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Fluorescence Lifetime for On-the-Fly Multiplex Detection of DNA Restriction Fragments in Capillary Electrophoresis

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This paper describes the first use of frequency-domain fluorescence lifetime for multiplex detection of DNA restriction fragments in capillary electrophoresis (CE). The fragments were labeled with monomeric intercalating dyes that can be excited by either the 488- or 514-nm line of an argon ion laser and have lifetimes in the range of 0.5−2.5 ns. We were able to achieve multiplex lifetime detection in the CE separation of a restriction fragment digest and a DNA size ladder in the same run, for fragments shorter than 700 bp. Different gel buffer systems, including a modified polyacrylamide gel and several tris−borate−EDTA/hydroxyethylcellulose (TBE/HEC) gels, were investigated for separation and detection of the dye-labeled DNA fragments. Best results for both electrophoretic resolution and lifetime detection were obtained using a gel containing 1% high molecular weight (90 000−105 000) HEC and 0.3% low molecular weight (24 000−27 000) HEC in TBE buffer.

A widely accepted tool for DNA analysis is restriction fragment length polymorphism (RFLP), in which DNA is cut by restriction enzymes. These enzymes recognize certain base sequences in the DNA and clip it near the recognition site. When DNA from different sources is cut using the same restriction enzyme, DNA from each source will be "digested" into a series of fragments. The fragment pattern of the DNA depends on the number of bases between recognition sites and is therefore a unique characteristic, or fingerprint, of DNA from that source.

The accepted medium for electrophoretic separation of DNA fragments in RFLP analysis is the slab gel. In recent years, however, capillary electrophoresis (CE) has gained popularity over traditional slab gel electrophoresis for DNA separations.[1−3] Slab gels are tedious to prepare, prone to error and poor reproducibility. Slab gel separations are slow, often requiring hours to complete, because of poor dissipation of Joule heat, which limits them to low-potential electric fields. Capillary electrophoresis, in contrast, offers high-performance separation. The narrow-bore capillaries used in CE have a high surface-to-volume ratio which provides efficient dissipation of Joule heat, allowing the use of high electric fields in order to achieve short separation times. CE requires only a miniscule sample volume, typically in the nanoliter range, which is important for applications in which total sample amount is limited.

The use of CE with UV−visible absorption or fluorescence spectral detection to analyze DNA restriction fragments has previously been demonstrated.[4−7] Multiplex detection of dye-labeled DNA restriction fragments has been achieved by using fluorescence color detection.[8] Fluorescence lifetime detection in CE has been described in both the time[9] and frequency domains[10] and has been successfully applied to multiplex detection of fluorescent-labeled DNA fragments[11] and sequencing.[12] The advantages of lifetime over intensity or color for multiplex CE detection of fluorescent-labeled DNA fragments, as well as the advantages of frequency domain over time domain for lifetime detection, have been discussed elsewhere.[10,11]

A particularly important feature of frequency-domain lifetime detection for on-the-fly detection is its speed. Complete lifetime profiles can be acquired at intervals of several tens of milliseconds during the CE run. Using in-house software, both fluorescence intensity and lifetime are extracted from the same signal to provide lifetime and intensity electropherograms in no more time than is required to obtain a steady-state intensity electropherogram alone.

We describe here the use of on-the-fly fluorescence lifetime detection for multiplex CE separations in RFLP analysis, in which a dye-labeled digest and a dye-labeled ladder (size standard) are

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simultaneously separated on the same column and the peaks of different origin are distinguished based on the fluorescence lifetimes of the dye labels. The fragments are labeled with intercalating dyes that bind by inserting between the base pairs in the fragments.[13] Intercalation is a simple alternative to the more labor intensive process of covalent attachment for labeling DNA fragments.

The use of intercalating dyes as labels for DNA fragments during electrophoretic separation has been previously reported.[14−16] For monomeric intercalating dyes such as TO and YO, it often is necessary to include the dye in the run buffer in order to obtain adequate signals due to the relative instability of the dye−DNA complex under electrophoretic conditions.[15] Unfortunately, free dye in solution may contribute significant background signal despite its low quantum yield. Furthermore, addition of dyes to the run buffer precludes multiplex analysis since it would not be possible to selectively label DNA fragments from different sources. DNA complexes formed with some dimeric dyes such as YOYO and TOTO are stable enough to allow detection without addition of dye to the run buffer; however, multiplex analysis can still be difficult due to exchange of dyes between fragments.[8,14]

The dye−DNA complexes formed by the monomeric intercalating dyes under the experimental conditions used in this work are sufficiently stable to provide measurable signals without adding dye to the run buffer, and exchange between DNA fragments is slow enough to allow multiplex analysis during a CE run. Monomeric dyes were used in these first studies despite their lower binding affinities for DNA order to avoid complications that may arise with dimeric, bis-intercalating dyes, such as binding of a single dye to two different fragments and heterogeneous fluorescence decay due to the increased number of possible dye−DNA binding modes.

EXPERIMENTAL SECTION

Reagents. The structures of the monomeric intercalating dyes are shown in Figure 1. The dyes include oxazole yellow (YO) and four novel dyes whose synthesis is described elsewhere.[13,17,18] Stock solutions of the dyes were prepared in DMSO and stored in the refrigerator. The stock solutions were diluted with Tris buffer (100 mM, pH 8.6). Dye concentrations of the injected samples ranged from 1 to 100 μM. DNA restriction fragment digests, including pBR322 DNA digested with the restriction enzyme *Bst*NI, pBR322 DNA digested with the restriction enzyme *Msp*I, and a 100-bp DNA ladder and undigested pBR322 DNA, were obtained from New England Biolabs (Beverly, MA). The 200- and 500-bp fragments were purchased from Gensura Laboratories, Inc. (Del Mar, CA).

Modified polyacrylamide gel (eCAP dsDNA 1000 kit, Beckman, Fullerton, CA) was rehydrated with 20 mL of deionized water and stirred overnight. The gel buffer contains <0.1% acrylamide, >75% polyacrylamide, >10% tris (hydroxymethyl) aminomethane, and >5% boric acid. Tris−borate−EDTA (TBE) buffer, (89 mM tris−borate, 2 mM EDTA, pH 8.3) was used as received from Sigma. The TBE/hydroxyethylcellulose gel buffers were prepared by dissolving appropriate amounts of high molecular weight (MW 90 000−105 000) and/or low molecular weight (MW 24 000−27 000) hydroxyethylcellulose (HEC, Polysciences) in TBE and stirring overnight. The gels were filtered through a 0.45-μm filter prior to use.

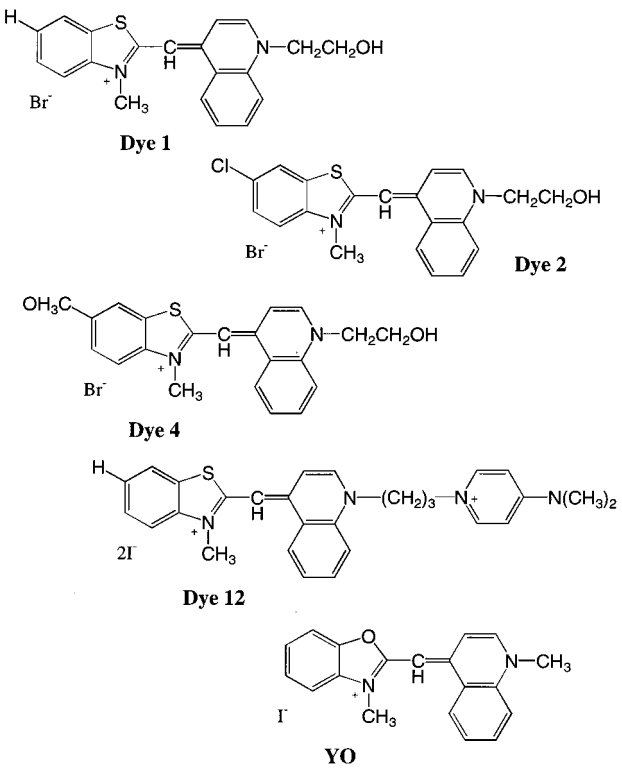

Figure 1. Structures of intercalating dyes.

Instrumentation. Absorption measurements were performed using a Perkin-Elmer Lambda 6 UV−visible spectrophotometer with tungsten and deuterium lamps as light sources. Fluorescence spectra of the dyes in batch solution were collected using an SLM phase-modulation spectrofluorometer 48000S (Spectronics Instruments, Inc., Rochester, NY), with a xenon arc lamp for excitation and PMT detection.

On-the-fly fluorescence lifetime detection of DNA fragments in CE was performed using a multiharmonic Fourier transform (MHF) fluorescence lifetime instrument interfaced to a Beckman P/ACE 5000 CE system, as previously described.[10] A double-stranded DNA separation kit (eCAP dsDNA 1000, Beckman) provided the coated DNA capillary column (100-μm i.d.). The column length was 47 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 labeled DNA samples (10^{-7} M total DNA fragments) onto the column. The volume injected was on the order of 10^{-10} L, corresponding to total of 10^{-12} mol of DNA. The CE was run in reverse polarity mode in which the inlet potential is negative and the outlet potential is positive. A constant voltage of 9.4 kV was applied to drive the dye-labeled DNA fragments to the detector. Average run time was 30 min.

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Table 1. Spectral Maximums of Intercalating Dyes in dsDNA Determined in Batch Solution (Tris-HCl Buffer) and Lifetimes Determined On-the-Fly in CE Using Various Gel Buffers

dye	excitation max (nm)	emission max (nm)	lifetime ± standard deviation (ns) PAG[a]	TBE/ 1% HEC	TBE/ 1% HMW HEC/ 0.3% LMW HEC
1	503	538	1.8 ± 0.2	*b*	*b*
2	507	530	2.2 ± 0.2	*b*	0.5 ± 0.01
4	512	549	0.7 ± 0.1	0.8 ± 0.01	1.0 ± 0.1
12	509	534	1.4 ± 0.1	*b*	1.3 ± 0.1
YO	475	508	1.7 ± 0.1	0.9 ± 0.01	1.8 ± 0.0

[a] Modified polyacrylamide gel. [b] Lifetime not determined.

Excitation was achieved using the 488-nm line of an air-cooled argon ion laser (OmniChrome). A 488-nm holographic filter was placed in the emission channel to reduce scattered laser light, and a 500-nm long-pass filter was used for emission wavelength selection. Scattered light, which has a fluorescence lifetime of essentially zero, provided the lifetime reference.

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. The fluorescence intensity was recovered from the MHF data using an in-house program.[19] The lifetime data were analyzed using nonlinear least-squares (NLLS) analysis software (Globals, Unlimited, Urbana, IL). Unless otherwise noted, single-component lifetime models (corresponding to monoexponential decay) were adequate to describe the data; lifetime values given in the text correspond to the lifetimes recovered at the apex of each peak.

RESULTS AND DISCUSSION

Fluorescence Properties of the Intercalating Dyes. There are several criteria for selection of dyes to be used in multiplex DNA fragment analysis. The dyes should have similar excitation maximums in order to be excited at a single laser line, they should be essentially nonfluorescent when they are not bound to DNA, and they must have resolvable fluorescence lifetimes so that the fragments from different digests can be distinguished. The emission spectral properties of the dyes are less important since resolution is based on lifetime rather than color, allowing us to use a broad emission window for detection.

As shown in Figure 1, the intercalating dyes used in this work share a common ring structure backbone, differing mainly in the type of substituents on the rings. These dyes are thought to intercalate into the stacked base pairs of the DNA, due to their planar ring structure. On the basis of reports for dyes including YO and YOYO-1,[20] it is possible that weaker associations such as groove binding also may occur.[13]

A summary of the fluorescence spectral properties of the dyes is given in Table 1. The dyes can all be excited at 488 or 514 nm. The spectra were measured for batch solutions of the dyes in Tris-HCl buffer containing calf thymus DNA (~1 dye/5 bp). The fluorescence of the dyes is negligible in the absence of DNA and greatly enhanced upon binding due to increased structural rigidity of the dye molecule upon intercalation. For this reason, measurements of the free dyes in the absence of DNA were not informative.

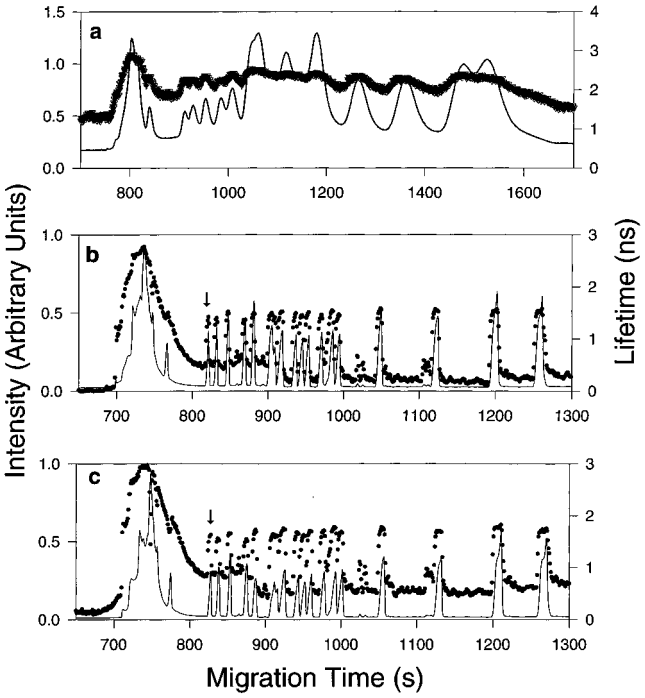

Figure 2. Electropherograms of dye 12-labeled pBR322DNA−*Msp*I digests: (a) 1 dye/15 bp; (b) and (c) 1 dye/1500 bp, duplicate runs. Arrows in (b) and (c) mark the 67-bp fragment as a reference point. Solid line is fluorescence intensity and dotted line is fluorescence lifetime.

The lifetimes of the dyes in dsDNA that were detected on-the-fly in the CE experiments described below are summarized in Table 1. The lifetimes are all monoexponential, but they vary among the different gel matrixes due to differences in the physical and chemical microenvironments experienced by the dyes in the various gels.

Separations Using Modified Polyacrylamide Gel. In the initial studies, we used a dye/DNA bp ratio of 1:15 in the pBR322DNA−*Msp*I digest labeled with dye 12. This digest has 26 fragments ranging from 9 to 622 bp in length. As seen in Figure 2a, peak resolution and lifetime detection were poor. Use of a 100-fold dilution of the dye (1 dye/1500 bp) yielded much better results for this dye (Figure 2b). Good resolution was obtained for all fragments longer than 60 bp. Even the 238- and 242-bp fragments that migrate at 986 and 995 s, respectively, could be resolved. Reproducibility between replicate runs was excellent (Figure 2b and c).

These results show the importance of dye concentration in the resolution of DNA fragments using this intercalating dye in this gel matrix. If the dye concentration is too high, other modes of interaction such as groove binding and electrostatic interactions can become important. These may result in uneven distribution of dyes among fragments of the same length, causing peak broadening due to variations in mass, as well as interactions between the weakly associated dyes and the capillary walls.

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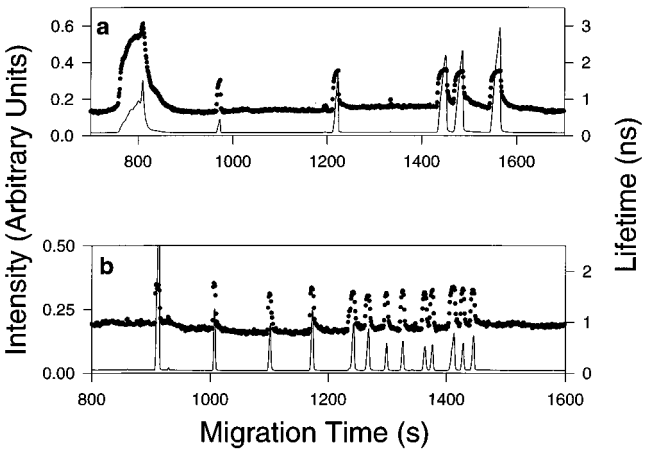

Figure 3. Electropherograms of DNA digests: (a) dye 1-labeled pBR322DNA−*Bst*NI (1 dye/95 bp); (b) YO-labeled 100-bp DNA ladder (1 dye/950 bp). Solid line is fluorescence intensity and dotted line is fluorescence lifetime.

In subsequent experiments, dye concentrations were adjusted to provide the best compromise between fluorescence signal and electrophoretic resolution. The best concentration thus varied among the different dyes as well as among the different gels for a given dye.

With the exception of the first peak, the fluorescence lifetime at each of the peaks is 1.5 ns. The longer lifetime of the first peak, which migrates around 725 s, is attributed to seven fragments below 60 bp in length that we could not resolve using this gel buffer. The cause of the longer fluorescence lifetime detected for this peak is unknown but may be due to higher dye loading of the smaller fragments. This could result in secondary binding interactions such as groove binding and electrostatic interactions, as well as physical and photophysical interactions among the bound dyes in a single fragment. There may also be aggregation of the short, unresolved fragments. These effects could cause the dye to experience microenvironments different from those experienced when the dye is more sparsely and evenly distributed within and among longer fragments.

Figure 3a shows the electropherogram for the pBR322DNA−*Bst*NI digest labeled with dye 1. This digest has 6 fragments that range in length from 13 to 1857 bp. The dye/DNA base pair ratio for this separation is 1:95. Baseline resolution of the fragments was achieved for the digest and a fluorescence lifetime of 1.8 ns was detected at all of the fragment peaks except the first peak that migrates around 800 s. As was discussed for the pBR322DNA−*Msp*I digest, this peak is attributed to fragments below 60 bp in length, which we could not resolve using this gel buffer.

Figure 3b shows the electropherogram of a 100-bp DNA ladder labeled with YO. This digest has 12 fragments, ranging from 100 to 1500 bp in length. The dye/DNA base pair ratio for this separation is 1:1236. Again, baseline resolution of all fragments was obtained. The fluorescence lifetime detected at all peaks is 1.7 ns. Unlike the pBR322DNA−*Bst*NI digest, the DNA ladder does not exhibit a large initial peak with a long fluorescence lifetime. This is probably because the shortest fragment of the ladder is 100 bp, which is longer than the unresolved fragments that gave the anomalous lifetime in the digest.

Note that we detected 13 rather than 12 fragments for this digest. The source of the extra peak is not known; however, it appears in all runs using this particular ladder and this particular gel. A possible explanation is that this is an impurity in the digest itself. Another possibility is that one of the fragments has two different migration times due to different possible binding sites for the intercalating dye. It has been shown that bis-intercalating dyes such as YOYO-1 and TOTO-1, when complexed to a fragment of DNA, can give rise to two components with different electrophoretic mobilities.[16] Although our dyes are not bis-intercalating dimers, it is possible that more than a single binding site exists in one or more of the fragments. It also has been proposed that band splitting observed in gel electrophoresis for dye−DNA complexes is due to the presence of DNA fragments with different numbers of bound dyes.[15] This is possible here if the distribution of dyes is not uniform among fragments even of the same size. Since this gel was replaced by a better gel matrix in subsequent studies, further investigation of the anomalous peak was not pursued.

In Figures 2b,c and 3a,b, the detected fluorescence lifetimes are readily distinguished from the background "lifetimes" recovered in baseline regions between peaks. It should be noted that fluorescence lifetime is undefined in the absence of detectable signal; therefore, the lifetimes recovered in the baseline regions of the electropherograms (regions of low signal) are meaningless manifestations of noise and systematic error.

We next attempted multiplex detection of a mixture of restriction fragment digest labeled with one dye and a ladder labeled with a different dye. Unfortunately, for all of the various mixtures examined, the observed lifetime for all peaks (ladder and digest) equaled the lifetime of one of the dyes. This indicated that one of the dyes was displacing the other from the DNA fragments in the mixtures.

Fluorescence emission spectra of digest mixtures in batch mode confirmed the occurrence of dye displacement. For example, the fluorescence emission spectrum of dye 12 in dsDNA does not change upon addition of YO, indicating that displacement of dye 12 does not occur. However, addition of dye 12 to YO in dsDNA causes a red shift in the emission maximum from 508 to 534 nm, which is the emission maximum of dye 12 in dsDNA, indicating that dye 12 has displaced YO from the dsDNA. Studies of the various binary combinations of dyes 1, 4, 12, and YO showed that YO can be displaced by each of the other dyes and does not displace any of the other dyes, dye 12 displaces dye 1 but only partially displaces dye 4, dye 4 partially displaces dye 1 and dye 12, and dye 1 partially displaces dye 4 but does not displace dye 12. These results indicate that YO forms the weakest complex and dye 12, which has two positive charges instead of one, forms the strongest complex.

In an attempt to slow the rate of exchange of dyes between DNA fragments, we decided to explore other gels as alternatives to the modified polyacrylamide gel for the CE separation. If the exchange could be slowed to a negligible rate on the time scale of an RFLP separation then it would not interfere with the analysis.

Separations Using Tris−Borate-EDTA/Hydroxyethylcellulose Gel. HEC was investigated as an alternative sieving matrix to modified polyacrylamide gel because of its previous use

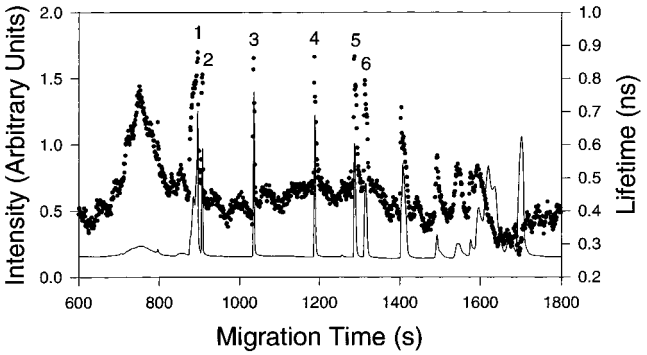

Figure 4. Electropherogram of mixture of dye 4-labeled pBR322DNA−*Bst*NI digest and YO-labeled 100-bp DNA ladder using TBE/1%HMW HEC. Peaks 1 and 3−5 are from the ladder, and peaks 2 and 6 are from the pBR322DNA−*Bst*NI digest. Solid line is fluorescence intensity and dotted line is fluorescence lifetime.

for separation of DNA fragments.[8,22] Both high molecular weight (HMW, 90 000−105 000) and low molecular weight (LMW, 24 000−27 000) HEC gels were investigated.

Figure 4 shows the separation of the fragments of a mixture of dye 4-labeled pBR322DNA−*Bst*NI fragments and YO-labeled 100-bp DNA ladder fragments on a TBE/1% HMW HEC gel. The dyes were present at similar concentrations (dye/base pair ratios of 1:8 for the dye 4-labeled DNA and 1:12 for the YO-labeled DNA). The peaks numbered 1 and 3−5 in Figure 4 are due to the YO-labeled DNA ladder. The lifetime detected across these peaks is 0.9 ns. Peaks 2 and 6 are due to fragments from the dye 4-labeled pBR322DNA−*Bst*NI digest and have lifetimes of 0.8 ns. Peak 6, which is due to the 383-bp fragment, migrates slower than the 400-bp peak of the DNA ladder (peak 5). Although this was reproducible, it did not occur for any of the other gel compositions and is probably due to effects of the different dyes on the mobility of the fragments in this gel. Beyond 1400 s, peak resolution and lifetime detection deteriorated.

Using the TBE/1% HMW HEC gel buffer, the peaks of fragments in the 60−600-bp range (800−1400 s migration time) are well resolved and the detected lifetimes are well above the background signal. We could not, however, detect all of the fragments from both digests (sample and ladder), particularly those above 600 bp. Therefore, other HEC gel compositions were investigated. Unlike the modified polyacrylamide gel, dye concentration and dye/DNA ratio did not affect the CE resolution of the peaks within the concentration ranges studied.

Figure 5 shows the separation of dye 4-labeled pBR322DNA−*Bst*NI digest fragments in four different gel buffers. Again, the separation of the DNA fragments in TBE/1% HMW HEC (Figure 5a) is not optimal. Although all fragments migrated in less than 1600 s, baseline resolution was not achieved for the two peaks migrating at ∼1400 s (due to the 929- and 1058-bp fragments). Decreasing the concentration of HMW HEC to 0.5% (w/v) decreased the total run time from 1600 s to less than 1200 s but also decreased overall resolution (Figure 5b). Figure 5c shows the separation of the DNA fragments using a mixture of high and low molecular weight HEC (0.3% HMW and 1% HMW). The total

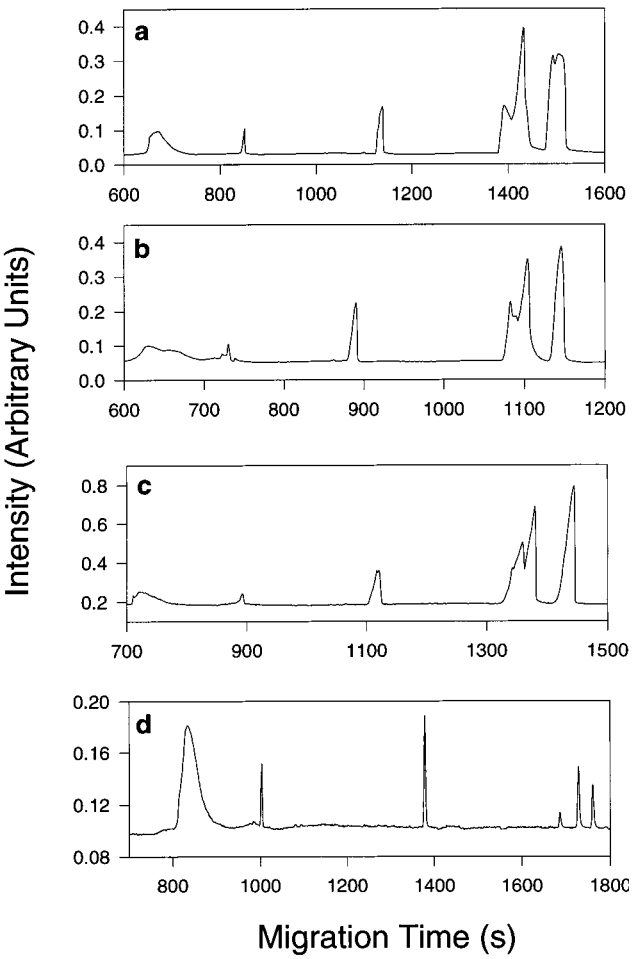

Figure 5. Electropherograms of dye 4-labeled pBR322DNA−*Bst*NI digest using TBE/HEC gel buffers: (a) 1% HMW HEC; (b) 0.5% HMW HEC; (c) 0.3% HMW/1% LMW HEC; (d) 1% HMW/0.3% LMW HEC.

run time was less than 1500 s, but the resolution of the 929- and 1058-bp fragments remained poor. Much better results were obtained using TBE/1% HMW/0.3% LMW HEC (Figure 5d), which provided baseline separation of all six fragments in the digest. The total run time increased to ∼1800 s, but the dramatic improvement in peak resolution outweighs the small increase in separation time.

Separation of a YO-labeled 100-bp DNA ladder using the same four gel buffers (not shown) gave results similar to those obtained for the pBR322DNA−*Bst*NI digest, with TBE /1% HMW HEC/0.3% LMW HEC providing the best peak resolution.

Multiplex detection in the TBE/1% HMW HEC/0.3% LMW HEC gel buffer was investigated using dye 4-labeled pBR322DNA−*Bst*NI digest and a reference fragment (YO-labeled 500 bp or dye 2-labeled 200 bp). Results are shown in Figure 6. The broad peak near 800 s (peak 1) is again due to unresolved fragments from the *Bst*NI digest. Peaks 2, 3, and 5−7 are from the digest and peak 4 is from the YO-labeled 500-bp fragment. The peaks are well resolved and the lifetimes are readily detected above the background; however, while the lifetime of the YO-labeled fragment is the expected value of 1.8 ns, the lifetime of the dye 4-labeled digest is 1.5 ns, which is higher than the expected value of 1.0 ns that was recovered for the dye 4-labeled fragments alone (Table 1). This can be explained by a relatively low affinity of YO

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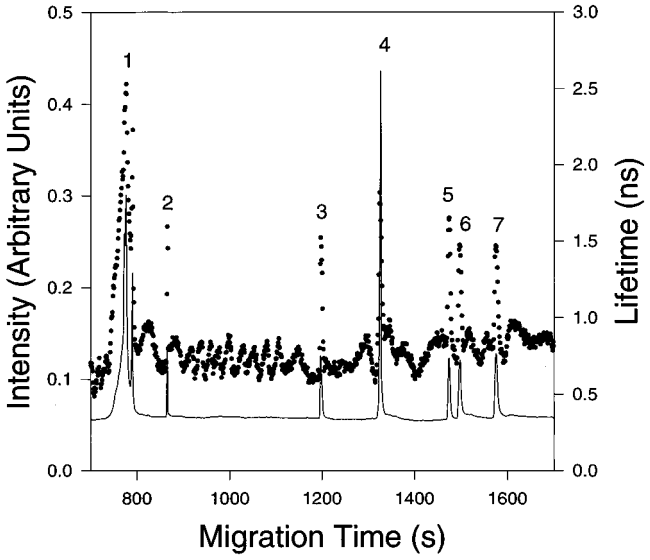

Figure 6. Electropherogram of mixture of dye 4-labeled pBR322DNA−*Bst*NI digest (1 dye/8 bp) and YO-labeled 500-bp DNA fragment (1 dye/12 bp) using TBE/1% HMW/0.3% LMW HEC. Peaks 1−3 and 5−7 are from the pBR322DNA−*Bst*NI digest, and peak 4 is from the 500-bp fragment. Solid line is fluorescence intensity and dotted line is fluorescence lifetime.

for DNA, resulting in its exchange between fragments. It does not appear to be displacing dye 4 in the digest, but simply occupying free binding sites. This causes the observed lifetime for the digest fragments to reflect contributions from both dye 4 and YO, resulting in the higher lifetime value.

Since YO interfered with detection of dye 4-labeled fragments, the experiment was repeated using dye 2 instead of YO. The results are shown in Figure 7. Peaks 2 and 4−7 are from the digest and peak 3 is from the dye 2-labeled 200-bp fragment. In this figure, only five contiguous lifetime points centered at the peak apex are shown for each peak, to focus on the data that would be used for peak identification. Due to the relatively low signals obtained in this run, background contributions to the detected lifetimes were not insignificant; a two-component model in which both lifetime components were allowed to vary in order to account for the background signal gave better results than a one-component model. Although the digest peaks are not as well resolved as in Figure 7a, they appear at the same migration times. Importantly, the lifetimes of the peaks are the expected values (1.0 ns for the digest peaks and 0.5 ns for the 200-bp fragment peak) and show no indication of dye exchange between fragments.

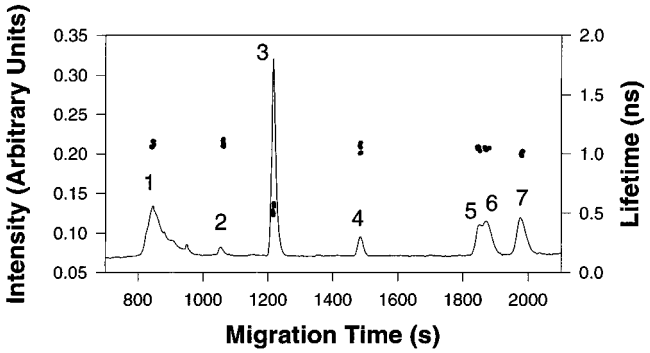

Figure 7. Electropherogram of mixture of dye 4-labeled pBR322DNA−*Bst*NI digest (1 dye/8 bp) and dye 2-labeled 200-bp DNA fragment (1 dye/79 bp) using TBE/1% HMW/0.3% LMW HEC. Peaks 1, 2, and 4−7 are from the pBR322DNA−*Bst*NI digest, and peak 3 is from the 200-bp fragment. Solid line is fluorescence intensity. Five contiguous lifetime points centered at the peak apex are shown for each peak.

These results demonstrate the ability to perform multiplex detection of CE-separated DNA fragments from different sources using DNA intercalating dyes and on-the-fly fluorescence lifetime detection. We were able to distinguish between DNA digest fragments and size standard fragments based on lifetime differences between intercalating dyes in the absence of any spectral ("color") discrimination. It was found that the optimal dye concentration is a compromise between detectability and electrophoretic resolution and therefore depends on both the dye and the gel buffer composition. Multiplex detection using intercalating dyes requires that the dyes remain bound to the fragments during the CE run—exchange of dyes between different fragments and between fragments and gel buffer must be negligible. The gel buffer composition was found to be a critical factor in preventing exchange of dyes and can affect the observed lifetimes of the dyes as well.

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