Electrophoresis with Polarized Fluorescence Detection. Application to Capillary Fluorescence Rejection

Shau-Chun Wang[a]* (王少君) and Michael D. Morris[b]
[a]*Department of Chemistry and Biochemistry, National Chung Cheng University, Chia-Yi 621, Taiwan, R.O.C.*
[b]*Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, USA*

In this paper, we integrate the apparatus of electrophoresis with polarized fluorescence detection to suppress the background fluorescence arising from illuminating the coating layer of channel containers with a laser excitation source. It has demonstrated moderate background suppression efficiency, close to the ideal 3-fold reduction on the surface doped with randomly distributed static fluorophores. In addition, when the fluorescence coverage is coated along the same orientation more uniformly, the background reduction is 10-fold in our experiments. This detection scheme is applicable to acquire the electrophoregrams of separating small organic molecules and biopolymers, such as nucleic acids, when the loading of staining dyes is not heavy. This apparatus is simple. Only adding a pair of polarizer optics is needed. This detection scheme should work equally well with an incoherent light source.

Keywords: Electrophoresis; Fluorescence rejection; Anisotropy.

INTRODUCTION

Fluorescence detection in capillary electrophoresis is conventionally performed in a section of capillary from which the protective polymer coating has been removed in order to eliminate the coating fluorescence. In glass or silica microchips there is no coating. In channels etched into those media fluorescence detection is performed through the non-fluorescent or weakly-fluorescent microchip material. Recently, there have been efforts to develop inexpensive microchips which can be mass produced from co-polyesters or other engineering polymers.[1-6] These materials are typically fluorescent. In this case, however, it is not easy to prepare a detection window in which the material fluorescence is absent.

It is possible to avoid capillary or microchip fluorescence by using fluorogenic reagents which are excited and emit in the red or near-infrared. However, most of the derivatizing reagents developed for analytical separations require green, blue or even ultraviolet excitation and detection at Stokes shifts of 20-100 nm. Typically, emission is detected in the 400-600 nm region, where polymer fluorescence is strong.

With laser-induced fluorescence, one can use confocal optics (spatial filtering) to reduce fluorescence from the electrophoresis capillary or chip. Confocal detection is more difficult if an incoherent source rather than a laser is used to excite fluorescence, or if imaging detection is required.

Fluorescence anisotropy has long been used to study rotational dynamics of large and small fluorescent molecules.[7] In this technique linearly polarized light is used to excite fluorescence. Emission is observed through a polarizer. Two polarization components are observable. Emitted light may be polarized parallel to or perpendicular to the plane of polarization of the exciting light.

Emission anisotropy (A) is defined by equation (1).

$$A = \frac{I_{vv} - I_{vh}}{S} \tag{1}$$

I_{vv} is the intensity of emitted light polarized parallel to the plane polarization of the exciting light. I_{vh} is the intensity of emitted light polarized perpendicular to the plane of intensity of emitted light, and S is the total fluorescence intensity.

In the present work we employ epi-illumination. In that case emission anisotropy is given by equation (2).

$$A = \frac{I_{vv} - I_{vh}}{I_{vv} + 2I_{vh}} \tag{2}$$

In cw illumination the anisotropy depends on the relative rates of rotation and excited state lifetime. If the molecule can change orientation before it emits, then fluorescence will be depolarized. Similarly, if the excited molecule remains stationary, then emission will be almost completely polarized. The anisotropy will be less than unity if the absorption and emission transition dipole moments are not exactly parallel to each other. This effect is usually small.

The situation is more complicated for polymeric molecules. Large polymers generally have rotational correlation times, which are long compared to fluorophore lifetimes. In that case, we would expect emission anisotropy of a fluorescent label or of intrinsic fluorescence to be quite close to one. In fact, depolarization is usually observed.

The most common depolarization mechanism is segmental twisting or bending.[8-10] More subtly, if the polymer contains many closely spaced fluorophores, Förster energy transfer from one to the next may occur.

Energy transfer depolarization has been demonstrated for nucleic acids heavily loaded with intercalators[8,9,11] because of the variety of depolarization mechanisms available for both large and small molecules or ions in solutions, which will exhibit low or zero anisotropy. In a rigid solid, fluorescence should be almost completely polarized. Only the noncoincidence of absorption and emission transition dipole moments causes the anisotropy to fall below unity.

However, static fluorophores, although stationary during the emission process, have orientation distributions, which diminish the ideal anisotropy of one to lower values. Emission anisotropy of static fluorophores follows equation (3),

$$A = \frac{(3 \cdot \langle \cos^2 \theta \rangle - 1)}{2} \quad (3)$$

In equation (3), θ is the angle between the direction of fluorophores' dipole and the orientation of incident polarized light. The expectation value $\langle \cos^2 \theta \rangle$ depends upon its distribution functions. In the case of completely polarized radiation such as scattering light, the angle θ is always or very close to zero. Anisotropy is, therefore, almost equal to the ideal value of one. In the case of randomly oriented fluorophores, however, $\langle \cos^2 \theta \rangle$ is equal to 0.6, whose anisotropy is 0.4. From the equation (3), when A is 0.4, the ratio is equal to three. In addition to residual anisotropy, strain birefringence and scattering may also be sources of fluorescence depolarization. Consequently, background suppression ratio with polarized fluorescence would be somewhat less than three.

On the other hand, when fluorophores are oriented more or less uniformly rather than randomly distributed, the anisotropy value should be somewhat greater than 0.4. The background suppression ratio would not be limited to three.[12] This case is demonstrated with a stretched film of nail polish, which is coated on the detection window of a capillary.

EXPERIMENTAL SECTION

Materials

Ethidium bromide (Molecular Probes, Eugene, OR) was diluted to 100 μg/mL. Propidium iodide (Molecular Probes) was diluted to 100 μg/mL. The reagents were used for free zone electrophoresis. Ethidium bromide was used as the intercalator for nucleic acids. The buffer in all CE experiments was 1X TBE, which is 8.9×10^{-2} M Tris, 8.9×10^{-2} M boric acid, and 5×10^{-3} M EDTA (1XTBE). The restriction digest fragments of one kbp DNA ladders (Gibco/BRL, Bethesda, MD, 1 kbp-12 kbp) were used at 50 μg/mL. The entangling polymer for nucleic acid separations was 0.19% (w/w) hydroxyethyl cellulose (HEC, Polyscience, Inc., Warrington, PA, Mn = 90,000-105,000). HEC 0.05% (w/w) was also used as a surfactant in free zone electrophoresis. A 3.5% (w/w) acrylmide solution was used to coat DNA electrophoresis capillaries to reduce electroosmotic flow.

Instrumentation

The capillary apparatus is illustrated in Fig. 1A. The electronics and data acquisition systems have been previously described.[13,14] Briefly, a personal computer with attached data acquisition board was used to drive a high voltage amplifier (Model 609, Trek, Inc., Medina, NY). A 543 nm HeNe laser was used to excite fluorescence, which was detected with a photomultiplier tube, digitized at 10 samples/sec and stored in the computer.

The polarized fluorescence system was arranged for epi-illumination. A plastic film polarizer (The Telescope Warehouse, Inc., NV, USA), P1, was placed between the laser and the 50/50 metallic beam splitter, BS. The input light was focused through a 20X/NA 0.35 microscope objective. Emitted light was collected by the same objective. The component passing the beam splitter was passed through film polarizer P2, which could be oriented parallel to or perpendicular to P1. The polarized emission was passed a 580 nm high pass filter (Kodak) to a Hamamatsu R928 photomultiplier tube.

Another optical train with the arrangement of 90° fluorescence collection system illustrated in Fig. 1B was em-

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ployed for the case of a detection window coated with nail polish. The same laser light was collimated with a lens (f = 180 mm, d = 40 mm). Fluorescence was collected through a 20X/NA 0.35 microscope objective before being projected to PMT.

Free zone electrophoresis was performed in a 25 cm capillary (20 cm to the detection window, ID 100 mm and OD 360 mm). In the detection window area, the polyimide coating was removed.

Polymer solution electrophoresis of DNA was performed in a similarly coated 75 mm ID capillary, 35 cm long (30 cm to the detection window), with an internal wall coating of polyacrylmide.[15] A thin layer of clear and stretched fingernail polish was coated on the detection window area.

Electrophoresis protocols

For small molecule separations, a capillary was filled with the running buffer solution (1XTBE). Mixed ethidium bromide (λ_{em} = 590 nm) and propidium iodide (λ_{em} = 610 nm) were introduced into the capillary by electrokinetic injection at 500 V/cm for 3 sec. After each injection, the sample vial was replace by a running buffer solution (1XTBE) reservoir. Separations were run at 500 V/cm.

Aliquots of restriction digest fragments of one kbp DNA ladders were intercalated with 10 μg/mL ethidium bromide prior to electrophoresis. The restriction fragment mixture was introduced into an internally coated, HEC-containing capillary at -114 V/cm for 5 sec. Separation was at -343 V/cm.

Anisotropy measurement of ethidium bromide

A spectrofluorometer (Model RF-5000, Shimadzu Co., Japan) equipped with polarizers in the excitation and emission optical train was employed for fluorescence spectroscopy and anisotropy measurement.

RESULTS AND DISCUSSION

Anisotropy measurement of ethidium bromide with spectrofluorometer

With the polarizers parallel and crossed orientation, four fluorescence spectra (580-700 nm) of ethidium bromide excited at 545 nm were taken. The integration of each spectrum from 580-700 nm was the measurement of fluorescence intensity. The anisotropy A was calculated according to the equation (4):

$$A = \frac{I_{vv} - I_{vh} \cdot G}{I_{vv} + 2I_{vh} \cdot G} \tag{4}$$
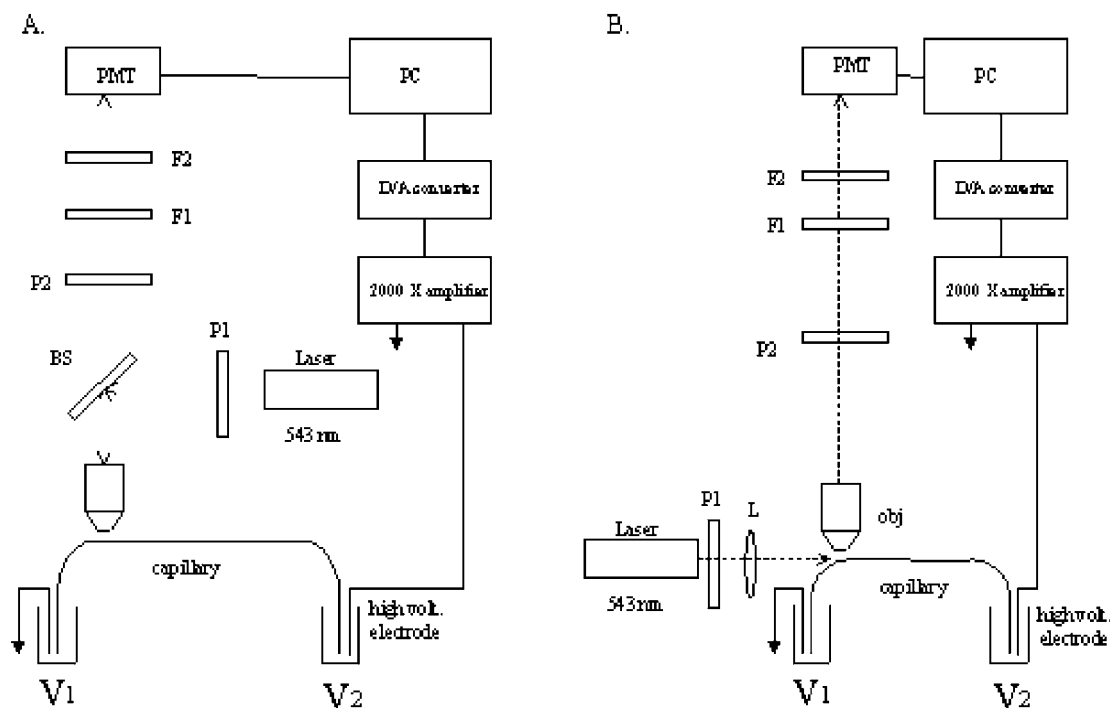

Fig. 1. Block diagrams of the electrophoresis apparatus with polarized fluorescence detection. BS: beam splitter; F1 and F2: high pass filters; P1 and P2: polarizers; V1 and V2: buffer reservoirs. High volt. electrode: HV electrode.

where the first and second subscript letters refer to the polarization planes of the excitation light and the emission light, respectively, and $G = I_{vh}/I_{hh}$ is an instrumentation correction factor.[16]

The zero calculation result shows the isotropic nature of the fluorescence of ethidium bromide. The rotational diffusion constant of a spherical molecule D_r follows,

$$D_r = \frac{k_B T}{6 \eta V} \qquad (5)$$

k_B is the Boltzman constant, T is absolute temperature, η is the solvent viscosity, and V is the molecule volume. Assuming the dye molecule to be a sphere with diameter 2.5 nm and using the water viscosity at 298 K, 0.894 cP, as η, one obtains the $D_r = 8.25 \times 10^{10}$/sec at 298 K. The time resolved anisotropy of fluorophores is an exponential decay function with a time constant equal to one sixth of the inverse of its rotational diffusion constant hV/kT. Accordingly, the time constant is 2 ps. The time constant of the anisotropy decay of a dye molecule is much smaller than the lifetime of ethidium bromide 20-30 ns.[17] The emission is expected to be isotropic.

Small molecule free zone electrophoresis

Fig. 2 shows electropherograms of ethidium bromide and propidium iodide. The upper trace shows the parallel polarization component of the detected emission. The lower trace shows the perpendicular polarization component of the detected emission. The background at the lower trace was close to one third as much as that at the upper one. However, the integrated intensities of electrophoretic peaks at the lower trace are about 1.3 times as great as those at the upper one.
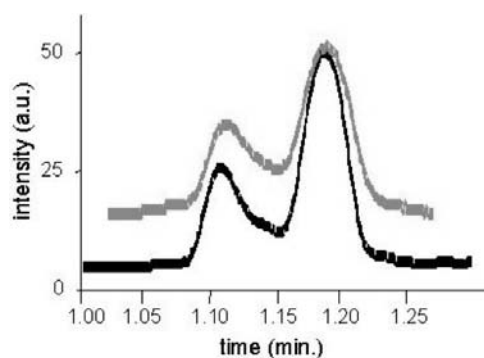

Fig. 2. Free zone electropherograms of ethidium bromide (100 μg/mL, first peak) and propidium iodide (100 μg/mL, second peak); Upper trace: electropherogram acquired without polarizers. Lower trace: electropherograms acquired with polarizers.

Transmission correction procedures and anisotropy calculation of small molecules

We filled the capillary with ethidium bromide solution. Without application of the electric field, we measured both the parallel component (I_{vv}) and perpendicular component (I_{vh}) of the fluorescence with an excitation source with polarization plane parallel to the capillary.

The anisotropy value of ethidium bromide solution should be zero. However, the intensity ratio (I_{vv}/I_{vh}) measured from capillary detection window is 0.80. We used this ratio as the instrumentation correction factor G of the fluorescence detection system of the CE instrument.

We performed several free zone electrophoreses of ethidium bromide with polarized fluorescence detection (electropherograms not shown). We calculated the anisotropy values. The average value is -0.09. A similar anisotropy was obtained from the electrophoretic data in Fig. 2.

Ethidium bromide solutions are mainly composed of organic cations and bromide anions. During zone electrophoresis, the fluorophores may align along the electric field. Accordingly, the assumption of complete rotational freedom is not valid. A non-zero anisotropy of its fluorescence is expected.

Nucleic acid CE separations with polarized fluorescence detection

Fig. 3 shows the electropherograms of the separations of a nucleic acid sample, one kbp DNA ladders restriction digest fragments. The upper trace and the lower trace were re-
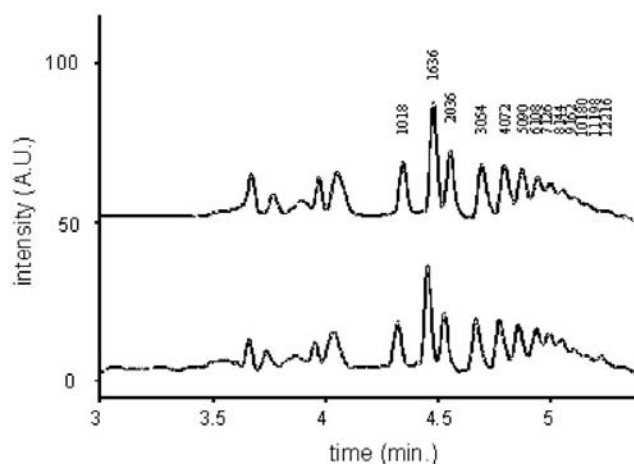

Fig. 3. Electropherograms of 1 kbp DNA ladder fragments in 0.19% HEC (M_n = 90,000-105,000). Upper trace: electropherogram acquired without polarizers. Lower trace: electropherograms acquired with polarizers.

Table 1. Capillary electrophoresis separation results of six DNA fragments in the 1 kbp ladder sample, including 1018, 1636, 2036, 3054, 4072, and 5090 base pair

Fragment size (bp)	Migration time (min.)		Efficiency (No. of theoretical plates)		Signal-to-noise (S/N) ratio	
	Upper trace	Lower trace	Upper trace	Lower trace	Upper trace	Lower trace
1018	4.3	4.3	58000	57000	> 100	> 100
1636	4.5	4.4	80000	80000	> 100	> 100
2036	4.6	4.5	72000	81000	> 100	> 100
3054	4.7	4.7	68000	67000	> 100	> 100
4072	4.8	4.8	50000	55000	> 100	> 100
5090	4.9	4.9	46000	46000	> 100	> 100

corded with the polarizers parallel and crossed orientation, respectively.

As similarly observed in previous publications,[18,19] because DNA ladder samples are not of 100% purity, some fragments smaller than 1 kpb are also seen in both electropherograms.

The CE separations of the first six fragments in the 1 kpb ladders are successfully achieved. The migration times and separation efficiencies (the numbers of theoretical plates) of these fragments in both electropherograms are illustrated in Table 1. The signal-to-noise (S/N) ratios of these fragments are all greater than 100.

There is no S/N ratio difference between the electrophoretic peaks acquired via polarizers of either parallel (upper trace) or crossed (lower frame) orientation. The background upper trace is of moderate level when the operational voltage of PMT is at typical setting (600 volt). The signals on the upper trace maybe reach the saturation level when the PMT voltage becomes somewhat higher. On the other hand, there is more room to increase the PMT voltage to acquire

peaks of higher sensitivity when the crossed-orientation polarizer is employed. The background obtained via cross-orientation polarizer does not reach the saturation level when we increase the PMT voltage to 1050 volt, where the PMT gain has become 100-fold higher.

The calculated anisotropy values based upon the integrated intensities of several peaks of one kbp DNA ladders in electropherograms (1-5 kbp) are shown in Fig. 4. The anisotropies are 0.10-0.20. Because the 1 kbp ladder samples were intercalated by ethidium bromide in a high density (0.2 dye/bp), the fluorescence depolarization is mainly due to energy transfer. The simulation of Carlsson and co-workers show that DNA intercalated at 0.2 dye/bp caused depolarization 0.4.[11] Since the anisotropy data of the 1 kpb ladder samples at zero intercalation are unknown, the anisotropy calculations from Carlsson's model cannot be accomplished. Comparison with the data in Fig. 4 is not feasible.

CONCLUSION

This work demonstrates the moderate effects of container background reduction by polarized fluorescence. Background reduction is limited by the orientation distribution of static fluorophores. While a laser is used for efficient illumination of a capillary, laser illumination is in fact not required in the experiment. Background reduction by polarization should work equally well with an incoherent light source.
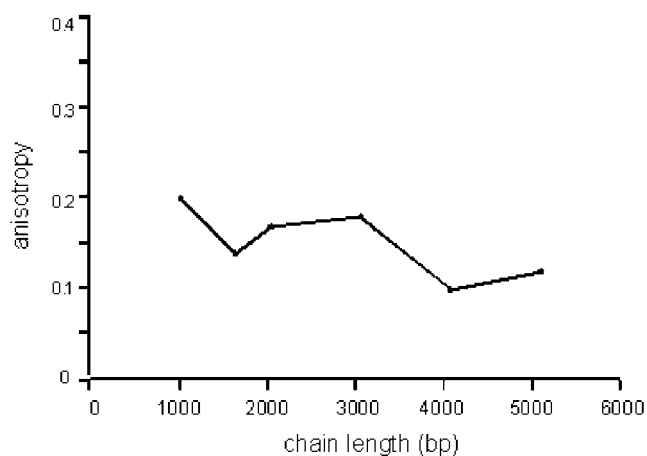
Fig. 4. Measured anisotropy values of several DNA ladder fragments during electrophoresis.

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