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Journal of Chromatography A, 841 (1999) 95–103

JOURNAL OF CHROMATOGRAPHY A

Effects of gel material on fluorescence lifetime detection of dyes and dye-labeled DNA primers in capillary electrophoresis

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Received 16 October 1998; received in revised form 18 February 1999; accepted 25 February 1999

Abstract

Investigations of fluorescence lifetimes of the dye 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (NBD-HA) and of DNA M13 primers labeled with NBD-HA, Cy3, rhodamine green and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY-FL) dyes in polyacrylamide gels of various degrees of crosslinking and different crosslinkers, and in a cellulose sieving buffer with different organic modifiers, are described. The dependence of fluorescence lifetime on gel matrix and on experimental conditions was studied in order to identify which factors may be important for optimization of multiplex fluorescence lifetime detection. Lifetimes were determined in both batch solution and on-the-fly, on-column in CE. Results show that lifetimes of the primer-attached dyes remain constant in gels of different composition. Additionally, multiexponential fluorescence decays are observed for primer-attached dyes in batch solutions of the cellulose sieving buffers but are reduced to monoexponential decays when measured on-the-fly, on-column in CE. Lifetime detectability can be improved by addition of an organic modifier to the gel matrix.

Keywords: Detection, electrophoresis; Fluorescence detection; Dyes; DNA

1. Introduction

A new detection scheme for multiplex DNA sequencing has recently been described which uses fluorescence lifetime instead of spectral wavelength to discriminate among the four base-specific fluorescent labels [1–4]. This four-decay fluorescence lifetime detection method has the potential to increase accuracy and resolution of base calling as well as to indicate the presence of impurities and matrix effects. On-the-fly, real-time detection of fluorescence lifetime on capillary electrophoresis (CE) has been demonstrated [1] and applied to the detection and resolution of electrophoretically separated DNA primers labeled with four different visible dyes [2–4].

One of the challenges in fluorescence lifetime detection for DNA sequencing is selection of dyes. The dyes should exhibit high molar absorptivity, high fluorescence quantum yield and similar absorption maxima. Their lifetimes must be resolvable, which requires a lifetime difference of at least 20% between two dyes of predetermined lifetime using the multiharmonic Fourier transform lifetime instru-

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00292-7

ment [5]. Most of the commercial fluorescent dyes are designed for color, not lifetime, discrimination. It is therefore difficult to find an ideal set of four dyes that meet all of the criteria for lifetime resolution and detectability. However, since fluorescence characteristics of some dyes are sensitive to the microenvironment [6], it may be possible to enhance lifetime detection and resolution through appropriate selection and modification of the CE packing material.

Another challenge lies on performing lifetime measurements in the gel-filled capillary. The gel matrix can produce a large scattering background and introduce fluorescent background due to impurities. Fortunately, the matrix effects show up directly in lifetime analysis through the recovery of unexpected lifetimes, leading to better experimental design, improved data analysis, and more accurate interpretation of sequencing data.

This paper describes the evaluation of polyacrylamide gels and a cellulose sieving buffer as candidates for the CE packing material in four-decay fluorescence detection scheme for DNA sequencing. The dependence of fluorescence lifetime on experimental conditions were investigated, including dependence on the composition of polyacrylamide gels (various degrees of cross-linking and different cross-linkers), on the lifetime reference and on the fluorescence emission window. The hydroxyethyl cellulose sieving buffer with different organic additives was also explored using a set of four dyes as the probes.

2. Experimental

2.1. Preparation of samples and gels

The stock solution of 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (NBD-HA, Molecular Probes) was prepared in absolute ethanol and stored in a refrigerator. NBD-HA, Cy3, rhodamine green (RG), and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY-FL) dye-labeled M13-forward sequencing primers d(5′-TGTAAAACGACGGCCAGT-3′) were custom-synthesized by Midland Certified Reagent using succimidyl esters of the dyes that were purchased from Amersham (Cy3) or Molecular Probes (NBD-HA, RG and BODIPY-FL). Acrylamide, *N*,*N*′-methylenebisacrylamide (Bis), *N*,*N*′-(1,2-dihydroxyethylene)-bisacrylamide (DHEBA) and ethylene diacrylate (EDA) were purchased from Aldrich.

For batch lifetime measurements, polyacrylamide gels were prepared in 5-ml aliquots from the degassed mixture of acrylamide and crosslinker (Bis, DHEBA or EDA) in 1× TBE (Tris-borate ethylenediaminetetraacetic acid) buffer (diluted from 10× TBE, Life Technologies) and 7 *M* urea (EM Sciences). Polymerization was initiated by addition of 2 μl of *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED, Bio-Rad) and 20 μl of 10% ammonium persulfate (Bio-Rad). A stock solution of NBD-HA was added to the gel solution before polymerization to make a final dye concentration of 1 μ*M*. The gel solution was transferred to a disposable polystyrene cuvette and stored overnight for completion of polymerization before use.

The cellulose sieving buffer hydroxyethylenecellulose (HEC, molecular mass approx. 720 000, Aldrich) was prepared by dissolving 1.25 g cellulose and 22.5 g urea in 50 ml HPLC grade water. The solution was stirred overnight with 0.5 g Amberlite MB-150 ion-exchange resin (ICN Biochemicals). The solution was then transferred to 25-ml tubes, centrifuged in a tabletop centrifuge for 30 min and the matrix was decanted off the pelleted resin. 1.5 ml 10× TBE was then added to 12 ml purified matrix. The final solution was stirred for 30 min, then dispensed into 1.5-ml microcentrifuge tubes and centrifuged for 10 min. The top 800 μl of the solution (2% HEC, 6 *M* urea in 1× TBE) in each tube was used for on-the-fly CE and batch lifetime measurements. The solvent modulated HEC sieving buffers had a composition of 2% HEC, 1× TBE, 6 *M* urea and 10% (v/v) organic solvent, either dimethyl sulfoxide (DMSO), dimethylformamide (DMF), formamide, glycerol or methanol. Solutions of dye-labeled primers in cellulose sieving buffers were equilibrated overnight and not degassed prior to use.

2.2. Capillary preparation

CE separations in both cellulose sieving buffer and linear polyacrylamide gel were performed in a polyacrylamide-coated capillary (360 μm O.D.×75

μm I.D., Polymicro Technologies) that was 48 cm in length and 40 cm from the inlet to the detection window.

To prepare cross-linked polyacrylamide gel capillaries, the 50 cm fused-silica capillary column (360 μm O.D.×75 μm I.D., Polymicro Technologies) was pretreated by flushing it with 1 *M* HCl, HPLC grade water, 1 *M* NaOH and HPLC grade water, each for 10 min. The column was then filled with 0.4% 3-methacyloxypropyltrimethoxysilane solution (pH 3.5, adjusted by adding acetic acid) and stored overnight at room temperature. After a final wash with water and drying, the column was ready for gel polymerization. The gel solution of acrylamide and cross-linker in 1× TBE, 7 *M* urea buffer, was degassed and polymerization was initiated by adding 2 μl TEMED and 20 μl 10% ammonium persulfate. The pretreated capillary was immediately filled with the gel solution by means of a gas-tight HPLC syringe (in-laboratory modified). After overnight polymerization, the gel-filled capillary was ready to use.

2.3. Instrumentation

A multiharmonic Fourier transform phase modulation spectrofluorometer (MHF, Model SLM 4850S-MHF, Spectronics Instruments) was used for both batch-mode and on-the-fly fluorescence lifetime measurements. An air-cooled argon ion laser (Model 543R-AP-A01, Omnichrome) provided 100 mW excitation at 488 nm. A 488 nm bandpass filter with a 10 nm bandwidth was used in the excitation beam to remove the majority of the plasma background in the laser light. For batch measurements, the emission signal was selected using either a 500 nm longpass filter in combination with a 600 nm shortpass filter, a 550 nm bandpass filter with an 80 nm bandwidth, or a 515 nm longpass filter. The 515 nm longpass filter was used for the on-the-fly experiments. In all cases, a 488 nm holographic filter (Notch-Plus, Kaiser Optical Systems) was placed in the emission beam to greatly reduce the contribution from scattered laser light. In addition, a 10× or 40× microscope objective was added in the emission path to improve the collection of emitted light for on-the-fly CE detection. Fluorescein (Sigma) in phosphate buffer (pH=7.4, τ=4.1 ns) or scattered light (τ=0 ns) provided a convenient lifetime reference.

For batch measurements, each lifetime measurement contained phase-modulation information at 50 frequencies, collected using a base frequency of 4.1 MHz, a correlation frequency of 5.0 Hz, and 50 internal averages per measurement. Ten pairs of sample and reference measurements were collected for each lifetime determination.

For on-the-fly detection, the MHF instrument was interfaced in the laboratory to a Beckman P/ACE 5000 which was equipped with a CE–MS interface to provide an external power supply [1]. A cross-correlation frequency of 10 Hz was used, resulting in 10 lifetime measurements per second. Electrokinetic injections were made for 1–2 s at −7.5 kV or −10 kV, and electrophoresis was continued at −7.5 kV or −10 kV to generate a 150 V/cm or 200 V/cm field strength.

2.3.1. Data analysis

Lifetime data for batch and on-the-fly experiments were analyzed using non-linear least-squares analysis (Globals Unlimited) or the maximum entropy method (Maximum Entropy Data Consultants). Non-linear least-squares analysis (NLLS) fits the phase and modulation data to a priori models of discrete lifetime components; selection of the most accurate solution is based on the χ^2 goodness-of-fit parameter and randomness of the fitting residuals. In some cases, a very short, fixed lifetime was added to the fitting model to account for scattered light contribution. The maximum entropy method (MEM) imposes a dual constraint of minimum χ^2 and maximum statistical entropy in order to recover a unique lifetime distribution without assumptions about the fluorescence decay of the sample [7,8]. Therefore, MEM provides unbiased recovery of contributions from impurities and scattered light and is a good indicator of matrix effects.

3. Results and discussion

3.1. Investigation of polyacrylamide gels

In previous work on the characterization of dyes

for four-decay fluorescence detection in DNA sequencing, it was observed that primer-attached NBD-HA showed an increase in lifetime from 1.3 ns in batch solution, which was Tris–borate/7 *M* urea buffer, to 2.1 ns in the capillary column, which contained polyacrylamide gel [3]. This lifetime increase was attributed to the interaction between the gel and the dye, leading to the present investigation of the effects of gel material on dye lifetimes.

In the present work, polyacrylamide gels of various degrees of cross-linking and constructed using different cross-linkers were studied. First, the total acrylamide concentration was varied from 2.5% to 10% (w/v) in non-crosslinked gels. Next, the total monomer concentration (acrylamide plus cross-linker) was kept constant at 6% (w/v) and the cross-linker concentration was varied from 0.5% to 10% (w/w) of the total monomer concentration. The three cross-linkers used were Bis, EDA and DHEBA. Free (unconjugated) NBD-HA dye was selected to probe the different gel matrices because of the earlier results, as well as prior reports of the susceptibility of NBD-HA fluorescence to microenvironmental polarity, hydrogen-bonding ability and viscosity [6]. The NBD-HA was incorporated into the gel matrix during the preparation of the gel in order to facilitate uptake of the dye into the matrix.

Lifetime results for batch solutions of free NBD-HA dye in the various polyacrylamide gels are shown in Table 1, as well as results for NBD-HA in plain buffer with and without urea. The results shown in Table 1 were recovered using MEM analysis, but the results of NLLS analysis were very similar. The major lifetime component, which is in the range of 1.0 ns to 1.5 ns, is attributed to dye molecules which may not be incorporated into the gel matrix. The longer component of 4–5 ns is significant only in the presence of gel and may be due to effects of the gel matrix on the individual dyes or aggregates of dye molecules. Its contribution to the total intensity is generally 10–20% in the gel matrices, with the exception of 2.5% total acrylamide concentration with no crosslinking, for which the long lifetime component dominates the decay. The short lifetime component increases in all cases from 1.05 ns in plain buffer to values of 1.3 ns in the presence of urea. Urea is added as a denaturing agent and decreases the polarity of the buffer and gel matrices, which may contribute to the lifetime change.

Table 1
Fluorescence lifetimes (τ) and fractional intensity contributions (α) of batch solutions of free NBD-HA in buffer and in the presence of linear and cross-linked polyacrylamide gels of different total polyacrylamide concentration (T) and % crosslinking (% C)[a]

Sample medium	τ_1 (ns) (α_1)	τ_2 (ns) (α_2)
Buffer	1.0 (0.99)	6.0 (0.01)
Buffer with urea	1.3 (0.97)	5.4 (0.03)
2.5% *T*, 0% *C*	1.4 (0.17)	4.0 (0.82)
5.0% *T*, 0% *C*	1.3 (0.80)	4.0 (0.19)
10% *T*, 0% *C*	1.5 (0.72)	4.6 (0.28)
6.0% T		
0.5% *C* Bis	1.4 (0.87)	4.9 (0.13)
5.0% *C* Bis[b]	1.4 (0.77)	4.7 (0.10)
10% *C* Bis[b]	1.3 (0.53)	4.6 (0.09)
6.0% T		
0.5% *C* EDA	1.3 (0.78)	3.7 (0.22)
5.0% *C* EDA	1.3 (0.87)	4.2 (0.12)
10% *C* EDA	1.4 (0.89)	4.7 (0.11)
6.0% T		
0.5% *C* DHEBA	1.4 (0.88)	4.2 (0.11)
5.0% *C* DHEBA	1.3 (0.84)	3.9 (0.15)
10% *C* DHEBA	1.3 (0.86)	4.0 (0.14)

[a] 100 m*M* Tris–borate buffer (pH 8.7) with 7 *M* urea buffer. Lifetime results are mean values of triplicate determinations, recovered by MEM analysis. Standard deviations of the triplicate runs were approximately 7% for τ_1 and 9% for τ_2.

[b] In these cases a third, very short component was recovered and attributed to scattered light.

These results show that addition of urea to NBD-HA in buffer increases the lifetime of the short decay component and the same value is obtained for the dye in gels in the presence of the urea-containing buffer. The fractional intensity contribution of the long, minor lifetime is significant only in the gels, but its contribution decreases in the presence of crosslinking. This may reflect differences in chemical microenvironments among the different matrices or decreased uptake of the dye into the crosslinked matrices.

On-column experiments were performed in polyacrylamide gel matrices using the dye-labeled primers M13-NBD-HA and M13-Cy3 as probes. Both fluorescence intensity and lifetime were recovered

from the same dynamic MHF data to provide an lifetime-intensity electropherogram. The lifetime value was constant across a given peak and so the average lifetime across the peak was used to compare the different gel matrices. The lifetime of M13-NBD-HA detected on-the-fly, on-column is in the range of 1.8–2.0 ns regardless of the composition of the polyacrylamide gel matrix. This is longer than the lifetime of approximately 1.2 ns that was obtained for M13-NBD-HA in batch solutions of simple buffer and may be due to interactions of the primer with the gel. Interestingly, the long lifetime component of 4–5 ns that was observed for free NBD-HA in batch solutions of the gels was not observed for the primer-attached dye in the gel-filled capillaries.

The lifetime of M13-Cy3 also increases from approximately 0.8 ns to 1–1.5 ns in going from batch buffer solution to on-column detection in gel-filled capillaries. Although the lifetime variation is greater for M13-Cy3 than M13-NBD-HA among the different matrices, there are no discernible trends.

Based on the above observations, the lifetime increase of NBD-HA from batch solution gels to on-column detection in a capillary containing the same polyacrylamide gel as in the batch solutions cannot be attributed to gel matrix effects alone. It was therefore necessary to investigate other possible causes such as effects of experimental conditions and the data analysis process. These experiments were performed using NBD-HA in buffer.

The use of different emission windows, including a 500 nm longpass/600 nm shortpass combination, a 550 nm (80-nm bandwidth) bandpass interference filter, and a 515 nm longpass filter, did not significantly affect the lifetimes recovered for free NBD-HA dye in batch solution. The broad emission window created by the 515 nm longpass filter was therefore selected to give the highest signal intensity for on-column detection.

Fluorescein in phosphate buffer served as the lifetime reference in batch solution experiments, while scattered light from the detection window of the capillary was used as the lifetime reference for on-the-fly, on-column detection in CE. In order to determine if the choice of lifetime reference has any effect on the lifetime results for the primer-attached dyes, studies were performed using both fluorescein and scattered light references in both batch solution and on-column detection. For on-column detection, fluorescein solution was pumped into an empty capillary to get a reference lifetime relative to scattered light. The dominant lifetime component was found to be independent of the lifetime reference. However, longer lifetimes were recovered using on-column detection relative to batch solution, regardless of lifetime reference, especially for Cy3.

These experiments show that neither the emission window nor the lifetime reference are responsible for the differences observed between the lifetimes in batch solution and on-column. Since the on-column lifetimes show little systematic dependence on the particular polyacrylamide gel matrix, it is likely that the increase in lifetime in going from batch to on-the-fly, on-column measurements is due to more general effects of the interaction of the dye-labeled primers with the polyacrylamide gel matrix.

3.2. Investigations of cellulose sieving buffers and organic additives

Since the lifetimes of the primer-attached dyes show no systematic dependence on polyacrylamide gel composition, a study of the effects on lifetime detection of small amounts of organic solvents in an appropriate separation medium was undertaken. The cellulose sieving buffer HEC was chosen for these studies rather than the polyacrylamide gels in order to determine the effects of a different type of matrix; cellulose sieving buffers offer several advantages including low viscosity and ease of manipulation. A set of four dye-labeled M13 primers that can be excited at 488 nm (M13-Cy3, M13-NBD-HA, M13-RG and M13-BODIPY-FL) were used in these investigations.

Lifetimes of M13-Cy3 and M13-NBD-HA in batch solutions of 2% HEC containing 1× TBE, 6 *M* urea and 10% of the organic additive analysis are shown in Table 2. Both labeled primers exhibit multiexponential decay, as they did in the polyacrylamide gels. M13-RG and M13-BODIPY-FL exhibited essentially monoexponential decay (>90% fractional intensity of the major component), giving lifetimes of approximately 3.8 ns and 5.4 ns, respectively, in all of the solutions.

Table 2
Fluorescence lifetime results for M13-Cy3 and M13-NBD-HA in batch solutions of 2% HEC sieving buffer containing 10% (v/v) water or organic modifier[a]

Modifier	τ_1 (ns) (α_1)	τ_2 (ns) (α_2)	τ_3 (ns) (α_3)	τ_4 (ns) (α_4)
M13-Cy3				
Water	0.7 (0.46)	2.2 (0.41)	5.2 (0.13)	–
Glycerol	0.5 (0.27)	1.4 (0.49)	4.0 (0.22)	12.2 (0.02)
DMSO	0.9 (0.65)	2.9 (0.31)	–	8.2 (0.04)
Formamide	0.5 (0.35)	1.4 (0.45)	4.4 (0.19)	14.2 (0.01)
Methanol	0.6 (0.45)	2.0 (0.40)	5.0 (0.14)	13.5 (0.01)
DMF	1.0 (0.68)	–	3.6 (0.30)	11.1 (0.02)
M13-NBD-HA				
Water	1.1 (0.46)	–	3.4 (0.32)	–
Glycerol	–	1.4 (0.63)	4.3 (0.25)	12.2 (0.02)
DMSO	0.6 (0.19)	1.7 (0.67)	5.2 (0.14)	–
Formamide	–	1.5 (0.68)	4.8 (0.24)	–
Methanol	1.0 (0.51)	2.4 (0.36)	5.6 (0.11)	–
DMF	0.4 (0.15)	1.5 (0.58)	4.8 (0.35)	–

[a] Lifetimes (in ns) and fractional intensity contributions (in parentheses) for each sample performed in triplicate and recovered using MEM analysis. Standard deviations of the triplicate runs were approximately 12% for the major lifetime component.

Lifetime-intensity electropherograms containing all four dye-labeled primers were generated by sequential injection of the dye-labeled primers. Fig. 1 shows the lifetime-intensity electropherograms of the four, sequentially injected labeled primers in 2% HEC in buffer containing 10% water, 10% formamide or 10% DMF. The lifetimes in the figure were recovered using NLLS fits to a single lifetime component model. It should be noted that the lifetimes recovered in baseline regions of the electropherograms are due to background noise and scattered light. Lifetime is undefined in the absence of signal, resulting in recovery of noisy, meaningless values. These contributions are a negligible fraction of the total intensity in peak regions and should have little impact on the results.

Fig. 2 shows the lifetime results obtained using the self-modeling MEM analysis of the same lifetime data that was used to generate Fig. 1. In order to minimize the background noise in baseline regions between peaks, an in-laboratory program was used to multiply the fractional intensity of each lifetime cell in MEM lifetime results by the steady-state intensity at that migration time [1]. This weighting scheme serves to enhance the lifetime features associated with real fluorescence signals while minimizing the contribution from undefined lifetime ''garbage'' that is recovered in the absence of detectable signal.

The lifetime electropherograms of the dye-labeled primers in 10% water and 10% formamide are essentially identical, indicating that any differences in lifetime that are observed in batch mode are eliminated in the capillary. Also, the self-modeling MEM results for these two electropherograms show that there is essentially a single lifetime component, in contrast to the multiple components observed in batch solution.

The results for 10% DMF differ from those for water and formamide. The lifetimes of the primer-attached dyes are longer and the MEM results show that there is considerable heterogeneity for both M13-Cy3 and M13-NBD-HA, as was also observed in batch solution. This may be due to modification of the cellulose sieving buffer in the presence of DMF in such a way as to promote multiexponential decay of the dye or to solubilization of the dye-labeled primers in two or more different chemical microenvironments in the gel medium.

3.3. Signal background

A high signal-to-background ratio (S/B) is an

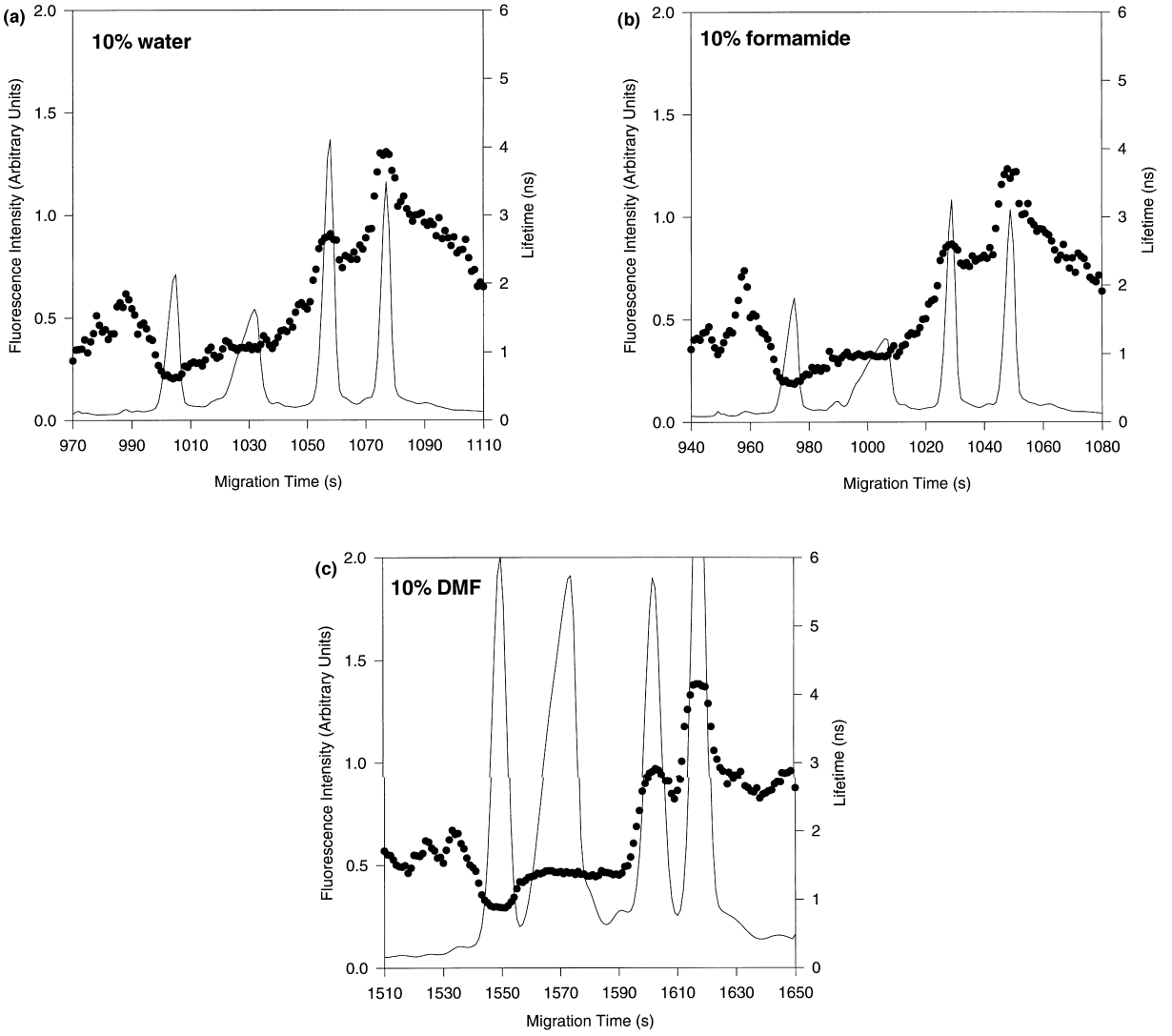

Fig. 1. Fluorescence lifetime-intensity electropherograms of four dye-labeled primers, sequentially injected and detected on-the-fly, on-column, in 2% HEC sieving buffer containing 10% (v/v) water or organic modifier. Lifetimes were recovered from NLLS fits using a monoexponential decay model. Peaks and concentrations of injected solution, in order of migration: M13-Cy3 (5 μM), M13-NBD-HA (20 μM), M13-RG (10 μM), M13-BODIPY-FL (20 μM). Injection times of 1 s for all except M13-NBD-HA (4 s), 10 kV, approximate injection volume is 1 nl.

important factor in maximizing lifetime detectability. The S/B for on-the-fly, on-column detection of fluorescence intensity in different gel matrices was investigated using M13-RG. The results are shown in Fig. 3. Better S/B values were obtained in the presence of methanol, formamide, or glycerol than in DMSO, DMF or 4% (w/v) non-crosslinked, linear polyacrylamide gel. The S/B for on-the-fly detected, dye-labeled primers depends on the purity and viscosity of the sieving buffer, and on the signal enhancement provided by the organic additives. Thus, it is important to select or design a sieving

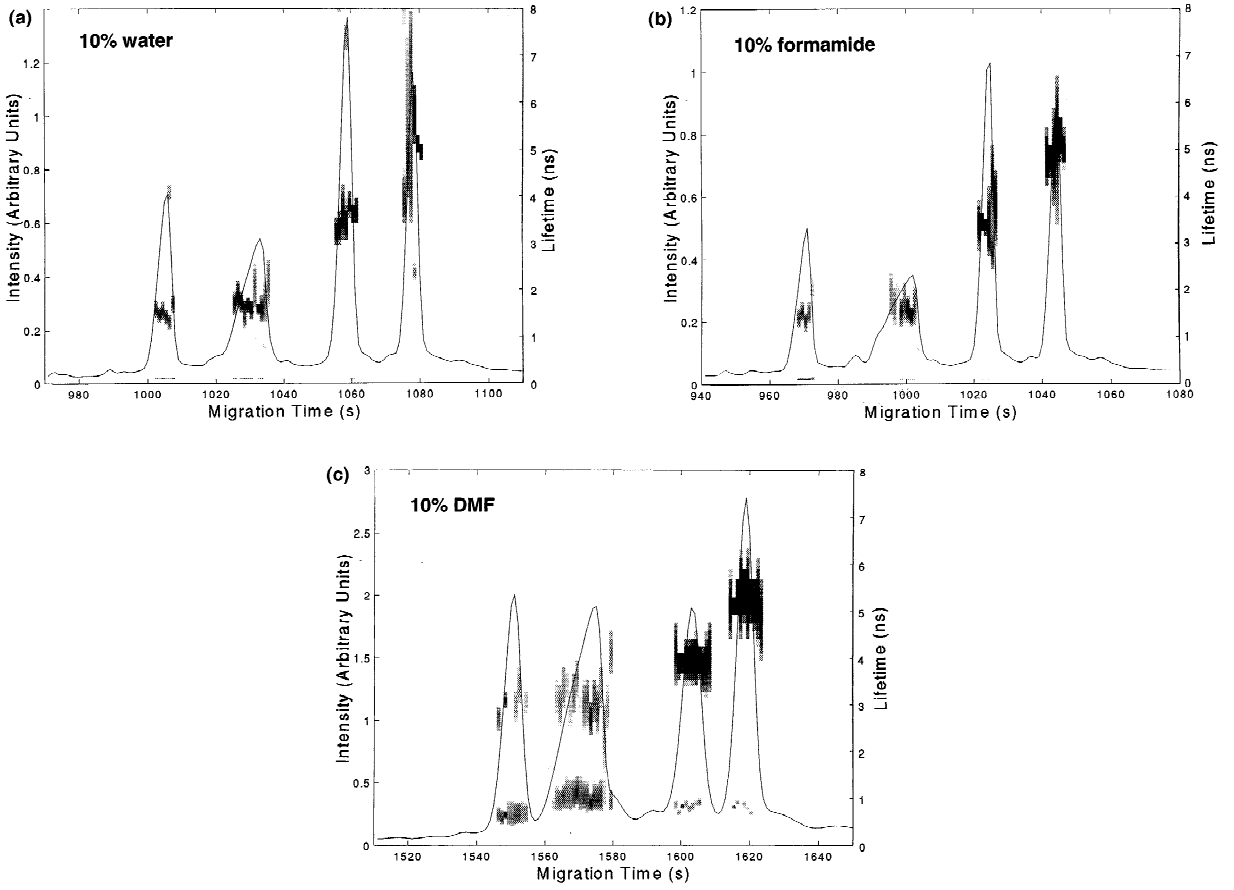

Fig. 2. Fluorescence lifetime-intensity electropherograms for the same data used for Fig. 1, but using the self-modeling MEM analysis to recover lifetimes.

matrix that contributes minimal background and enhances the fluorescence without loss of separation efficiency or lifetime resolution.

4. Conclusions

Lifetimes of the primer-attached dyes remain constant in polyacrylamide gels of varying composition and in cellulose sieving buffers as well. Thus, varying the gel composition will be of limited value in optimizing the lifetime detection of labeled DNA fragments, but on the positive side, this means that fluctuations in gel composition will not adversely affect the analysis. Organic additives can improve the signal-to-background ratio and, in some cases, appear to affect the lifetimes as well. Batch solutions of the gels are not reliable models for on-the-fly, on-column detection. In fact, primer-attached dyes exhibiting multiexponential decay in the batch solutions generally exhibit monoexponential decay on-column in the same gel medium. This is important since multiexponential decay would seriously complicate analysis of on-the-fly lifetime data. Care must be taken, however, to evaluate a matrix including any additives before using it since in the case of DMF, multiexponential decay was observed on-column for the shorter lifetime primer-attached dyes.

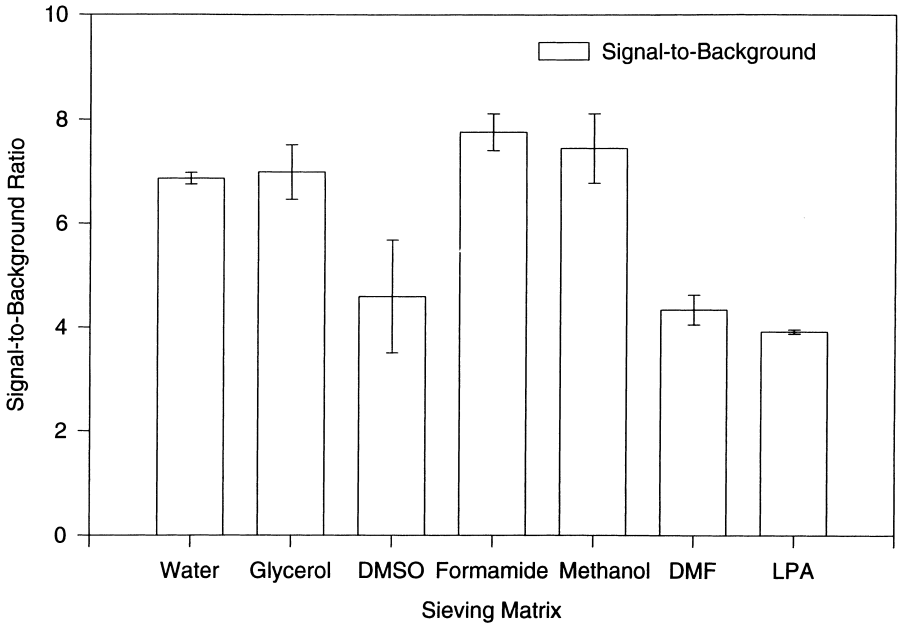

Fig. 3. Signal-to-background ratios and standard deviations obtained from electropherograms run in triplicate for on-the-fly, on-column detection of M13-RG (0.1 μ*M*) in different sieving matrices. Other conditions as in Fig. 1.

Acknowledgements

This work was supported by the National Institutes of Health (Grant 2R01-HG-01161).

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