Mono- and bis-intercalating dyes for multiplex fluorescence lifetime detection of DNA restriction fragments in capillary electrophoresis

The use of novel intercalating dyes as labels in DNA restriction fragment analysis by capillary electrophoresis with frequency-domain fluorescence lifetime detection is described. The dyes, including one mono-intercalating dye with three positive charges and three bis-intercalating, homodimeric dyes with four positive charges, were excited by the 488 nm line of an argon ion laser and exhibited lifetimes in the range of 1–3 ns. The separations were performed using a gel containing 1% high-molecular-weight (HMW) hydroxyethylcellulose (HEC) (90 000–105 000) and 0.3% low-molecular-weight (LMW) HEC (24 000–27 000) in Tris-borate-EDTA buffer (TBE). Multiplex lifetime detection of mixtures of dye-labeled DNA restriction fragment digests and size standard fragments was achieved. Compared to previous results obtained with several mono-intercalating dyes of lesser charge (McIntosh, S. L., Nunnally, B. K., Nesbit, A. R., Deligeorgiev, T. G., Gadjev, N. I., McGown, L. B., Anal. Chem. 2000, 72, 5444–5449), the present dyes provided a wider range of lifetimes and better lifetime discrimination in multiplex detection. There was no evidence of dye exchange during the capillary electrophoresis experiment.

Keywords: DNA restriction fragment analysis / Intercalating dyes / Multiplex lifetime detection
based on the fluorescence lifetimes of the dye labels. The DNA fragments were labeled with novel, mono- or bis-intercalating dyes containing three and four positive charges, respectively [15]. Free dye was not added to the run buffer, which consisted of a mixture of high- and low-molecular-weight hydroxyethylcellulose (HEC) in Tris-borate-EDTA (TBE) buffer.

2 Materials and methods

2.1 Reagents

The structures of the intercalating dyes are shown in Fig. 1. They include three bis-intercalating, homodimeric dyes (referred to as Dyes 55, 57, 58) and one mono-intercalating dye (Dye 50). Their synthesis is described elsewhere [15]. Stock solutions of the dyes were prepared in DMSO and stored in the refrigerator. The stock solutions were diluted with Tris-HCl buffer (100 mM, pH 8.6). The DNA restriction fragment digest pBR322/BstNI, and a 100 bp size standard ladder, were from New England Biolabs (Beverly, MA, USA). The 200 bp and 500 bp size standard DNA fragments were from Gensura Laboratories (Del Mar, CA, USA). Dye-DNA mixtures were prepared by placing 10 μL of a 3 × 10⁻⁷ M solution of DNA into a plastic sample vial and adding 0.5 μL of a dye solution of appropriate concentration. TBE buffer, (89 mM Tris-borate, 2 mM EDTA, pH 8.3) was used as received from Sigma (St. Louis, MO, USA). The TBE/HEC gel buffer was prepared by dissolving appropriate amounts of high-molecular-weight (HMW; M, 90,000–105,000) and low-molecular-weight (LMW; M, 24,000–27,000) HEC (Polysciences, Warrington, PA, USA), in TBE and stirring overnight. The gels were filtered through a 0.45 μm filter prior to use and a new gel was prepared each day. These gels served as the run buffer without further modification; free dye was not added.

2.2 Instrumentation

Fluorescence spectra of the dyes in batch solution were collected using a phase-modulation spectrofluorometer (SLM 48000S; Spectronics Instruments, Rochester, NY, USA), with xenon arc lamp excitation and photomultiplier tube (PMT) detection. On-the-fly fluorescence lifetime detection of DNA fragments in CE was performed using a MHF fluorescence lifetime instrument (SLM 48000 MHF; Spectronics Instruments) interfaced to a P/ACE 5000 CE system (Beckman Coulter, Fullerton, CA, USA), as previously described [7]. The coated DNA capillary column (internal diameter of 100 μm) was purchased from Beckman (Cat. No. 477477). The column was 47 cm in total length and 39.5 cm to the detector. All experiments were performed at room temperature. Electrokinetic injection, with an injection voltage of 9.0 kV and an injection time of 10 s, was used to introduce the dye-DNA mixtures onto the column. The CE was run in reverse polarity mode using a separation voltage of 9.4 kV (200 V/cm). The typical run time was 35 min. Excitation was achieved using the 488 nm line of an air-cooled argon ion laser (OmniChrome). A 488 nm holographic “notch” filter was placed in the emission beam to reduce scattered laser light, and a 515 nm long-pass filter was used for emission wavelength selection. Scattered light, which has a fluorescence lifetime of essentially zero, provided the lifetime reference.

2.3 Data analysis

In on-the-fly fluorescence lifetime detection, both fluorescence intensity and fluorescence lifetime are recovered from the same dynamic MHF data to provide intensity and lifetime electropherograms [13]. The lifetime data were analyzed using nonlinear least squares (NLLS) analysis software (Globals, Unlimited, Urbana, IL, USA). Lifetime values given in the text correspond to the lifetimes recovered at the apex of each peak. Single-component lifetime models (corresponding to mono-exponential decay) were adequate to describe the data.

3 Results and discussion

3.1 Dye structures

As shown in Fig. 1, Dye 50 is a monomeric dye with three positive charges. It contains several aromatic moieties groups linked by carbon chains, which make it larger
than most common mono-intercalators such as oxazole yellow (YO) or thiazole orange (TO). The homodimeric, bis-intercalating dyes (Dyes 55, 57, and 58) have four positive charges. They all contain the same monomeric units and differ only by the linker joining the monomers to form the homodimer. In a study of the effects of linker structure on the binding properties of the homodimeric intercalating dye TOTO, it was found that longer linkers enabled the dye to bi-intercalate into sites separated by two base pairs, instead of the usual single base pair separation [16]. Longer linkers significantly increased the DNA binding strength of TOTO and its analogs and increased the fluorescence quantum yields of the intercalated dyes.

### 3.2 Fluorescence properties of the intercalating dyes

Intercalating dyes for multiplex fluorescence lifetime detection of DNA fragments in CE should meet several photophysical criteria. The dyes should have similar excitation maxima so that a single laser line can be used for excitation. They should be essentially nonfluorescent when nonintercalated so that the contribution from free dye to the fluorescence background is negligible. Finally, they must have resolvable fluorescence lifetimes so that fragments from different digests can be distinguished. Fluorescence spectra of the dyes in the Tris-HCl buffer were collected in the absence and presence of dsDNA (~1 dye per 5 bp). The fluorescence of the dyes was negligible in the absence of DNA and greatly enhanced upon DNA binding due to the increased structural rigidity of the dye molecule upon intercalation. Spectral characteristics of the dyes in the presence of dsDNA are summarized in Table 1. The dyes have similar excitation maxima (505–507 nm) and can all be excited by the 488 nm or 514 nm line of an argon ion laser. The homodimeric dyes 55, 57 and 58 have almost identical emission maxima (533–534 nm), which would preclude their use in multiplex detection based on fluorescence color. The emission maximum of monomeric Dye 50 is slightly longer (537 nm) but still too close for color discrimination. The homodimeric dyes have very similar molar absorptivities that are more than twice that of the monomeric dye 50. The quantum yields in the presence of dsDNA show more variation, decreasing in the order Dye 57 > Dye 55 > Dye 58. The fluorescence lifetimes range from 1–3 ns, decreasing predictably in the same order as the quantum yields.

### 3.3 Dye exchange studies

An advantage of using bis-intercalating dyes is their high affinity for dsDNA. This is a particularly important consideration for multiplex detection, in which dye exchange among fragments must not occur on the time scale of the separation. The DNA binding constants have not been determined for the dyes used in this study, but studies of other bis-intercalating, homodimeric dyes have shown that the bis-intercalators generally have association constants in the range of $10^{12}$–$10^{13}$ M$^{-1}$, nearly double those of their corresponding monomeric intercalators [17]. A possible problem with bis-intercalating dyes is the appearance of double electrophoretic bands for certain DNA fragments due to variations in dye loadings among fragments of the same length [18].

In the previous study of monomeric intercalating dyes [8], it was found that dyes can be exchanged among fragments. If one of the dyes has a stronger DNA binding affinity than the other, it may replace the other dye in the fragments. The distribution of dyes among fragments is presumed to depend upon the relative DNA binding affinities of the dyes, so that fragments may end up with equal numbers of both dyes or a preponderance of one dye in all fragments. Dye exchange was indicated by spectral shifts in the emission maxima of dye-DNA solutions upon addition of the second dye.

In the present work, the emission maxima of the dyes are useful only in cases involving Dye 50 and one of the homodimeric dyes, since the homodimeric dyes have essentially identical emission maxima. Therefore, it was necessary to use changes in the intensity of the emission signal as an indication of possible exchange of dyes between fragments. Based on changes (or lack of changes) in spectra of dye-DNA solutions for each dye upon addition of a second dye it appears that Dye 50 does not replace other dyes in the fragments: Dye 58 replaces Dye 55 and perhaps Dye 50; Dye 57 replaces Dyes 50 and 58; and there is exchange between Dyes 55 and 57 that favors Dye 57 if the solution is left overnight.

### Table 1. Characteristics of dyes in dsDNA

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation maximum (nm)</th>
<th>Emission maximum (nm)</th>
<th>$\varepsilon^0$</th>
<th>$\Phi^0$</th>
<th>Lifetimes (ns)$^a$</th>
<th>Dye: bp$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>507</td>
<td>537</td>
<td>78 300</td>
<td>–</td>
<td>1</td>
<td>1:1900</td>
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<tr>
<td>55</td>
<td>506</td>
<td>533</td>
<td>162 300</td>
<td>0.69</td>
<td>2.5</td>
<td>1:2900</td>
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<tr>
<td>57</td>
<td>505</td>
<td>533</td>
<td>169 000</td>
<td>0.94</td>
<td>3</td>
<td>1:2200</td>
</tr>
<tr>
<td>58</td>
<td>506</td>
<td>534</td>
<td>170 100</td>
<td>0.48</td>
<td>2</td>
<td>1:4600</td>
</tr>
</tbody>
</table>

a) Molar absorptivity (Lmol$^{-1}$cm$^{-1}$)
b) Fluorescence quantum yield, from [15]
c) Lifetimes typically have standard deviations in the range of $\pm 0.01$ ns. Relative standard deviations in lifetimes recovered across a given peak in an electropherogram all were within 10%.
d) Approximate ratios at which best results for peak resolution and lifetime detection were obtained.
Since Dye 50 is a mono-intercalator, it is reasonable to expect that it could be replaced by, but not replace, a bis-intercalator. Of the bis-intercalators, Dye 57 has the longest linker. Linker length, along with the ability of the linker to cross the minor groove such that the positively charged nitrogen atoms are close to the negatively charged phosphate groups in the DNA backbone, has been reported to increase binding strength [16]. The ability of Dye 57 to replace the other dyes is consistent with its longer linker.

3.4 CE separations of dye-labeled DNA fragments

Figures 2–4 show electropherograms of pBR322/BstNI labeled with the four different dyes. In all cases, fragments below 60 bp in length were not resolved. These fragments migrated as a large peak near 700 s for Dye 50 and 800 s for the bis-intercalators. A similar lack of resolution of short fragments was previously observed for the same digest that was labeled with mono-intercalating dyes [8] and is attributed to limitations of the gel matrix. In both the present and previous studies, an anomalously long lifetime is recovered across the broad peak of unresolved fragments. Also, for all of the dyes in the present work, a small peak with an anomalous lifetime appeared near 830 s for Dye 50 and right before 1000 s for the bis-intercalators. It should be noted that the migration times for the mono-intercalating, dye:DNA base pair (bp) ratio affects both CE resolution of fragment peaks and lifetime detection [8]. At a ratio of 1 dye per 20 bp (Fig. 2a), the three longest fragments (929 bp, 1058 bp, and 1857 bp) migrated as a single, broad peak around 1700 s. The lifetime detection was also poor, with only a very short lifetime (0.15 ns) detected for the peak composed of the three longest fragments. At a ratio of 1 dye per 1982 bp (Fig. 2b), the three longest fragments were resolved and a lifetime of 1 ns was detected across all of the peaks above 60 bp. The lifetime detected across the peaks was well-resolved from the background lifetime.

Figure 2 shows electropherograms of Dye 50-labeled pBR322/BstNI at several different dye loadings. It has been demonstrated for mono-intercalating, monomeric dyes that dye:DNA base pair (bp) ratio affects both CE resolution of fragment peaks and lifetime detection [8]. At a ratio of 1 dye per 20 bp (Fig. 2a), the three longest fragments (929 bp, 1058 bp, and 1857 bp) migrated as a single, broad peak around 1700 s. The lifetime detection was also poor, with only a very short lifetime (0.15 ns) detected for the peak composed of the three longest fragments. At a ratio of 1 dye per 1982 bp (Fig. 2b), the three longest fragments were resolved and a lifetime of 1 ns was detected across all of the peaks above 60 bp. The lifetime detected across the peaks was well-resolved from the background lifetime.
Figure 4. Fluorescence intensity (solid line) and lifetime (circles) electropherograms of (a) Dye 57-labeled pBR322/BstNI (1 dye per 2215 bp), and (b) Dye 58-labeled pBR322/BstNI (1 dye per 4613 bp).

Figure 5a shows the electropherogram of Dye 57-labeled pBR322/BstNI (1 dye per 2215 bp) and a Dye 55-labeled 200 bp DNA fragment (1 dye per 2907 bp). A lifetime of 2.5 ns was detected for the 200 bp fragment (marked with an arrow, at 1200 s), which is consistent with the lifetimes observed for the Dye 55-labeled digest in Fig. 3. The Dye 57-labeled pBR322/BstNI fragments longer than 60 bp are well-resolved and the peak lifetimes are well-resolved from the background lifetime; a lifetime of around 3 ns was detected for these peaks, which is consistent with the lifetimes detected for the Dye 57-labeled digests alone. Figure 5b shows the electropherogram of a Dye 57-labeled pBR322/BstNI (1 dye per 2215 bp) and a Dye 58-labeled 500 bp size standard fragment (1 dye per 4631 bp, marked by an arrow). Lifetimes of about 2 ns and 3 ns were detected for the Dye 58-labeled 500 bp fragment and the Dye 57-labeled digest fragments, respectively, which are consistent with the lifetimes recovered for Dye 58- and Dye 57-labeled DNA fragments alone. The peaks due to fragments longer than 60 bp in length from the Dye 57-labeled pBR322/BstNI are well-resolved, with the exception of the 929 bp fragment around 1750 s that is affected by tailing of the 500 bp fragment peak. The 500 bp fragment is easily distinguished from the digest fragments based on fluorescence lifetime. The electropherogram of Dye 50-labeled pBR322/BstNI (1 dye per 1982 bp) and a Dye 55-labeled 500 bp size standard fragment (1 dye per 2907 bp) is shown in Fig. 5c. All fragments longer than 60 bp in length are well-resolved. The 500 bp fragment, marked with an arrow and migrating around 1500 s, has a lifetime of approximately 2.5 ns. This lifetime is consistent with that detected for this dye in previous runs. The lifetime at the apex of the peak is well-resolved from the background lifetime. The peaks due to the Dye 50-labeled digest have a lifetime of approximately 1 ns, which is consistent with the lifetime for the digest alone. The lifetimes of the two dyes are significantly different and the differently labeled fragment peaks can be easily distinguished from each other.

Figure 6 shows the electropherogram of a Dye 55-labeled 100 bp size standard ladder (1 dye per 2907 bp) (Fig. 6a) and replicate runs of a mixture of the ladder with Dye 57-labeled pBR322/BstNI (1 dye per 1982 bp) (Fig. 6b and c). The longer lifetimes of the Dye 57-labeled digest are easily distinguished from the shorter lifetimes of the Dye 55-labeled ladder, except where peaks from the two sources overlap; for overlapping peaks, the detected lifetime is dominated by the more intense contribution of the digest fragments. Good agreement was obtained between the replicate runs of the mixture, with the excep-
Figure 5. Fluorescence intensity (solid line) and lifetime (circles) electropherogram of (a) Dye 57-labeled pBR322/BstNI (1 dye per 2215 bp) and a Dye 55-labeled 200 bp DNA fragment (1 dye per 2907 bp), (b) Dye 57-labeled pBR322/BstNI (1 dye per 2215 bp) and a Dye 58-labeled 500 bp DNA fragment (1 dye per 4631 bp), (c) Dye 50-labeled pBR322/BstNI (1 dye per 1982 bp) and a Dye 55-labeled 500 bp DNA fragment (1 dye per 2907 bp). The arrow in each figure indicates the 200 bp or 500 bp size standard fragment.

Figure 6. Fluorescence intensity (solid line) and lifetime (circles) electropherogram of Dye 55-labeled 100 bp size standard ladder (1 dye per 2907 bp) (top), and replicate runs of a mixture of the ladder and Dye 57-labeled pBR322/BstNI (1 dye per 1982 bp) (middle and bottom).

3.6 Comparison with previous results

Multiplex fluorescence lifetime detection of CE-separated DNA fragments was previously described using mono- or bis-intercalating dyes containing one or two positive charges [8]. The purpose of the present work was to determine if it would be advantageous to use more highly charged (3+ or 4+) mono- and bis-intercalating dyes. In this study, as in the previous study, it was found that peak resolution and lifetime detection are strongly influenced by the dye:bp ratio. The optimal ratio for use in the TBE/1%...
HMW HEC/0.3% LMW HEC gel varied among the dyes, but was generally in the range of 1 dye per 2000–5000 bp for the dyes used in the present work, which is approximately 100-fold more dilute than the dye loadings that were required to provide sufficient signals for detection of the dyes used in the previous study. Low dye loadings are desirable to minimize the background fluorescence from free dye in solution, the effects of the dyes on resolution of the fragments, and the occurrence of multiple peaks for fragments of the same length due to uneven dye distribution.

The lifetimes of the dyes used in the present work are longer, and the lifetime range is broader (1–3 ns vs. 0.8–1.8 ns), compared to the previously used dyes. These are important advantages since longer lifetimes can be measured more precisely, and a wider lifetime range allows better discrimination among lifetimes of different dyes in multiplex analysis. The results for multiplex detection of digest fragments and standard fragments or fragment ladders using the combinations of Dyes 57/55, 57/58 or 50/55 demonstrate better discrimination between the lifetimes of the differently labeled fragments compared to the previous results. Visual comparison indicates better lifetime precision within a run. There was no indication of dye exchange among fragments during an electrophoretic run, indicating that dye exchange is absent or slow for the dyes used in the present study. We conclude that the larger, more highly charged mono- and bis-intercalators are superior to the dyes used in the previous study for multiplex lifetime detection of DNA fragments in CE.

Received October 10, 2002

4 References