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Liquid Core Waveguide for Full Imaging of Electrophoretic Separations

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We employed a liquid core waveguide to image both DNA electrophoresis separations and isoelectric focusing of proteins. The utility of the system is demonstrated for DNA fragment sizing and protein separations. The system utilizes the liquid-core waveguide as an efficient window for both the excitation of separated samples and the collection of light through total internal reflectance, with an ability to detect target molecules in the zeptomolar range. Scanning the excitation laser along the length of the electrophoresis capillary excites individually separated analyte bands, while the fluorescence is collected end-on by an optical fiber coupled to a photomultiplier, thus, creating an image of the separation along the length of the capillary.

Electrophoresis has become the standard technique in DNA separation for fragment sizing and sequencing. Although ultrathin gel systems for slab-gel electrophoresis are proving to be a good alternative, the small sample quantity, low thermal gradients, and separation speed make DNA separation in capillaries and etched channels preferable to the slab-gel process.[1] Furthermore, the high sensitivity and selectivity of laser-induced fluorescence (LIF) detection together with the small volume required for detection makes LIF an excellent detection method for electrophoresis in these media.[2]

Traditionally LIF detection in capillary electrophoresis and etched channels is implemented with a fixed-detector format; i.e., the fluorescence is induced and observed at a particular point or "window" which the analytes flow past as they traverse the separation channel. Imaging or scanning the electrophoretic process along the complete separation channel length offers an attractive alternative. Nilsson et al. expanded on the attractiveness of such a detection scheme.[3] Their method includes early monitoring of the sample injection; termination of the separation at an optimal time (i.e., maximizing resolution while minimizing separation and detection time), rapid detection of separation failures, and improved data quality (via improved signal-to-noise ratio by revisiting weak bands). In addition, physical parameters affecting the separation can be easily determined, the condition of the capillary along the entire length can be monitored, and this monitoring can be used for educational purposes. In their work, Nilsson et al.[3,4] demonstrated the imaging of the electrophoretic process for DNA fragments by fluorescence detection from a decoated capillary with a CCD camera and laser illumination of the entire capillary. Similarly, Wu and Pawliszyn,[5,6] demonstrated the imaging of the isoelectric focusing process in bare capillaries using a CCD camera and light illumination for refractive index gradient imaging, absorption imaging, and fluorescence imaging of separated proteins.

To scan the capillary image, the electrophoretic separation, and isoelectric focusing of proteins, Beale and Sudmeier[7] used a confocal lens detection scheme on a bare capillary positioned on a translational table. Handling decoated/bare capillaries (i.e., where the external protective polyimide coating has been removed) poses a technical problem in that these systems become very fragile. Therefore, Kim et al.[8] used the translucent properties of Teflon-coated capillaries, in a capillary pulling machine that rolled the capillary past the excitation laser beam and orthogonal light collection optics. This use of the Teflon-coated capillary eliminates the fragility of decoated/bare capillaries, but Kim et al. found very low sensitivity for fluorescence detection in DNA sequencing. Fluorescence light collection from bare/decoated capillaries is low in efficiency because light escapes in all directions from the capillaries; thus, large numerical-aperture optics must be used to increase sensitivity. On the other hand, the low refractive index of the Teflon coating on the capillaries, used by Kim et al.,[8] caused the electrophoresis capillary to act as a liquid-core waveguide, trapping much of the fluorescence emission inside the capillary and letting very little light escape for detection, which, in retrospect, is the most likely cause of the low sensitivity observed in their system. Teflon AF-coated capillaries have been demonstrated, in capillary electrophoresis, as an excellent detection medium for fluorescence via total internal reflectance (TIR) in the liquid core.[9] Recently, such a system was used to demonstrate the detection of fluorescence from DNA sequencing

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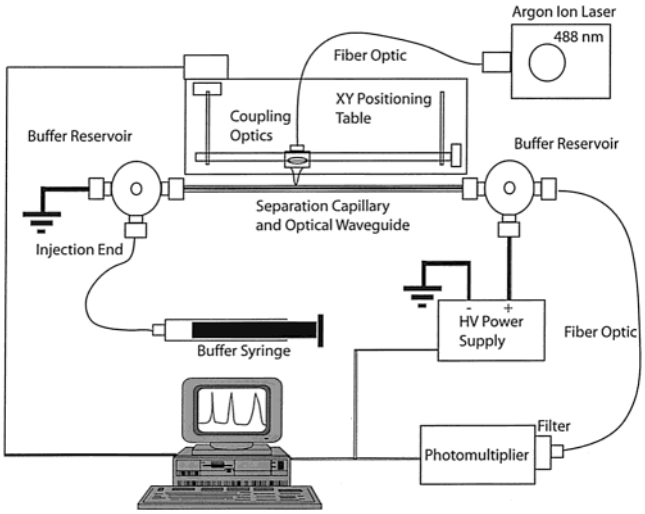

Figure 1. Schematic diagram of the capillary electrophoresis/laser-induced fluorescence detection and imaging system.

separations in 30-cm capillaries.[10] In this paper, we demonstrate the use of Teflon AF as a platform for scanning and imaging the complete electrophoretic separation. We demonstrate this new detection-and-imaging scheme both on short separation channels with modified sieving gels for DNA fragment sizing and on gels for the isoelectric focusing of proteins.

EXPERIMENTAL SECTION

Apparatus. The instrumental setup is schematically shown in Figure 1. A 300-μm-o.d. by 100-μm-i.d. by 12-cm-long Teflon AF-coated capillary (Polymicro Technologies, Phoenix, AZ) was securely positioned between two modified Peek Ts (Valco Instruments Co, Houston, TX). The Peek Ts have been drilled through with a 0.5-mm drill to allow the ends of the capillary to be inserted up to the middle of the T. A 1-mm orifice connecting to the T channel was drilled on the top of each structure to make small reservoirs ($\sim$5 μL) that serve as injection-end and termination-end buffer reservoirs. A platinum electrode, connected to electrical ground potential, was inserted into one leg of the injection-end T. A syringe (not shown) with running buffer was connected to the second leg of the injection-end T. The buffer syringe was used to wash the buffer reservoir and maintain it full. A second platinum electrode, connected to a 5000-V dc power supply, was inserted into the leg of the terminating-end T coupling. A 400-μm core diameter fiber-optic (model FVP400440480UVM, Polymicro Technologies) was inserted into the leg directly opposite the Teflon AF-coated capillary. The fiber-optic end and capillary end were aligned under a stereomicroscope by viewing their positions through the reservoir opening. The spacing between the two ends was $\sim$500 μm. The detection fiber optic was coupled to a 510-nm band-pass filter (model 510DF20, Omega Optical, Brattleboro, VT) and an analog photomultiplier (model HC120−05, Hamamatsu, Bridgewater, NJ).

A fiber-optic coupled argon ion laser (model Reliant 50S, Laser Physics, Salt Lake City, UT), 488-nm emission line, was coupled to a 15-mm focal length lens and focused to 50-μm spot. The fiber-optic and lens mount was mounted on a stepper motor XY translational table (model MD-2, Arrick Robotics, Hurst, TX). The table had a resolution of 125 μm/step and was typically run at 2 steps/acquisition and 3.98 steps/s. A LabView program (National Instruments, Austin, TX) was created to control the XY table and acquire the photomultiplier output data from a 12-bit ADC board (model PCI-MIO-16E-4, National Instruments, Austin, TX). Data were typically acquired with 0.25-s integration time per two motor steps. The XY table allowed the positioning of the fiber optically coupled laser along the entire length of the Teflon AF-coated capillary. A full scan of the 12-cm capillary was performed in 120 s using this setup.

Sieving Matrix. For DNA fragment analysis, the two 5-μm electrode reservoirs and the 12-cm separation capillary were each loaded with a mixture containing 0.5% (w/v) poly(vinylpyrrolidone) (PVP, 1 000 000 M_r, Polyscience, Warrington, PA) and 0.5% (w/v) hydroxypropylmethyl cellulose (HPMC, Sigma Chemical, St. Louis, MO). These materials were prepared in electrophoresis grade 1× Tris−boric acid−EDTA (TBE, Fisher Scientific, Fairlawn, NJ). Combined in this manner, these polymers served both as a physical sieving medium for the DNA electrophoretic separations and to deactivate the surface charge on the inner wall of the 100-μm-i.d. Teflon-coated separation capillary, thus stopping the electroosmotic flow of the buffer/sieving matrix. This combination of HPMC with PVP provided a reduction in the overall viscosity of the sieving matrix, while allowing high-resolution DNA separations normally achievable only with the use of higher concentrations and consequently higher viscosity, cellulose-based sieving matrixes.

For capillary isoelectric focusing experiments, an inner capillary solution was prepared containing 2.5% (v/v) pH 3−10 ampholytes (Bio-Rad Laboratories, Hercules, CA), 8 M urea (Sigma Chemical), 2% (w/v) (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate) (CHAPS, Sigma Chemical), 50 mM DL-dithiothreitol (DTT, Sigma Chemical), 0.025% (w/v) PVP, and 0.025% (w/v) HPMC. These additives were necessary to form a dynamic coating to reduce EOF and minimize bulk flow, to minimize interactions between the analytes and the capillary wall, and to enhance protein solubility. Prior to performing isoelectric focusing, the Teflon AF-coated capillary was filled with this solution. At the cathodic end of the capillary, a 20 mM NaOH catholyte solution was used in the 5-μL electrode reservoir, and similarly, at the anodic end a 10 mM H_3PO_4 anolyte solution was utilized. Under the influence of an applied electric field, a pH gradient was induced by the ampholytes, with the high- and low-pH sides set up at the cathode and anode, respectively. The sample components then focus according to their isoelectric points in the pH gradient between the sodium hydroxide and phosphoric acid electrolyte reservoirs.

Sample. Fluorescently labeled DNA samples for capillary electrophoresis experiments were prepared by contacting a 100-base pair (bp) DNA ladder (Promega Corp., Madison, WI) with PicoGreen nucleic acid stain (Molecular Probes, Eugene, OR). Briefly, this involved preparing a 1/20 dilution of the PicoGreen reagent in 1× TBE and adding 30 μL of this solution to 75 μL of the Promega DNA ladder. The Promega ladder consists of 11 fragments ranging in size from 100 to 1000 base pairs in 100-bp increments, with an additional fragment at 1500 base pairs. The

(10) Hanning, A.; Lindberg, P.; Westberg, J.; Roeraade, J. *Anal. Chem.* **2000**, *72*, 3423−3430.

500-bp fragment is present at approximately three times the concentration of the other fragments and was used as a reference indicator. The PicoGreen DNA stain is specific for double-stranded DNA and exhibits fluorescence excitation and emission maximums at 480 and 520 nm, respectively, making it ideal for use with the 488-nm line from an argon ion laser.

Isoelectric focusing experiments were conducted using fluorescently conjugated, high molecular weight protein markers (Sigma Chemical), which were added to the capillary inner filling solution to achieve a 2.5% (v/v) final concentration. These molecular markers consisted of six proteins ranging in molecular weight from approximately 20 000 to 200 000 and come prelabeled with fluorescein isothiocyanate (FITC), making them ideal for excitation using the 488-nm emission line of an argon ion laser.

Sample Injection Procedure. For DNA fragment analysis, the Teflon-coated capillaries were prepared for capillary electrophoresis experiments by first cutting the bulk capillary to 12 cm and polishing both ends. With the capillary carefully positioned in the modified PEEK fittings, the capillary was loaded with the PVP/HPMC sieving mixture by closing off the injection buffer reservoir and applying pressure with the syringe containing the sieving material at the injection end of the capillary. The injection- and terminal-end buffer reservoirs were also each filled with the PVP/HPMC sieving matrix. A 3000-V potential was applied for 1 min to precondition the capillary and to monitor the current, typically 10−20 μA. Next, the PVP/HPMC sieving material was removed from the injection-end buffer reservoir using a Pasteur pipet and replaced with the PicoGreen-labeled Promega 100-bp DNA ladder. A 1500-V potential was again applied for 5 s to electrokinetically inject the sample into Teflon capillary. The DNA ladder was removed from the injection port reservoir and replaced with the PVP/HPMC buffer solution. The potential was again set to 1500 V, and a LabView VI was started to monitor the electrophoresis current and voltage, laser position, time, and photomultiplier response.

Similarly, for the protein isoelectric focusing experiments, a 12-cm Teflon AF-coated capillary was prepared and loaded with the inner capillary filling solution containing the labeled proteins and was subjected to a 3000-V potential (250 V/cm). With the voltage kept constant during the isoelectric focusing, the current is known to decrease during the focusing step due to the increasing resistance of the generated pH gradient. The end of the focusing step was estimated by monitoring the decrease in current, and the separation was terminated (voltage off) when the current reached roughly 15−20% of its initial value. Utilizing the entire capillary as an optical waveguide allowed elimination of the mobilization step typically employed to read the analytes by conventional detection methods. At the end of the isoelectric focusing step, the LabView VI was initiated, laser rastering of the separation capillary commenced, and the positions of the protein bands were determined by monitoring the fluorescence signal of the analytes as a function of laser position along the capillary.

RESULTS AND DISCUSSION

Making the electrophoresis separation channel function as an optical waveguide provided several advantages to our detection system. As shown by Dasgupta et al.[9] and Hanning et al.,[10] the liquid-core optical waveguide that is formed in the separation channel provides an effective method for capturing the fluorescence emitted by the excited analytes through total internal reflectance. A properly mated and filtered light detector, e.g., a photomultiplier, a CCD camera, or a photodiode, can then detect this signal light. The detection limit observed for fluorescein, using an analog photomultiplier and simple light filter, is below 40 zmol. This detection limit can be improved with the use of spatial filtering to remove laser scattered light, which promulgates primarily through the fused-silica core of the capillary.[10]

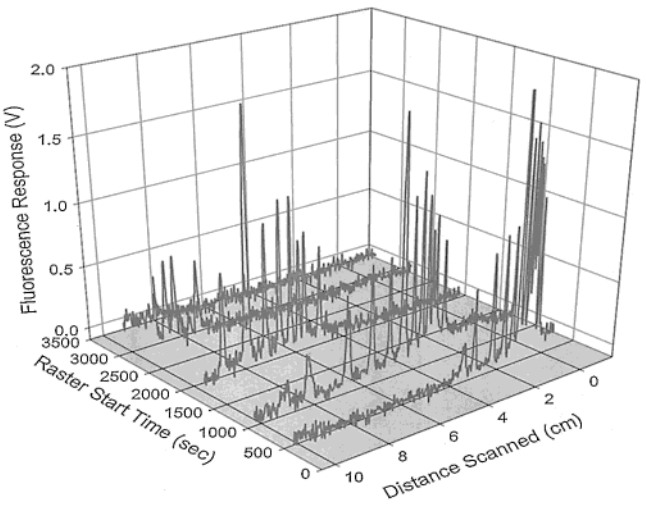

Figure 2. Selected imaging scans of a DNA fragment ladder at various times during the electrophoresis run.

An estimate of the efficiency of light collection can be made from the critical angle for light reflection due to the change in the index of refraction between the liquid core and the Teflon AF coating. The index of refraction for gels is ∼1.4 while for Teflon AF it is 1.31.[9,10] The critical angle is given by $\sin \theta_c = n_2/n_1$, and for this system it is 69°.[11] Thus, the calculated light collection efficiency for the current system is ∼12%. Adding an additional detection fiber optic at the injection end should further improve the sensitivity by a factor of 2. Nevertheless, imperfections in the Teflon coating, such as scratches and impurities, will cause further light losses.

In addition to this excellent sensitivity, the light waveguide provides the ability to observe the electrophoresis separation at any point in the capillary. This is done by scanning the length of the capillary with the excitation beam, as shown in Figure 1, thus producing a full image of the separation inside the capillary. The separation can be imaged several times, as long as the laser power is low enough so as not to cause significant photobleaching of the analyte. Although the laser system used in this work was capable of 20-mW power output, it was typically used at its minimum setting estimated to be 5 mW. Nevertheless, only a small portion, ∼<1 mW, of the beam actually makes it into the capillary since much of the laser light is reflected by the outer surface of the capillary. The slow scan speed of our XY table required that the electrophoresis voltage be turned off while a full image of the separation channel is gathered. This start-and-stop action could have some minor effects on the overall performance of the separation due to diffusion, heating, and high-voltage spikes. Such effects were not investigated in this work. A faster XY table would

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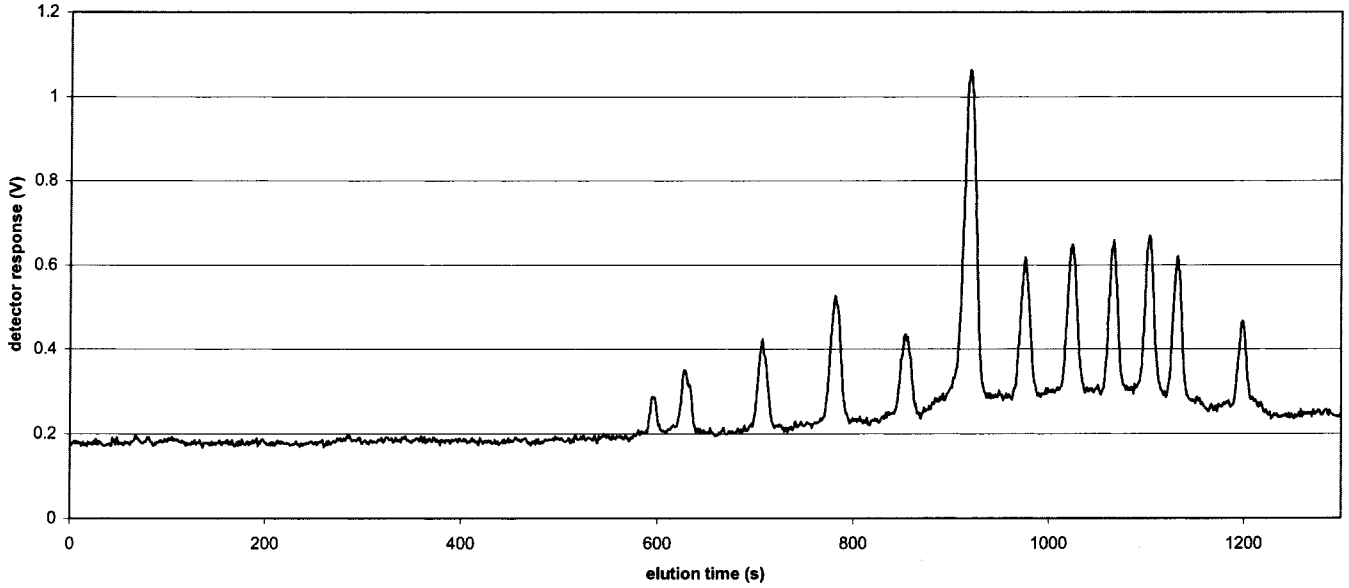

Figure 3. Fixed-detector (10 cm from injection end) separation of a 1500-bp DNA fragment ladder.

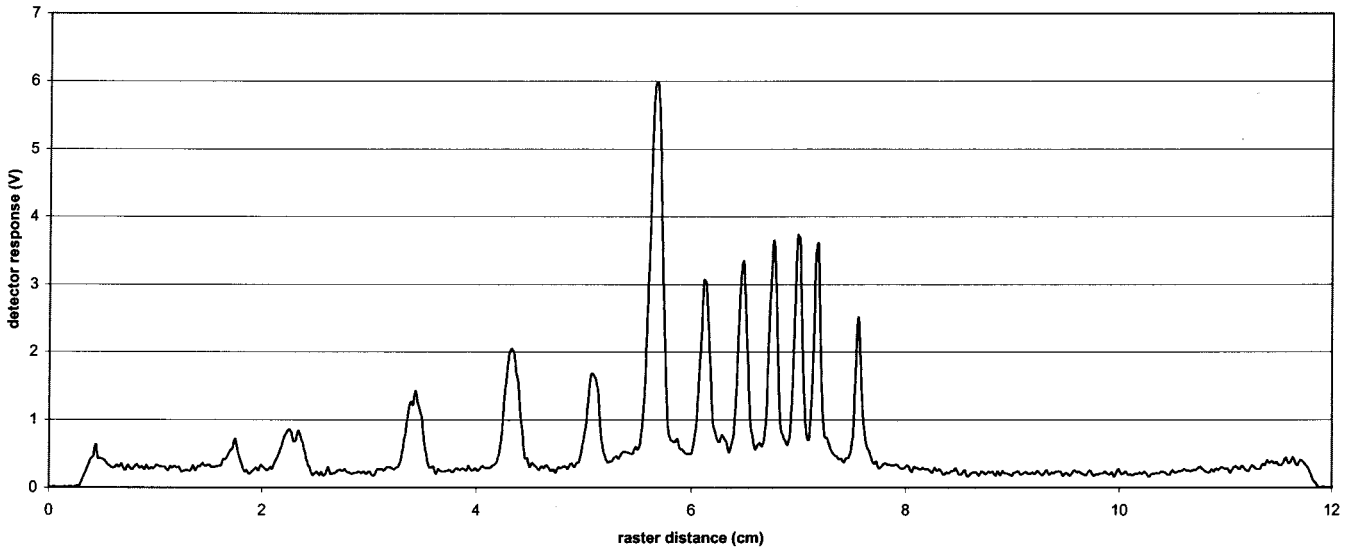

Figure 4. Raster image of the capillary electrophoresis separation shown in Figure 3 after 600 s of electrophoresis time.

overcome this requirement. Nevertheless, with this system, we were able to monitor full separations as they occurred in time, as shown in Figure 2. Early scans would allow for the fast determination of the quality of the injection, availability of signal, and adjustment of the separation electrical field strength in order to maximize the resolution while minimizing the analysis time.

By fixing the excitation laser near the end reservoir we were able to duplicate a traditional electrophoresis experiment, in which all analytes must migrate through the detection window in order to be detected. Such a separation is shown in Figure 3, where the *X* axis corresponds to elution time for each DNA fragment. Note that, for this particular separation, the first fragment was observed at 10 min after injection and the last fragment was observed at ~20 min after injection. When the electrophoresis separation was stopped after 10 min from injection, the scanned electropherogram image shown in Figure 4 was observed. The signal difference between Figures 3 and 4 is due to different amounts of PicoGreen dye used in the injected DNA samples. In both these electropherograms, a small peak of unknown origin that is possibly an impurity in the sample precedes the 100-bp DNA ladder. The two electropherograms, shown in Figures 3 and 4, are related to each other according to the elution time and migration position of each fragment, i.e., according to their mobility. This relationship is given in Table 1. The measured mobilities for both electropherograms are very similar, even though no attempt was made to control the temperature of the system or to exactly reproduce all of the separation conditions. The measured resolutions are calculated according to eq 1 from

$$R = [2 \ln 2]^{1/2} (t_2 - t_1)/(\mathrm{hw}_1 + \mathrm{hw}_2) \qquad (1)$$

the full width at half-maximum (hw) and the elution times or positions for each fragment (t) assuming Gaussian peak shapes.[12]

As expected, the resolution for the scanned detection was slightly worse than that for the fixed detection. This is due to the

(12) Luckey, J. A.; Norris, T. B.; Smith, L. M. *J. Phys. Chem.* **1993**, *97*, 3067−3075.

Table 1. Measured Mobilities and Resolution for the CE-LIF System

	fixed detection (at 11 cm)			scanning detection (at 610 s)			ΔR	
fragment size (bp)	elution time (s)	mobility ($cm^2/V \cdot s$)	measured resolution	position (cm)	mobility ($cm^2/V \cdot s$)	measured resolution	measd	theor
100	630	1.40×10^{-4}	3.97	9.71	1.27×10^{-4}	3.43	1.16	1.06
200	707	1.24×10^{-4}	3.51	8.58	1.12×10^{-4}	3.23	1.09	1.13
300	782	1.13×10^{-4}	3.37	7.67	1.01×10^{-4}	3.07	1.10	1.19
400	854	1.03×10^{-4}	2.86	6.92	9.07×10^{-5}	2.63	1.09	1.26
500	919	9.58×10^{-5}	2.58	6.34	8.31×10^{-5}	2.46	1.05	1.32
600	975	9.03×10^{-5}	2.52	5.88	7.70×10^{-5}	2.29	1.10	1.37
700	1023	8.60×10^{-5}	2.30	5.53	7.25×10^{-5}	1.97	1.17	1.41
800	1065	8.26×10^{-5}	2.05	5.24	6.87×10^{-5}	1.69	1.21	1.45
900	1103	7.98×10^{-5}	1.71	5.01	6.57×10^{-5}	1.34	1.28	1.48
1000	1131	7.78×10^{-5}	4.38	4.82	6.32×10^{-5}	3.02	1.45	1.51
1500	1198	7.35×10^{-5}		4.45	5.84×10^{-5}			

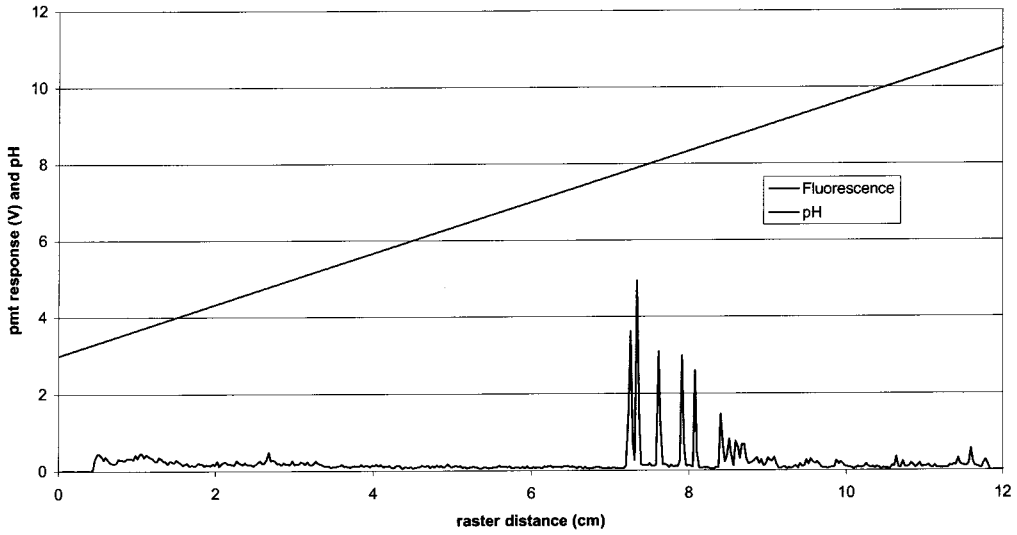

Figure 5. Raster image of the isoelectric focusing of FITC-labeled high molecular weight proteins. The diagonal line indicates the estimated linear pH gradient within the capillary.

fact that, in the scanned detection system, the fragments have not traveled as far as in the fixed detection system. In fact, if band broadening due to diffusion and injection-area variance are accepted as the main contributors to loss of resolution, the loss in resolution factor (ΔR) of the scanned detection system can be predicted by eq 2, derived from eq 31 in ref 12, where L_{D1} and

$$\Delta R = L_{D1}{L_{D2}}^{1/2}/L_{D2}{L_{D1}}^{1/2} \quad (2)$$

L_{D2} are the lengths that the analyte bands have traveled to the detector in the fixed detection mode and the scanned detection mode, respectively. As can be seen in Table 1, this loss in resolution should vary from a factor of 1.06 to 1.51 for this system. The measured loss in resolution was higher than predicted for the 100-bp fragment but lower for the remainder of the fragments. This could be due to additional heating of the column for the longer separations required with the fixed detector.

Notwithstanding the loss of resolution observed with the scanned detection system, the quality of the separation is still acceptable at half the time required for analysis. Thus, the scanning system lends itself very well toward achieving the optimal desired resolution while minimizing the total analysis time. Another effect of the scan detection mode observed in the electropherograms in Figures 3 and 4 is the increased noise observed in the scan mode. This is mainly due to the following two factors: (1) our experimental XY table utilizes stepper motors to move the laser's fiber-optic head along the separation capillary (which is also mounted on a long arm, thus causing the fiber-optic head to shake as it is moved to a new position); and (2) in order to decrease the scan time required, we lowered the integration time per step from 1 to 0.2 s. Use of brushless dc motors and better design of the fiber-optic mount should easily allow us to minimize the first factor. The second factor can be integrated into an advantage, that is, to minimize scan time and maximize signal-to-noise ratio, the system can be programmed to spend less time on large peaks and more time on smaller peaks; or low-intensity signals can be rescanned several times to increase signal-to-noise.

Another useful application of the scanning system is the imaging of capillary isoelectric focusing separations. This is demonstrated in Figure 5, where a set of FITC-derivitized molecular weight marker proteins were separated in an isoelectric focusing medium. If a fixed detector is used to observe this type of separation, a mobilizing agent must be added after isoelectric focusing in order to elute the analyte bands past the detection window. Using the liquid-core waveguide detector, full images of

the isoelectric focusing separation can be obtained during the focusing process itself. The proteins used in this work were soybean trypsin inhibitor, myosin, β-galactosidase, bovine serum albumin, alcohol dehydrogenase, and carbonic anhydrase. The theoretical p*I*'s for these proteins were found in the SWISS-PROT database and range from 4.90 to 6.63 in the order listed above. Since these proteins were FITC derivitized, their p*I* was sifted toward more basic values. Thus, the actual p*I* and order of the proteins may also be affected since the FITC derivatization may bond to more than one amine site on each protein. Therefore, assignment of peaks to particular proteins cannot be performed without further study as to the quantitative changes caused by the FITC derivatization in individual proteins. Moreover, in this paper, we demonstrate the use of the liquid-core waveguide by direct fluorescence. Indirect fluorescence (where the separation buffer carries a fluorescent dye) can be applied to isoelectric focusing, thus minimizing the effect of derivatization on isoelectric point measurements. A similar setup could be used to observe an isotachophoresis separation.

CONCLUSIONS

A liquid-core waveguide has been used to demonstrate imaging of electrophoresis and isoelectric focusing separations. We have demonstrated the utility of this system to observe DNA fragment sizing and protein separations. The system utilizes the liquid-core waveguide as an efficient window for the excitation of separated samples and the collection of light through total internal reflectance, with zeptomolar detection limits. Scanning the excitation laser along the length of the electrophoresis capillary excites individually separated analyte bands, while the fluorescence is collected end-on by an optical fiber coupled to a photomultiplier. Further improvements in the scanning system to maximize scanning speed and minimize induced noise by the XY scanning table should allow for a system that can be used to predict separation parameters that can be used to minimize separation time and maximize the separation efficiency. Due to the predictability afforded by observing separations in their early stages, we anticipate that this type of system can be utilized to speed up analysis time, yielding higher throughput for sample analysis. Since the analyte never leaves the capillary, the separation may be rescanned and imaged several times, with or without the electrophoresis voltage, to maximize separation between critical analyte bands, increase signal-to-noise ratio, or verify the analysis. Although the system is demonstrated using direct fluorescence in electrophoretic and isoelectric focusing separations, it can be applied to isotachophoresis, micellar electrokinetic chromatography, and open tubular capillary chromatography, among others. Finally, like most capillary electrophoresis systems, the system can be multiplexed and should be amenable to microchip packaging and integration.

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