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Original Paper

Capillary electrophoresis of double-stranded DNA fragments using a new fluorescence intercalating dye EvaGreen

EvaGreen is a new DNA intercalating dye successfully used in quantitative real-time PCR. In the present work, we firstly apply EvaGreen to the analysis of dsDNA by CE with LIF detection. Comparisons of EvaGreen dye with the commonly used dyes SYBR Green I and SYBR Gold were preformed in dsDNA analysis by CE. The linear range of dsDNA using EvaGreen was slightly wider than that using SYBR Gold and SYBR Green I, and the detection limits of dsDNA were not significantly different for the three dyes. Good separations of dsDNA fragments were obtained using the three dyes. Reproducibility of migration time and the peak area of dsDNA fragments with EvaGreen were better than those for SYBR Green I and SYBR Gold. The RSD values were 0.24–0.27% for migration time and 3.45–7.59% for peak area within the same day, 1.35–1.63% for migration time and 6.72–12.05% for peak area for three days. Our data demonstrated that EvaGreen is well suited for the dsDNA analysis by CE with LIF detection.

Keywords: Capillary electrophoresis / EvaGreen / Intercalating dye

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1 Introduction

CE has become a powerful method for the analysis of nucleic acids mainly including the analysis of PCR products [1, 2] and restriction digest fragments [3–5], mutation detection and DNA sequencing based on the size-separation in gel media or polymer solutions [6–9], due to its speed, sensitivity, high resolution, automation, and so on. Originally, the UV-detection system is often used in CE analysis of nucleic acids. However, UV detection was of low sensitivity, and the concentration of the sample needs to be up to 10^{-6} M [10]. LIF detection is the most sensitive method, and is widely used in the analysis of DNA. Due to its very poor native fluorescence of DNA, fluorescent derivatization is needed in the LIF detection of DNA fragments. Usually, two methods are used to obtain fluorescent DNA derivatives. One is the covalent coupling of DNA with fluorophores. This method is mostly used in DNA sequencing using fluorescent labeled primers. The alternative method is to label DNA fragments using intercalating dyes. Some dyes easily intercalate between dsDNA chains and form dye–DNA complexes by noncovalent interactions. The commonly used intercalating dyes include ethidium bromide (EtBr), thiazole orange (TO), oxazole yellow (YO), and their homodimers such as TOTO and YOYO [11–14]. These dyes possess very low native fluorescence, but they emit very strong fluorescence when they intercalate in dsDNA chains.

Recently, a series of unsymmetrical cyanine dyes, such as SYBR Green I and SYBR Gold have been developed for the analysis of nucleic acids in slab gel electrophoresis and CE. These dyes have low intrinsic fluorescence, high-binding affinity to nucleic acids, and the DNA–dye complexes have high fluorescent quantum yield, and therefore, they are widely used in the analysis of nucleic acids by slab gel electrophoresis [15, 16] and CE [17–24]. The advantages and drawbacks of different dyes were compared using CE or slab gel electrophoresis [20, 25–29].

More recently, EvaGreen is emerging as a new alternative attractive fluorescent dye for quantitative real-time PCR. This dye is characterized by very little PCR inhibition, high fluorescence intensity, excellent stability in the real-time PCR, and its absorption and emission spectra were similar to that of SYBR Green I. To the best of our knowledge, there was no systematic study on the use of EvaGreen in CE.

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Abbreviations: PDMA, polymethylacrylamide; **TAPS**, *N*-tris-methyl-3-aminopropanesulfonic acid

In this paper, we systematically investigate the characteristics of EvaGreen in CE of dsDNA fragments as fluorescent intercalating dye, and compare this dye with the commonly used dyes SYBR Green I and SYBR Gold in the quantification of DNA by CZE and the separation of DNA fragments by capillary gel electrophoresis. Furthermore, the advantages and drawbacks of the three dyes are discussed.

2 Materials and methods

2.1 Materials

Fused capillaries with 75 μm id were purchased from Yongnian Optical Fiber Factory (Yongnian, Hebei, P. R. China). SYBR Green I and SYBR Gold were from Molecular Probes (Eugene, OR, USA; concentration not given). EvaGreen™ was purchased from Biotium (Hayward, CA, USA). The digests of λ-Hind III DNA were provided by Takara (Japan). The concentration of λ-DNA was measured on a Hoefer DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA). TEMED and ammonium peroxydisulfate (APS) were purchased from BioRad Laboratories (Hercules, CA, USA). Calf thymus DNA, acrylamide, and *N*-trismethyl-3-aminopropanesulfonic acid (TAPS) were purchased from Sigma (St. Louis, MO, USA). TAPS solution is a zwitterions buffer, and is adjusted to pH 7.6 with 1 M NaOH solution. All solutions were prepared with ultrapure water with 18.2 MΩ/cm (Millipore, Bedford, MA. USA), and filtered through 0.22 μm membrane filters prior to use.

2.2 Instrumentation

The Beckman P/ACE MDQ system (Fullerton, CA, USA) was equipped with an LIF detector, in which an argon ion laser operated at 488 nm was the excitation light source. A 520 nm bandpass filter was used as an emission cut-off filter. Beckman System Gold software (version 7.0) was used in system control and data collection.

2.3 Capillary coating

For the suppression of EOF and adsorption of DNA on the capillary inner surface, the inner wall of the capillary was coated with linear polyacrylamide using a modified procedure as described by Hiertén [30]. In brief, a new capillary was rinsed with methanol for 10 min, etched with 1 M sodium hydroxide for 15 min, leached with 1 M hydrochloric acid for 15 min, and washed with water for 15 min, respectively. The capillary was first filled and left standing for 2 h with 50% acetic acid solution containing 10% γ-methacryloxypropytri-methoxysilane, then for 2 h with degassed 3% acryamide solution (5 mL of 3% acrylamide solution containing 4 μL TEMED and 40 μL of 10% APS).

2.4 CZE procedure

A coated capillary was used for the quantification of DNA. Before electrophoresis, TAPS buffer solution (80 mM, pH 7.6) containing a given dye was pumped into the capillary by pressure. An LIF system (excitation wavelength 488 nm; emission wavelength 520 nm) was utilized for DNA detection. The DNA samples were introduced by pressure. Between each run, the capillary was rinsed with distilled water and TAPS buffer solution for 1 min successively. Reversed polarity was used in electrophoresis.

2.5 Capillary gel electrophoresis procedure

A new uncoated capillary was washed with 1 M sodium hydroxide for 10 min, with 1 M hydrochloric acid for 25 min, and with water for 15 min, respectively. Electrophoresis buffer was TAPS buffer solution (80 mM, pH 7.6). In the separation of DNA fragments, 3% polymer (PVP or polymethylacrylamide (PDMA)) solutions containing different concentrations of dyes were used as sieving media. Before each run, the capillary was rinsed with water for 2 min and then filled with separation buffer at a given pressure for 4 min. Samples were introduced by hydrodynamic or electrokinetic modes.

2.6 Fluorescent spectra of DNA–dye complexes

The fluorescence excitation and emission spectra of dsDNA–dye complexes were measured by incubating different concentrations of dyes with 1 μg/mL calf thymus DNA in 80 mM TAPS buffer solution (pH 7.6) in a final volume of 2 mL, for 10 min. Measurements were performed on a Varian Cary Fluorescence Spectrometer with 3 mL quartz cuvettes (1 cm optical path). All the measurements were performed at room temperature.

3 Results and discussion

3.1 Fluorescent spectra of dsDNA–dye complexes

The fluorescence excitation and emission spectra of the dsDNA–dye complexes are shown in Fig. 1 using EvaGreen, SYBR Gold, and SYBR Green I as intercalating dyes. The emission spectra of the three complexes were obtained with the excitation of 488 nm wavelength, and the fluorescence excitation spectra were detected at 540 nm. The excitation maximum of the dsDNA–EvaGreen complex was at 500 nm and the emission maximum was at 529 nm. The excitation and emission maxima of the dsDNA–dye complexes using SYBR Gold and SYBR Green I were 497/546 and 497/526 nm, respectively, which were in agreement with those reported in [31]. The data demonstrated that argon ion laser with 488 nm was a good excitation source for dsDNA–dye complexes.

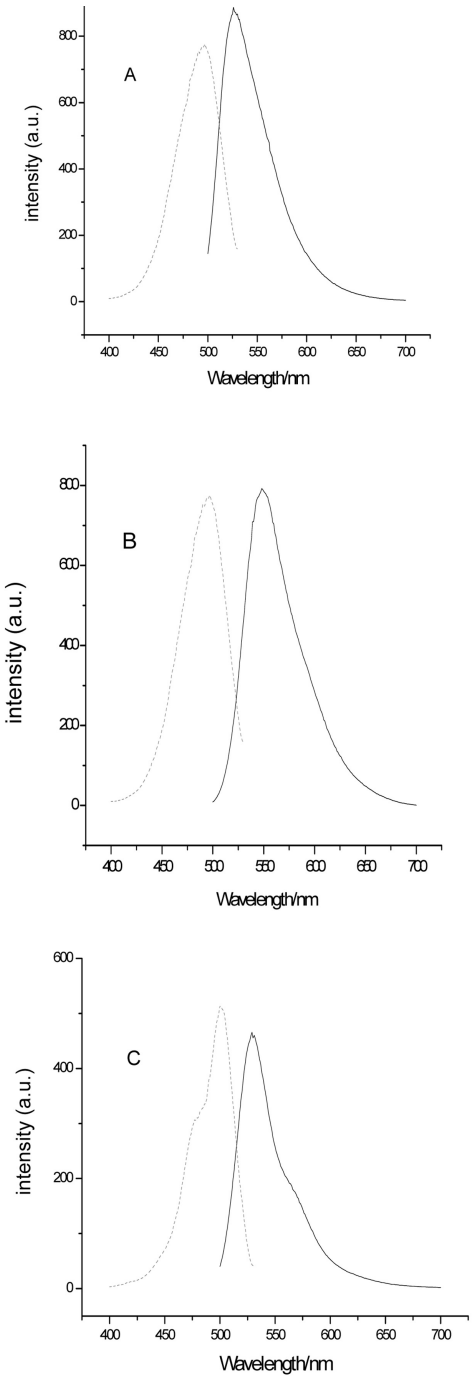

Figure 1. Fluorescence excitation (dashed line) and emission (solid line) spectra of the three DNA–dye complexes; (A) SYBR Green I, (B) SYBR Gold, and (C) EvaGreen. DNA sample was calf thymus DNA.

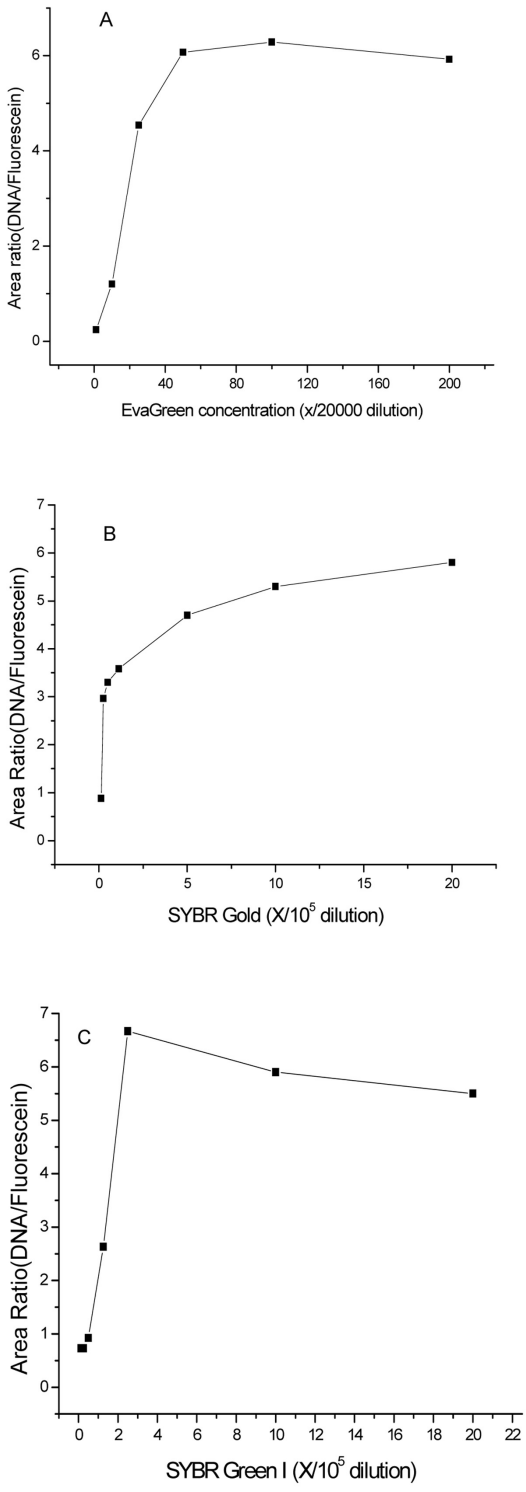

Figure 2. Effect of different dye concentrations on fluorescence intensity for λ-Hind III DNA. The λ-Hind III DNA at a total concentration of 240 ng/mL was injected by pressure (0.5 psi, 6 s). Coated capillary (20 cm effective length, 75 μm id) was used, filled with 80 mM TAPS solution, containing different concentrations of dyes. Electrophoresis buffer was 80 mM TAPS buffer solution (pH 7.6), the temperature was 25°C, and the applied voltage was −400 V/cm.

3.2 Quantitative analysis of DNA using EvaGreen, SYBR Gold, and SYBR Green I

In the quantitative analysis of DNA, the CZE mode was used. The concentration of the intercalating dye is very important to obtain the optimal fluorescence signal of

the DNA–dye complexes [32]. Therefore, we first investigated the effects of the concentrations of the three dyes on the fluorescence intensity (peak area) of dsDNA–dye complexes in CZE-LIF. In order to reduce the effects of some factors, such as laser power fluctuation and injection bias, which may influence accuracy of the results, an internal standard (ISTD, fluorescein) was used in our study. Figure 2 shows the effects of the dye concentrations on the peak area ratios of DNA–dye complexes and ISTD. Figure 2A illustrates that the fluorescence intensity increased with EvaGreen concentration up from 1/20 000 to 1/200 dilution of the stock solution (20 ×) and a dilution of 100/20 000 was the optimum concentration of EvaGreen. Figure 2B documented that the fluorescence intensity increased with the concentration of the SYBR Gold up to 1/5000 dilution of the stock solution. The higher dye concentration caused no significant increase in the fluorescence intensity. A dilution of 1/10 000 was used in the subsequent experiments. Using SYBR Green I, the fluorescence intensity increased with dilution from $1/10^6$ to 1/40 000 and the results are shown in Fig. 2C. At the lower concentration, the fluorescence intensity of the DNA–dye complexes was extraordinarily low. When the concentration of dye was up to a dilution of 1/40 000, the fluorescence intensity increased very rapidly. Higher fluorescence intensity may be the reason that dsDNA–SYBR Green I complexes have an excitation maximum/emission maximum of 496/522 which corresponds more closely to the argon ion laser with 488 nm (excitation wavelength) and 520 nm (emission wavelength) than SYBR Gold (496/540) and EvaGreen [31]. Furthermore, a higher concentration of SYBR Green I caused a decrease in fluorescence intensity.

3.3 Linearity and sensitivity using EvaGreen, SYBR Gold, and SYBR Green I

The optimal concentrations of EvaGreen (dilution 1/200), SYBR Gold (dilution 1/10 000), and SYBR Green I (dilution 1/40 000) were obtained at the concentration of 240 ng/mL λ-DNA. At the optimal concentrations of fluorescent dyes, we further investigated the dependence of the fluorescence intensity of dsDNA–dye complexes over the concentration range of λ-DNA (2.4–120 ng/mL) using CZE-LIF and determined the detection limits. We found that when the concentration of DNA was lower than 2.4 ng/mL the fluorescence intensity of DNA–dye complex became very poor for the three dyes, and when the DNA concentration was up to a certain value (EvaGreen:

Table 1. Linear range and detection limit of dsDNA analysis for EvaGreen, SYBR Gold, and SYBR Green I

Fluorescent dye	Detection limit (S/N = 3) (fg)	Linear detection range (ng/mL)
EvaGreen	10.7	2.4–60
SYBR Gold	11.3	2.4–40
SYBR Green I	13.5	2.4–30

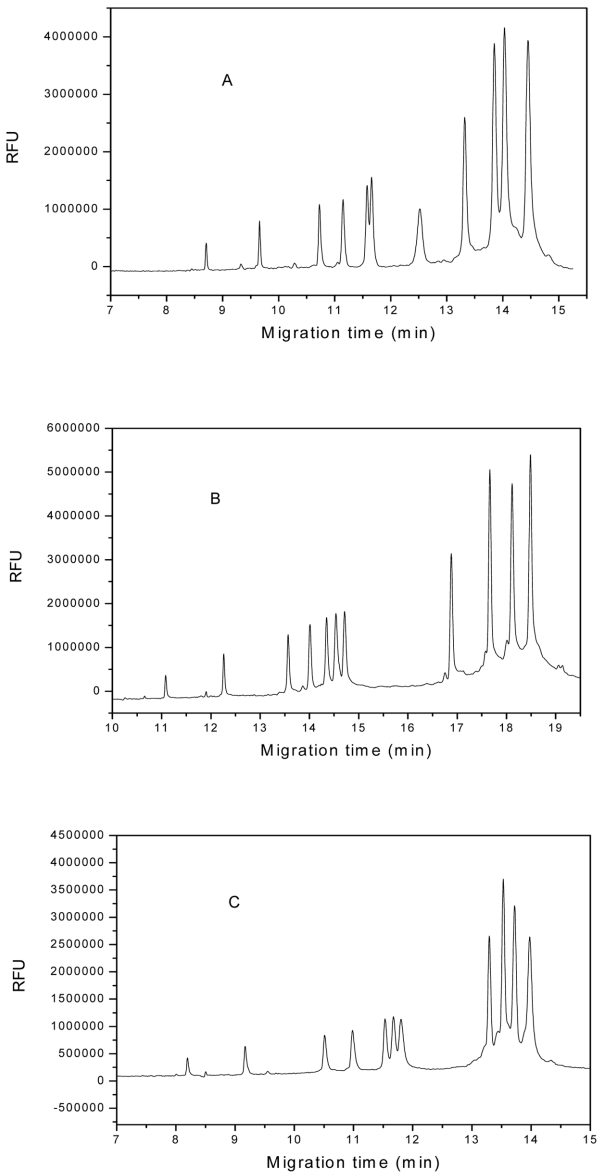

Figure 3. (A) Electropherogram of ϕX174 DNA fragments obtained with SYBR Green I. Uncoated fused-silica capillary was used with 50 cm of total length, 40 cm of effective length, and 75 μm id. Applied voltage was −400 V/cm, and running buffer was 80 mM TAPS buffer solution containing 3% PDMA at pH 7.5. Electrokinetic injection at −4 kV for 2 s was used, the temperature was 25°C and the applied voltage was −20 kV. RFU, relative fluorescence units. (B) Electropherogram of ϕX174 DNA fragments obtained with SYBR Gold. Electrokinetic injection at −4 kV for 7 s was used; other conditions were the same as those described in (A). (C) Electropherogram of ϕX174 DNA fragments obtained with EvaGreen. Electrokinetic injection at −4 kV for 5 s was used; other conditions were the same as those described in (A).

Table 2. Reproducibility of DNA analysis with EvaGreen, SYBR Green I and SYBR Gold.

Dyes	DNA size (bp)	RSD, migration time (%)		RSD, peak area (%)	
		Same day (n = 7)	Three days (n = 21)	Same day (n = 7)	Three days (n = 21)
EvaGreen	72	0.24	1.35	7.59	12.05
	118	0.27	1.43	3.45	6.72
	194	0.28	1.57	3.77	8.50
	234	0.27	1.63	4.30	10.74
SYBR Green I	72	1.39	1.45	8.37	10.96
	118	1.12	1.39	10.94	13.36
	194	1.20	1.60	9.13	11.53
	234	1.26	1.67	11.33	11.87
SYBR Gold	72	5.08	5.51	11.18	17.64
	118	5.12	5.53	11.04	13.99
	194	5.21	5.75	14.30	17.20
	234	5.30	6.00	11.20	11.28

Five ng/μl of the total DNA solutions was introduced by pressure (4 s at 0.5 psi). An uncoated fused silica capillary with 40 cm of total length, 30 cm of effective length and 75 μm id was used, applied voltage was −400 V/cm, and running buffer was 80 mM TAPS solution containing 3% PVP at pH 7.6, the temperature was 25°C.

60 ng/mL; SYBR Gold: 40 ng/mL; SYBR Green I: 30 ng/mL), the ratio of DNA-complex and fluoresein deviated from linearity. The detection limit was defined as the DNA sample concentration expected to give a signal (peak height) equal to three-fold of the noise. Using a 10 s low pressure injection (0.5 psi), we estimated that about 10 nL of samples were injected into the capillary and the mass detection limit was obtained. Table 1 summarizes the linear detection ranges and detection limits of dsDNA fragments obtained for the three dyes. We observed that the linear detection ranges of DNA–dye complexes with EvaGreen and SYBR Gold were slightly wider than that with SYBR Green I. The detection limits of dsDNA were not significantly different from those of the three dyes. This result illustrated that EvaGreen was well suited for the quantification of dsDNA with CZE-LIF as well.

3.4 Capillary gel electrophoresis of dsDNA fragments

PDMA can adsorb to the capillary inner surface, and can efficiently suppress the EOF and the adsorption of DNA fragments on the capillary surface. This polymer has also been successfully used as a sieving medium in CE of dsDNA fragments [33]. In this study, 3% PDMA solution was used as a sieving medium. Three dyes were added into 3% PDMA solutions at their optimum concentrations. Figure 3 shows the separations of dsDNA fragments using SYBR Green I (Fig. 3A), SYBR Gold (Fig. 3B), and EvaGreen (Fig. 3C). The results in Fig. 3 illustrate that SYBR Green I and SYBR Gold seemed to have a slightly higher separation efficiency than EvaGreen. In particular, the 271/281 fragments were completely resolved by analysis using SYBR Gold. The different migration times of DNA fragments with the three dyes were mainly attributed to the differences in the structures and molecular weight of the three dyes. When the dye binds to the DNA chain, the structure and the mass-to-charge ratios of DNA fragments are changed, which leads to the alteration of the electrophoresis behavior of DNA fragments.

3.5 Reproducibility of DNA analysis using EvaGreen, SYBR Gold, and SYBR Green I

Reproducibility of DNA analysis with EvaGreen, SYBR Gold, and SYBR Green I was measured using three different uncoated capillaries and 3% PVP sieving medium. The RSD values from the same day and three different days for the DNA fragments of 72, 118, 194, and 234 bp peaks are shown in Table 2. The data demonstrated that EvaGreen had better reproducibility in dsDNA fragment analysis compared to SYBR Green I and SYBR Gold. The RSD analysis of values with EvaGreen were 0.24–0.27% for migration time and 3.45–7.59% for peak area within the same day, 1.35–1.63% for migration time and 6.72–12.05% for peak area for 3 days. The good reproducibility of EvaGreen is probably attributed to the higher stability of the DNA–EvaGreen complex compared to the other two dyes.

4 Concluding remarks

In the present work, EvaGreen as fluorescent intercalating dye was successfully used in the analysis of dsDNA by CE with LIF detection. Compared to the commonly used dyes SYBR Green I and SYBR Gold, EvaGreen showed wider linear ranges in quantitative analysis and better

reproducibility in the separations of dsDNA fragments. Our data demonstrated that EvaGreen was also an attractive alternative DNA intercalating dye, and well suited for dsDNA analysis by CE with LIF detection.

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