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On-the-Fly Frequency-Domain Fluorescence Lifetime Detection in Capillary Electrophoresis

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On-the-fly fluorescence lifetime detection in capillary electrophoresis (CE) is demonstrated. Virtually continuous detection is achieved by interfacing a commercial CE instrument with a commercial, multiharmonic Fourier transform lifetime fluorometer (MHF). The CE capillary cartridge was modified to allow the capillary to pass through a specially constructed capillary column mount capable of micropositioning in the MHF sample chamber. Optimization of the CE/MHF interface was achieved through the incorporation of a focusing lens, appropriate alignment of the laser beam on the capillary, and use of appropriate optical filters in the emission beam. Both fluorescence intensity and lifetime are recovered from the dynamic MHF data, which is analyzed using either conventional nonlinear least-squares or the maximum entropy method, which allows for lifetime recovery without a priori knowledge of the system. Continuous, on-the-fly fluorescence lifetime detection using the MHF technology adds the dimension of fluorescence lifetime without sacrificing the resolution and speed of CE. Its application to the CE separation of a mixture of the fluorescent dyes NBD-hexanoic acid and fluorescein is demonstrated.

The importance of capillary electrophoresis (CE) as an analytical tool has grown steadily since its introduction.[1] The major advantages of CE over other techniques for chemical separation are speed and high resolution. CE requires only a minute volume or amount of sample, normally a few microliters, which is particularly important in applications such as biochemical analysis in which sample amount is often very limited. Numerous papers have described the theory, instrumentation, and application of the various CE techniques. A number of detection techniques, such as those based on absorbance,[2,3] fluorescence and laser-induced fluorescence,[4−7] conductivity,[8] electrochemistry,[9] mass spectrometry,[10] radioisotopes,[11] and refractive index,[12] have been successfully employed. UV−visible absorbance detectors are the most commonly used, while laser-induced fluorescence coupled with a sheath flow cuvette approaches the lowest detection limits.[13−15]

Although traditional, steady-state fluorescence techniques for CE detection offer high selectivity, sensitivity, and detectability, they have limitations. Detection at only one or a few wavelengths may be inadequate to resolve overlapping peaks, while the use of array detection to collect an entire spectrum can decrease sensitivity as a result of light dispersion. An alternative approach is the use of fluorescence lifetime to increase the information content and dimensionality of the detected signal. The entire emission window can be used in the lifetime measurement and lifetime can be used to resolve overlapping peaks. The benefits and limitations of fluorescence lifetime detection in chemical separations have only recently begun to be explored, with the advent of instrumental advances that allow on-the-fly detection of detailed lifetime information.

The ability of on-the-fly fluorescence lifetime detection (OFLD) using a frequency-domain fluorescence lifetime technology to detect, resolve, and reconstruct overlapping peaks in HPLC was previously demonstrated by our group.[16−18] In its most recent form, OFLD benefits from commercial, multiharmonic Fourier transform instrument (MHF) which allows simultaneous acquisition of multifrequency lifetime data.[16] The MHF provides the entire frequency response in a simultaneous measurement at intervals as short as milliseconds, allowing virtually continuous detection of lifetime during a single chromatographic run. This is a major advantage over other lifetime detection schemes which are often limited to a single lifetime determination for an entire peak, thereby prohibiting resolution of signals from multiple components over the course of overlapping peaks.

In this paper, we describe the application of the OFLD approach for CE detection. Measuring fluorescence from the very narrow capillary column presents particular challenges due to strong reflection and scattered light. The sheath flow cuvette[13] is capable of minimizing reflection and scattered light, thus providing very low detection limits, but current limitations such as high construction costs, difficulties in operation, and artifacts due to interference from the sheath flow with the capillary flow discourage its use. Although commercial laser-induced fluores-

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cence detectors are available, they were not readily able to be interfaced with the sample compartment of the MHF instrument. We therefore built an interface which incorporates a focusing lens and a custom-designed capillary mount to spatially minimize the background from reflection and scattering in a simple and economical way.

A second, unique aspect of this work is the application of the maximum entropy method (MEM) to the analysis of the OFLD data. MEM is a self-modeling method which does not require a priori knowledge or assumptions about the fluorescence decay of the sample. Recent studies of MEM have shown it to be a valuable tool in the recovery of fluorescence lifetime distributions from frequency-domain data.[19−23] Its application to OFLD should enhance the accuracy of lifetime recovery from on-the-fly data.

THEORY

In the frequency-domain, or phase-modulation, fluorescence lifetime measurement,[24] the excitation beam is intensity-modulated and the resulting ac component of the fluorescence emission is phase-shifted and demodulated relative to the ac component of the excitation beam. The phase shift (ϕ) and demodulation factor (m) can be used to calculate the fluorescence lifetime as follows:

$$\tau_p = \omega^{-1} \tan \phi \quad (1)$$

$$\tau_m = \omega^{-1}[((1/m^2) - 1)]^{1/2} \quad (2)$$

where τ_p and τ_m are the lifetimes calculated from phase and demodulation, respectively, and ω is the angular modulation frequency ($\omega = 2\pi F$, where F is the linear modulation frequency). For a single, exponential decay,

$$\tau_p = \tau_m = \tau \quad (3)$$

where τ is the true fluorescence lifetime. However, if the decay law is more complex, then the observed lifetimes τ_p and τ_m are only apparent values and measurements must be made at multiple modulation frequencies in order to resolve the lifetimes and fractional intensity contributions of the individual lifetime components.

The MHF instrument[25] generates a base frequency (F), which is then used by a comb generator to produce several harmonics (F, $2F$, $3F$, ..., nF). The sum of these harmonics is used by a Pockels cell to modulate the intensity of the exciting laser beam. A cross-correlation frequency, f, is combined with the base frequency ($F + f$, $2F + 2f$, $3F + 3f$, ..., $nF + nf$) to gate the PMTs, resulting in low frequency (f, $2f$, $3f$, ..., nf) signals which contain all of the high-frequency information. Conversion to low frequency allows the phase and modulation data to be easily detected by commercial electronics. In the MHF, the cross-correlation frequency determines the rate at which the lifetime measurements are made. For example, a cross-correlation frequency of 10 Hz corresponds to 10 lifetime measurements/s, or 1 lifetime measurement/0.1 s.

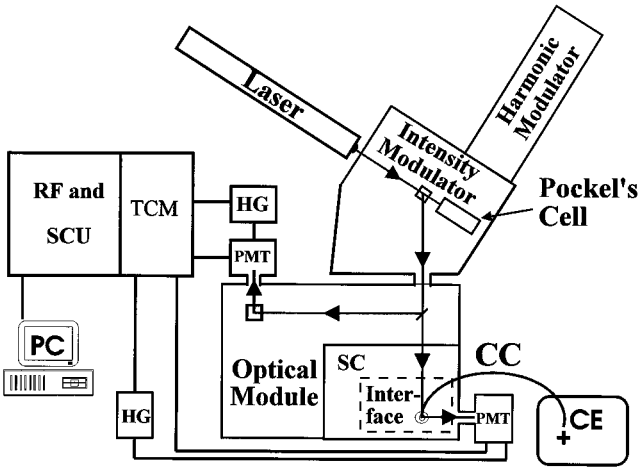

Figure 1. Schematic diagram of Model 4850MHF fluorescence lifetime instrument interfaced to a Model P/ACE 5000 CE system: SC, MHF sample chamber; HG, MHF harmonic generator; TCM, translator command module; PC, 386 personal computer; SCU, sampling control unit; CC, capillary column.

EXPERIMENTAL SECTION

Borate buffer was prepared from sodium tetraborate decahydrate (Aldrich) and boric acid (Mallinckrodt). The pH was adjusted with 1 N NaOH (Fisher) to 8.2, and the solution was then filtered through a 0.45-μm nylon filter (MSI). A stock solution (2.88 mM) of sodium fluorescein (Sigma) was prepared in 25 mM borate buffer and stored in the dark. A NBD hexanoic acid (NBD-HA, Molecular Probes, Eugene, OR) stock solution of 2.04 mM was prepared in the same borate buffer. Aliquots were removed and diluted to prepare solutions in a concentration range of 10^{-4} −10^{-7} M. A mixture of 4.62 μM fluorescein and 16.3 μM NBD-HA was prepared from their stock solutions.

A schematic diagram of the interfaced CE/MHF system is shown in Figure 1. The CE was a Beckman P/ACE 5000 equipped with a UV−visible detector. The low-pressure injection mode on the CE was used to introduce samples into a 75-μm-i.d., 375-μm-o.d. capillary column (Beckman) with 57 cm total length and 50 cm from the inlet to the detection window. A constant voltage (30 kV) was used to drive the analyte from the inlet to the window for the lifetime measurement.

OFLD was performed in the Kinetic Lifetimes mode of the multiharmonic Fourier transform fluorescence lifetime instrument (Model 4850 MHF, Spectronics). An air-cooled argon ion laser (Omnichrome) provided excitation at 488 nm. A Pockels cell is used in the MHF to electronically modulate the excitation beam intensity for dynamic-state measurements. The diameter of the excitation beam was then reduced with a focusing lens into a spot smaller than the diameter of the CE capillary. The emission beam was passed through another focusing lens and optical filters, and into a PMT for detection. The cross-correlation frequency (f) was set at 10.42 Hz, resulting in 1 lifetime measurement/0.096 s.

A scattering solution of kaolin was used as the lifetime reference ($\tau_{scatter} = 0$). In the batch experiments, the sample turret in the MHF alternates between the sample and the reference

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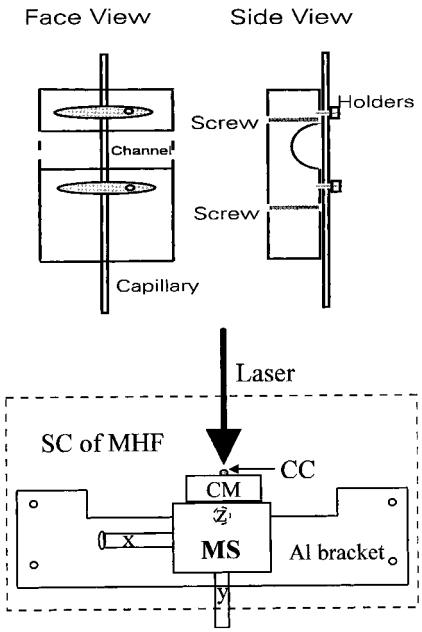

Figure 2. Components (top) and schematic diagram (bottom) of the capillary mount assembly. The capillary mount consists of three micropositioners, one for each axis. From the face view, one can see that the capillary is fixed onto the polyacrylate block with two small plastic holders. The assembly is then securely fastened in the MHF sample chamber with aluminum brackets: CC, capillary column; SC, sample chamber; CM, capillary mount; MS, micropositioning stages; *x*, *y*, *z*, micropositioners.

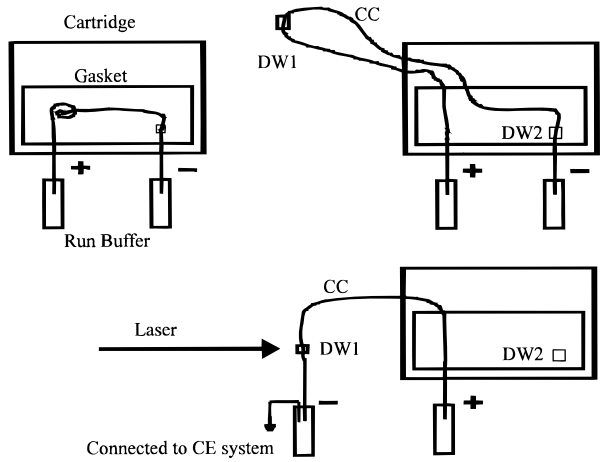

Figure 3. Modification of the CE cartridge. Top left: original assembly as provided with CE instrument. Top right: first modification approach. Bottom: second modification approach. CC, capillary column; DW1, detection window for OFLD; DW2, detection window for UV−visible absorption.

solutions. For on-the-fly detection in CE, the sample turret is removed, which disables the automatic reference lifetime acquisition. Instead, the reference is measured separately. This was done by placing neutral density filters in the emission beam instead of wavelength-selection optical filters and measuring the capillary column filled with the run buffer. The neutral density filters are necessary to reduce the intensity of the scattered light. After the reference lifetime measurement is finished, the neutral density filters are replaced with appropriate filters for wavelength selection of the sample emission.

An in-house program[16] is used to extract the intensity electropherogram from the dynamic MHF data. Another program was written and compiled to convert binary frequency data generated

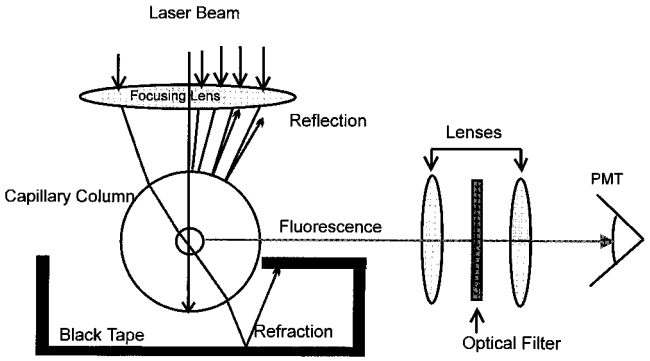

Figure 4. The vicinity of the detection window of the CE/MHF interface.

Table 1. Batch Mode Fluorescein Lifetime vs Scattered Light Reference Using 488-nm Laser Excitation and a 550-nm Long-Pass Filter

	replicates concn (μM)					
	115		4.62		0.462	
replicate	τ	χ^2	τ	χ^2	τ	χ^2
1	9.1	34	4.2	0.3	4.2	62
	9.1	15	4.2	1.5	4.2	67
	9.0	12	4.3	1.7	4.6	81
2	7.0	16	4.1	1.4	4.0	0.5
	7.2	8	4.1	4.7	4.0	0.1
	7.2	18	4.1	3.0	4.0	1.1
3			4.1	0.5		
			4.1	0.3		
			4.1	0.9		
av(SD)	8.10(1.1)		4.15(0.06)		4.17(0.2)	

by the MHF into text-formatted data readable by the MEM program. This program is also used to average the on-the-fly data over a designated time period, in order to smooth the data and to calculate "acquired errors", or standard deviations, of the phase and modulation measurements for use in the MEM analysis. In the absence of replicate CE runs, this is the only way of obtaining real measurement error. The alternative is to assume values for the errors associated with the phase and modulation measurements, such as constant (linear) error values, which is generally not as accurate as using true, acquired error in the data analysis.

Optimization of the CE/MHF interface was performed by alternately pumping the buffer and fluorescein into the capillary column and monitoring the dynamic (ac) and steady-state (dc) signals. The procedure was performed using different optical filters, focusing lenses, and alignments of the capillary column.

UV−visible absorbance spectra and fluorescence emission spectra of fluorescein were collected on a Lambda 6 spectrophotometer (Perkin Elmer) and an SLM 48000S spectrofluorometer (Spectronics), respectively.

RESULTS AND DISCUSSION

Design of the CE/MHF Interface. The capillary was mounted on a homemade device consisting of three micropositioners, a polyacrylate block, and two small pieces of polyacrylate (Figure 2). Polyacrylate was used because it is easy to work with and provides good electrical insulation between the CE and the MHF in case the weak detection window of the capillary column should break. The combination of the three micropositioners allows easy alignment of the capillary column. Alignment of the exciting laser beam onto the capillary is performed by adjusting

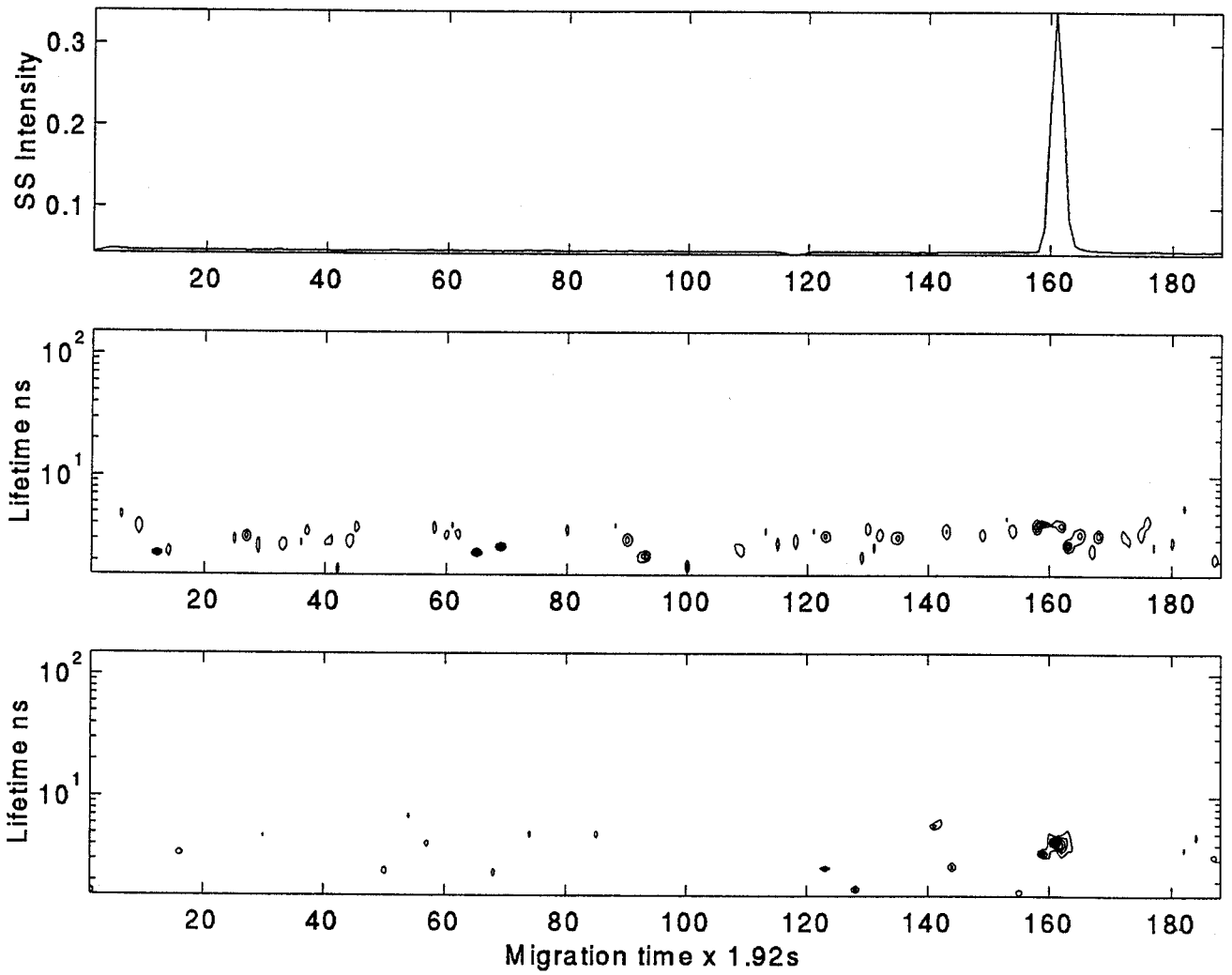

Figure 5. Intensity electropherogram (top) and lifetime electropherograms (middle and bottom) of 14.3 fmol of fluorescein in borate buffer (25 mM, pH 8.2): 30-kV separation, pressure injected, 57-cm column, 50-cm inlet to window, 550-mm long-pass optical filter, and 25-mm focusing lens.

the *x* and *y* micropositioners. The mount was securely fixed onto the MHF sample compartment using two heavy-duty aluminum brackets to maintain alignment. A channel having a depth of 7 mm was made to trap background light due to refraction and reflection.

It was necessary to modify the CE cartridge in order to interface the CE with the MHF. Figure 3 (top, left) shows the configuration of the original cartridge supplied with the CE instrument. The capillary extends from the high voltage (HV) end into the cartridge, winds around a bobbin, passes by the UV detection window, and then exits the cartridge. A large portion of the capillary is immersed in a mixture of fluoroorganic compounds which serves as a coolant. This configuration does not allow the capillary to pass through the MHF sample chamber.

Two approaches were used to route the capillary through the MHF sample chamber for OFLD. In the first approach (Figure 3, top, right), the capillary is extended from the CE cartridge into the MHF sample chamber, bypassing the bobbin. A window is burned onto the capillary for OFLD. The capillary re-enters the cartridge, where it passes by a window for UV detection that coincides with the original window in the capillary, and then exits from the cartridge. This design has two major drawbacks. First, the weak OFLD detection window in the capillary is subjected to a bending force and is easily broken in spite of extreme care in handling the capillary. Second, the capillary must be longer than 85 cm to be interfaced to the MHF due to the physical dimensions of the MHF and CE. The detection window for OFLD is approximately equidistant from the inlet and outlet of the CE, so that only half (15 kV maximum) of the applied high voltage is actually used in the separation. In addition, as the capillary is lengthened, the analysis time increases dramatically.

In the second approach (Figure 3, bottom), the capillary follows the same route into the MHF as in the first approach but uses the existing capillary window for OFLD and does not return to the cartridge. Instead, it terminates in the run buffer which is placed in the MHF sample compartment. To complete the circuit, an electrical wire is used to connect the ground of CE with the copper electrode in the run buffer in the MHF. In this configuration, the capillary can be as short as 40 cm and the detection window is free from loop strain. It is even more secure once it is fixed onto the capillary mount. This superior design was selected for OFLD. A drawback is that this design bypasses the UV−visible absorption detector in the CE instrument.

Minimization of Background from Reflected Light. The vicinity of the CE/MHF interface is shown in Figure 4, which illustrates the need to focus the laser beam that is incident upon the capillary column. When a surface of fused silica is exposed to a laser beam, reflection and refraction take place simulta-

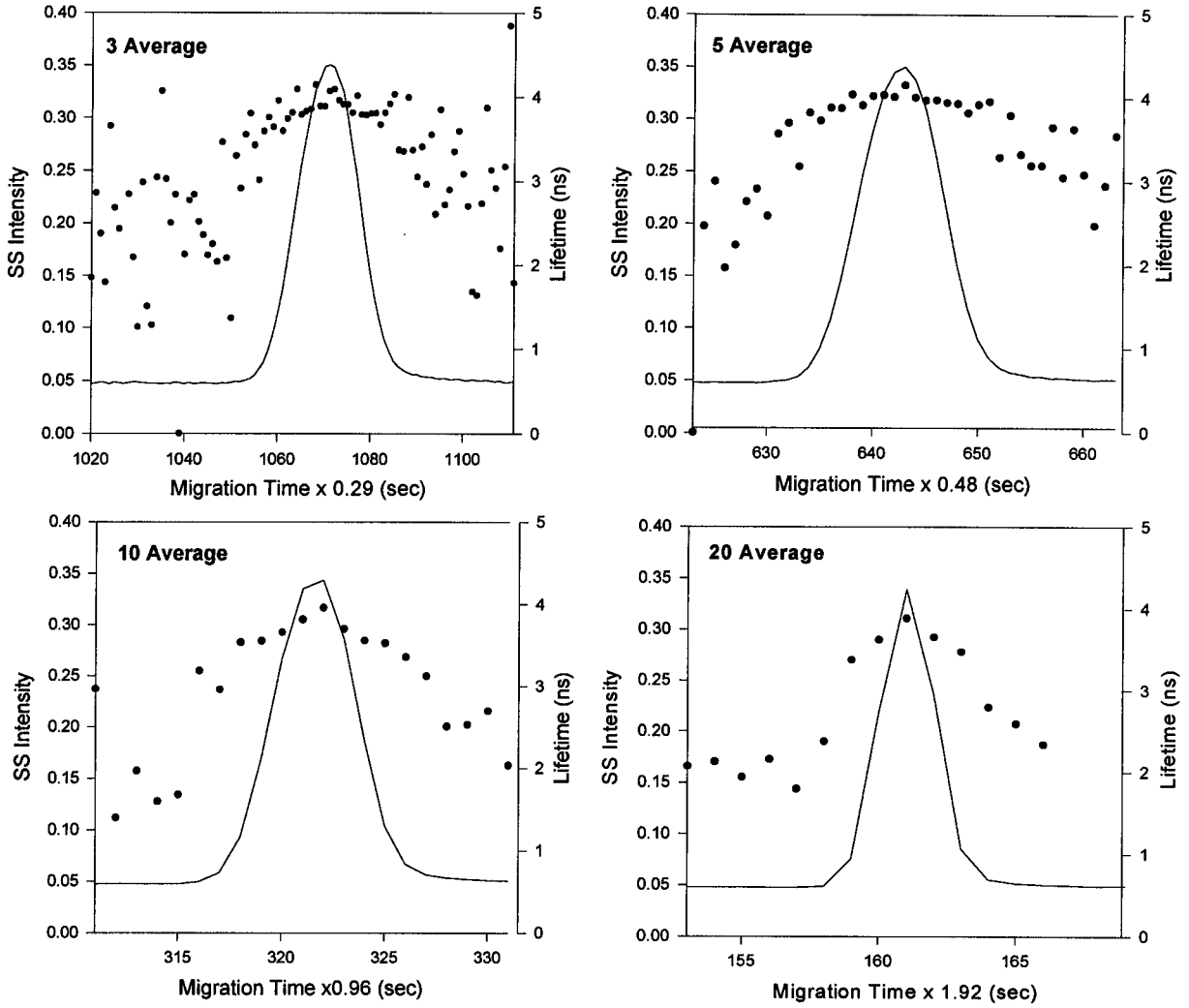

Figure 6. Intensity (solid line) and lifetime (circles) electropherograms of fluorescein for various averaging interval lengths.

neously. In a traditional spectrofluorometer with a rectangular, 1-cm fluorescence cuvette, the incident light is perpendicular to the flat cuvette surface and the detector is placed at 90° to the excitation beam, in order to minimize any signal from reflected light. However, if the light is incident on a curved surface such as a narrow capillary column, reflection is not as easily eliminated. The reflection from the capillary column spreads out and forms a plane of light since the diameter of the laser beam is larger than that of the column. If the incident light is perpendicular to the longitudinal axis of the capillary, then light rays that fall on the center of the capillary are either reflected at 180° or transmitted through the capillary without any change in direction. The angle between the incident and the reflected ray increases as the incident light ray is moved away from the center of the capillary. For example, an incident ray that is $^1/_4$ circle from the center is reflected at 90° to the incident ray. Incident light that is off-center on the capillary will contribute to the detected signal that is measured at 90° to the incident beam.

In order to minimize background from reflected light in the CE/MHF system, a focusing lens was placed in the path of the laser beam in order to converge the 1-mm parallel beam into a spot smaller than the diameter of the capillary (Figure 4). In addition, part of the CE mount was covered with black tape to block out refracted light.

Optimization of the CE Interface. Optimization of the CE interface was performed using measurements of fluorescein. The

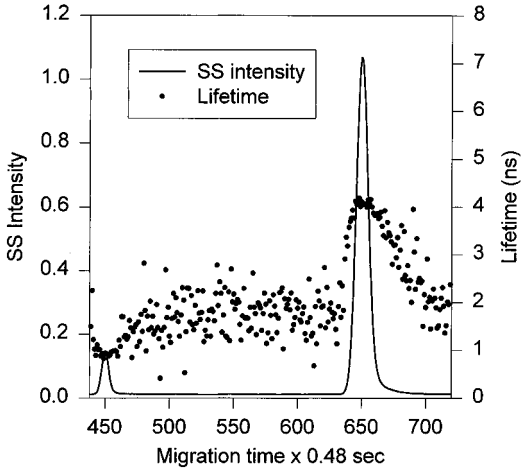

Figure 7. Intensity and lifetime electropherograms of fluorescein/NBD-HA mixture. Conditions as in Figure 5.

absorption spectrum of 4.62 μM fluorescein in borate buffer, pH 8.2, has four local absorbance maxima and a global maximum at 489 nm. The 488-nm line of argon ion laser is the best source for fluorescence excitation. The emission spectrum ranges from about 475 to 620 nm with a maximum at 516 nm.

The lifetimes of fluorescein were determined at various concentrations under batch conditions in rectangular, 1-cm cuvettes. Results are shown in Table 1 for analysis of the data using

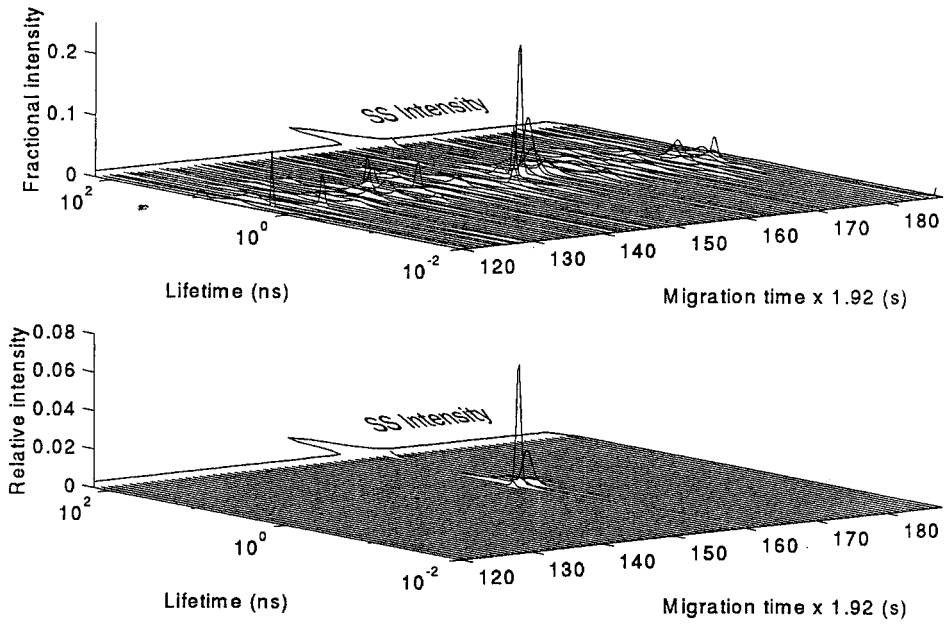

Figure 8. Three-dimensional image of OFLD electropherogram of fluorescein before (top) and after (bottom) steady-state intensity adjustment. Conditions as in Figure 5.

nonlinear least-squares (NLLS) fits to a one-component model. Good precision and consistency were obtained except for the 115 μM solution, for which results were very imprecise and yielded high χ^2 values, indicating poor fits. This is probably due to photobleaching, which was evidenced in stopped-flow CE experiments as well. Aggregation of fluorescein at this high concentration may also be a factor. The lifetime was found to be 4.16 ns using the results from the two lower concentrations, which agreed within a standard deviation.

Experiments were performed to examine the effects of different focusing lenses, optical filters, and positions of the laser beam on the capillary, the signal magnitudes, and signal-to-background ratios (S/B) in both dynamic and steady-state measurements. The steady-state S/B is the ratio of the dc intensity of fluorescein to that of the run buffer. The background dc signal is primarily due to scattered light and leakage of external light through the sample compartment. The dynamic-state S/B is the ratio of the ac amplitude of the fluorescein signal to that of run buffer. Several conclusions were drawn from the results of these experiments. First, best results were obtained when the laser beam was focused onto the center of the capillary. The purpose of an off-axis measurement is to divert most of the reflected light away from the detector. The off-axis detection resulted in a dramatic decrease in signal due to reduced measurement volume, while the decrease in reflection was not large enough to increase the S/B. Second, there was no difference between results using focusing lenses with focal lengths of 45 and 25 mm. Third, although a 520-nm long-pass filter appeared to give the highest signals, this filter also gave the worst S/B due to reflection of the strong 488-nm excitation line which is not entirely eliminated by the filter. The 530-nm band-pass filter and the 550-nm long-pass filter provided better S/B than either the 520-nm band-pass filter or the 520-nm long-pass filter. Further improvements in dynamic S/B were achieved by increasing the applied voltage. The setting with the 45-mm focusing lens and the 550-nm long-pass filter gave the best overall performance in both dynamic and steady states and was therefore selected for OFLD for use with 488-nm excitation.

On-the-Fly Lifetime Detection. Figure 5 shows a typical fluorescence steady-state (SS) intensity electropherogram and replicate fluorescence lifetime electropherograms of fluorescein. The three electropherograms were collected under the same conditions except that the 45-mm focusing lens was used for the bottom graph while the 25-mm lens was used for the others. The lifetime distribution recovered from MEM is shown at each migration time. At 307.2 s (160 $\times$ 1.92 s), fluorescein is detected and the lifetime is well-defined, giving a maximum fractional intensity at the expected lifetime of 4.1 ns. Fluctuations in lifetime (Figure 5, middle) or undefined lifetime components (Figure 5, bottom) are observed in the absence of a measurable fluorescence signal in between peaks.

Figure 6 shows the effect of the data-averaging interval on the lifetime results across the fluorescein peak. Four different interval lengths were examined, including 3, 5, 10, and 20 measurements per average. This corresponds to 0.29, 0.48, 0.96, and 1.92 s in the CE run since the cross-correlation frequency was 10.42 Hz. The results show that, if the averaging interval is too short, the lifetime results across the peak become imprecise due to noise in the raw data. If the averaging interval is too long, the lifetime results deteriorate at the peak peripheries. A five-point average (0.48 s) appears to give best results. This is different from the optimum of 10 points per average which was found for OFLD in HPLC.[16] The difference is due to the much shorter peaks in CE relative to HPLC (8 s in CE vs 30−60 s in HPLC in this comparison). Moreover, if data are overaveraged, then only a few points will be obtained per CE peak and resolution of lifetime information across the peak will decrease.

OFLD of a Fluorescein/NBD-HA Mixture. Figure 7 shows the intensity and lifetime electropherograms for a mixture of fluorescein and NBD-HA that was pressure-injected into the CE. NBD-HA, which has a lifetime of about 1 ns, migrates faster than fluorescein. These lifetime results were recovered by NLLS analysis. Although the molar intensity of NBD-HA is much lower than that of fluorescein, its lifetime is just as well-defined across the peak. This demonstrates one of the advantages of using fluorescence lifetime to characterize samples: since lifetime is

an intrinsic, normally constant, feature of a fluorescent analyte, the characteristic lifetime should be obtained once the signal exceeds the detection limit for lifetime detection. Thus, because OFLD is relatively insensitive to fluctuations in the analog intensity signal, it is a robust indicator of the analyte.

Again, as for the MEM analysis of fluorescein, fluctuations in lifetime or undefined lifetime components are recovered between peaks, in the absence of analyte intensity. In order to minimize such fluctuations, an in-house 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. 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. This method is very powerful in removing the noise in the fluorescence lifetime electropherograms, as shown in Figure 8 for fluorescein. Before the steady-state correction, noise dominates the electropherogram and the real lifetime signal is obscured. After the correction, the noise is greatly reduced and the real signal due to fluorescein emerges.

In order to further increase the detected signal without increasing the accompanying background, it may be feasible to use the 520 LP filter to obtain more fluorescence in combination with minimization of the background due to scattered and reflected light. This is accomplished in the data analysis by fixing a component to a very short (ps) lifetime to account for the background in NLLS or by assigning a compensating background channel in MEM. This is one of the advantages of lifetime-resolved detection and is currently under study.

CONCLUSIONS

The results demonstrate the successful recovery of fluorescence lifetimes of fluorescein and NBD-HA on-the-fly in CE using a custom-designed interface to the MHF lifetime instrument. The MHF has great potential as a CE detector since the entire frequency response of phase and modulation are acquired simultaneously with fluorescence intensity from a single, on-the-fly measurement during electrophoretic separation. These measurements can be made at millisecond intervals for virtually continuous detection, allowing many measurements per electrophoretic peak without sacrificing either the completeness of the lifetime information or the speed of the CE analysis. No a priori assumptions about the fluorescence decay are needed to perform the lifetime analysis by MEM, which can therefore serve as a useful guide to model selection for subsequent NLLS analysis. Data analysis strategies for OFLD in CE are currently under further investigation.

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