M* borate buffer, pH 10.2, containing 1 μ*M* TP3; 20 kV/15 μA; detection, 635 nm excitation, 670 nm emission. (b) Electropherogram of a ZFY PCR product of 307 bp. Conditions as in (a).

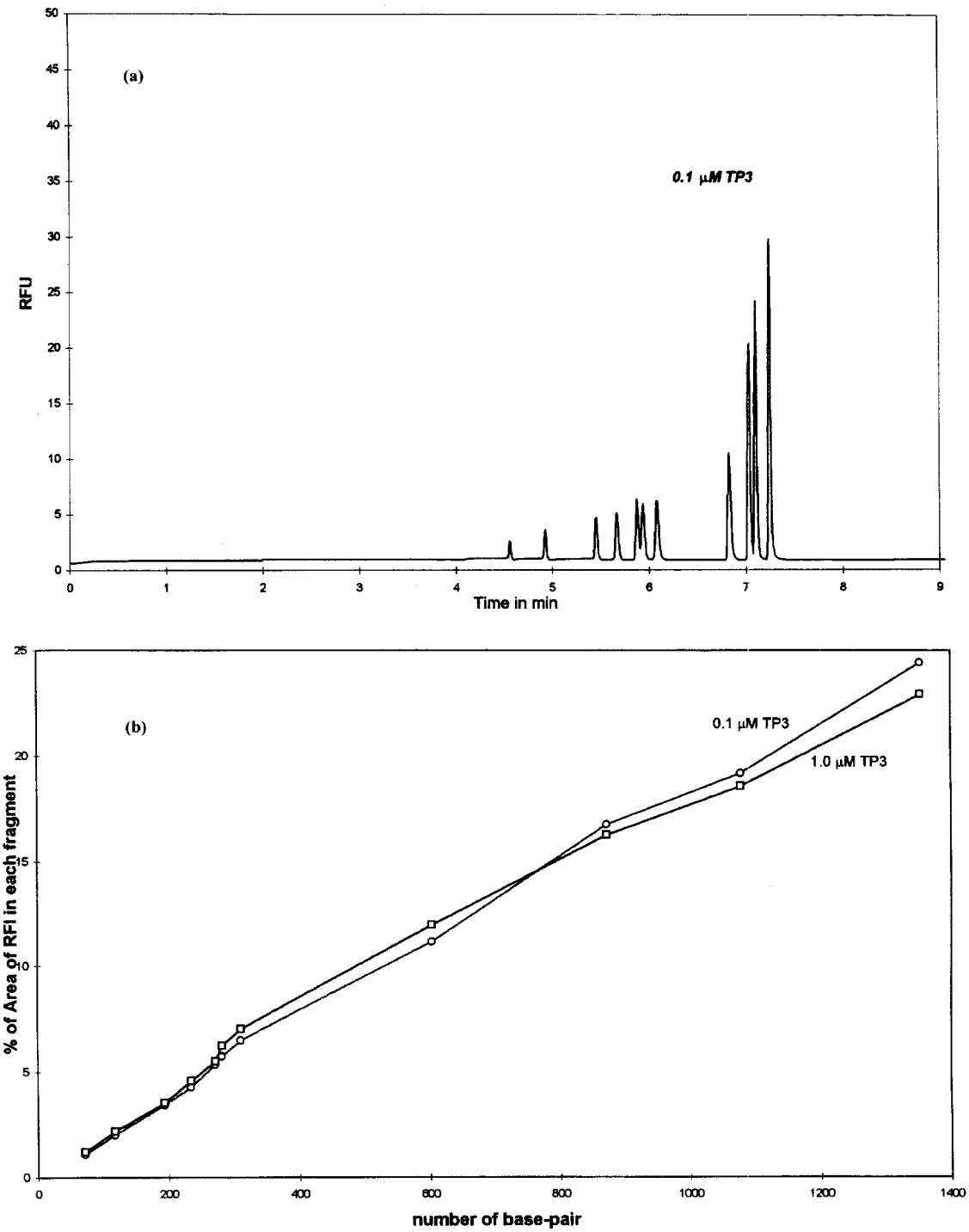


Fig. 4. (a) Electropherogram of ΦX174/*Hae*III restriction fragements in 3% LPA with 0.1 μ*M* TP3. Conditions: 27 cm×75 μm coated capillary; 8 kV/23 μA; detection, 635 nm excitation, 670 nm emission. (b) Relative fluorescence unit of each ΦX174/*Hae*III restriction frgaments vs. number of their basepair.

the TP3 at 10−7 *M* is insignificant; however, when both species were present, there was a drastic increase in TP3 fluorescent intensity due to the formation of dsDNA of the $pd(A)_{40-60}$ and $pd(T)_{20-40}$, consistent with the specificity of the TP3 intercalation with the dsDNA (Fig. 2a).

The relative fluorescence intensity of the intercalation complex of TP3 at 0.1 and 1.0 μ*M* in the presence of varying amounts of dsDNA is presented in Fig. 2b.

3.2. Analysis of dsDNA by CE–LIF using TP3

3.2.1. Capillary zone electrophoresis

Free zone electrophoresis with a UV detection at 254 nm of oligonucleotide mixtures $d(A)_{18}$ and

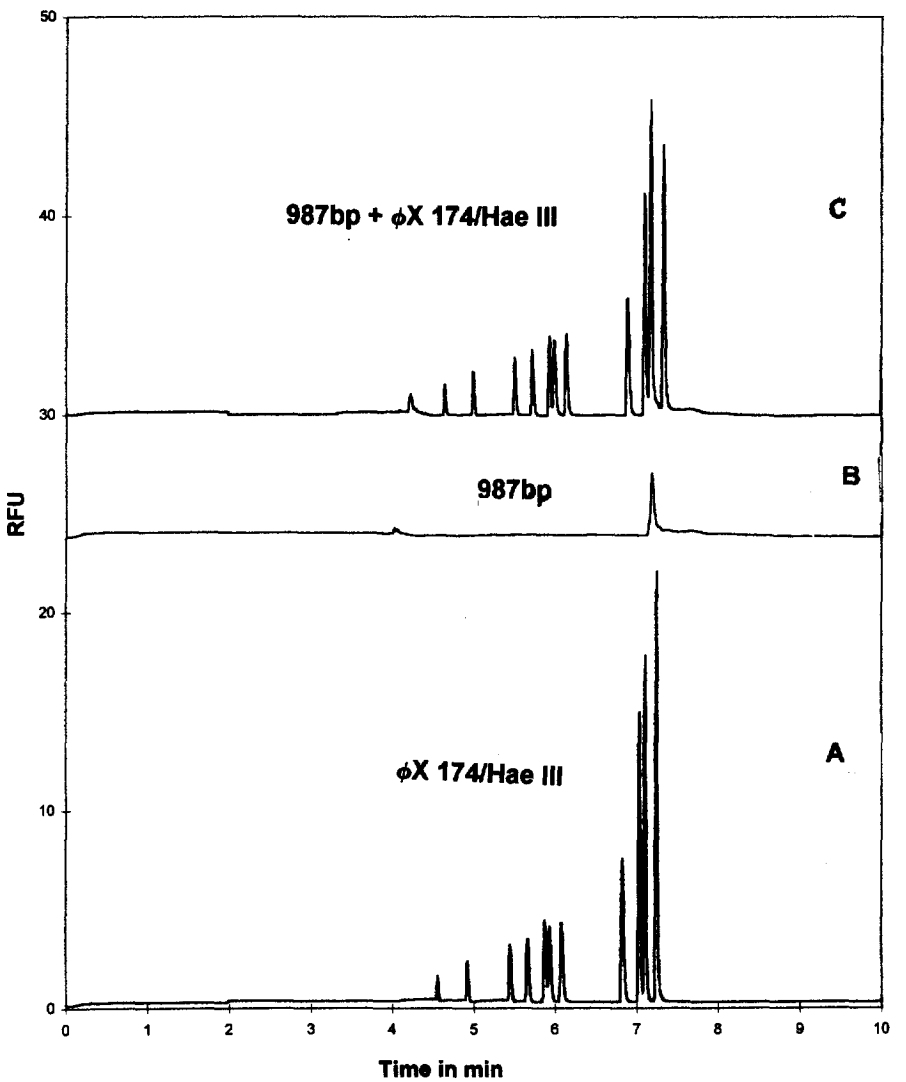


Fig. 5. (A) Electropherogram of ΦX174/*Hae*III restriction fragements. (B) Electropherogram of a PCR product of 970 bp. (C) Electropherogram of ΦX174/*Hae*III restriction fragements and a PCR product of 970 bp in 3% LPA with 0.1 μ*M* TP3, 27 cm×75 μm coated capillary; 8 kV/24 μA; detection, 635 nm excitation, 670 nm emission.

$d(T)_{18}$ in borate buffer containing 1.0 μM TP3 showed the discrete electrophoretic mobility of the $d(A)_{18}$ and $d(T)_{18}$ complex, a distinctly new species with higher electrophoretic mobility towards anode than either one of the species, presumably due to the hybridized $d(A)_{18}/d(T)_{18}$ complex (data not shown).

In the presence of the LIF detection system, however, neither $d(A)_{18}$ nor $d(T)_{18}$ produced any fluorescent signal in CE–LIF analysis, while the combination of $d(A)_{18}$ and $d(T)_{18}$ at an equal molar concentration (0.2 μM each) exhibited very intense fluorescent signals with the hybridized dsDNA–TP3 complex (Fig. 3a). This result is consistent with the characteristic of fluorescence enhancement of TP3 binding to dsDNA.

This unique characteristic makes TP3 applicable to the analysis of PCR products where the dsDNA product can be readily separated from the excess amount of oligonucleotide primers.

Fig. 3b shows a real sample of the PCR-amplified ZFY DNA gene of 307 bp [11,19]. The excess primers (which migrate at 2.15 min) exhibit a relatively low fluorescent intensity, while the PCR product of 307 bp (which migrates at 2.77 min) appears as the major fluorescent species.

3.3. Capillary gel electrophoresis

TP3 in the running buffer at concentrations from 0.05 to 0.5 μM in the CE system produces a relatively low fluorescence intensity at 670±10 nm. At concentrations higher than 1.0 mM, TP3 tends to cause the precipitation of dsDNA.

Fig. 4a shows the electropherogram of the ΦX174/*Hae*III digest in 3% LPA [3] with 0.1 μM TP3. The present method of dsDNA analysis by CE–LIF in TP3 is reproducible in migration time with an R.S.D. of 0.7% or less in six consecutive runs, providing that, between runs, the capillary is thoroughly rinsed with running buffer.

Inspection of the relative intensity of each peak with respect to the number of basepairs (bp) indicates that the distribution of TP3 in dsDNA is dependent on the number of basepairs, as shown in Fig. 4b.

A sample of the PCR reaction product, with an anticipated size of 970 bp, was analyzed by the present method using 0.1 μM TP3 as an intercalating agent. The result showed the 970 bp comigrated with a 1021-bp restriction fragment of the ΦX174/*Hae*III (Fig. 5A).

4. Conclusions

In summary, the utility of a diode laser-induced fluorescence detection for dsDNA using TP3 as an intercalator was demonstrated.

The restriction fragments from ΦX174/*Hae*III and DNA produced by PCR amplification could be readily separated by the present CE–LIF system with a buffer containing TP3.

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