Universal detection in capillary electrophoresis with a micro-interferometric backscatter detector

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An optically simple, inexpensive, micro-volume refractive index detector was applied to capillary electrophoresis (CE), allowing universal solute detection at the sub-picogram level. The micro-interferometric backscatter detector (MIBD) employs direct, side illumination of an unmodified capillary by an He–Ne laser, producing a 360° fan of scattered light that contains a set of high contrast interference fringes. These light and dark spots are viewed on a flat plane in the direct backscatter configuration. A slit–photodetector assembly accomplishes signal interrogation of the time-dependent fringe shifts, produced or imparted by refractive index (RI) changes. Using an unfocused laser beam to prove the unmodified separation capillary produces a detector volume of 4.7×10^{-9} L. The separation and quantification of a mixture of organic dyes and simple sugars demonstrate the system's utility. Submicromolar concentration detection limits of 0.46, 1.1 and 0.72 μM for Bromothymol Blue, Thymol Blue and Bromocresol Green, respectively, are achievable with CE-MIBD in the simplest configuration. The 3σ RI concentration detection limits are 2.5 times superior to those obtained by UV/VIS detection performed under the same conditions. Several carbohydrates (maltose, lactose and D-ribose) are separable and detectable at the ppm level, using no active thermal stabilization. Further demonstrating the utility of MIBD for universal detection with CE.

Introduction

The refractive index (RI) detector is a workhorse detector in conventional high performance liquid chromatographic (HPLC) separations because it is a concentration sensitive, bulk property, non-destructive sensor. These detectors are reasonably sensitive, providing a signal for essentially all analytes. However, except for use in basic research,[1–10] RI measurement schemes have found little acceptance in either capillary HPLC or in capillary electrophoresis (CE).[1] The lack of a commercial device and the limited use of RI detection in CE stem not from opulence, as universal detection for micro-volumes is surely needed, but from the inherent difficulty in reducing detection volumes to 1 nL or smaller without a loss of sensitivity.

The properties of the laser, such as high spatial coherence and monochromaticity have led to spectacular improvements in detection limits for spectroscopic measurements.[11,12] Using a laser's high spatial coherence, the beam can be focused to a very small spot without loss of power[13–15] or launched into a fiber optic efficiently.[12,16] Because of these and other optical properties, lasers have become the 'source of choice' in the effort to construct micro-volume RI detectors.[2–10,17–23] Most state-of-the-art RI measurements involve some form of interferometry, a measurement technique that is critically dependent on the characteristics of laser light. In general, very small phase changes caused by optical path length differences, in response to the analyte, allow for high sensitivity.[12,24] While the use of interferometric techniques has produced some impressive results toward measuring RI changes in small volumes,[2–5,16] sensitivity dependence on pathlength has persisted. For example, although Woodruff and Yeung[5] demonstrated a Fabry–Perot interferometer with excellent sensitivity and detection volumes in the low microliter region, flow cell volume constraints limit this method to schemes larger than capillary-based techniques.

Other RI detection methods have been investigated, including a concentration gradient method, which probes the on-axis optical perturbation produced by a transient solute band,[20,22] and the use of a tapered fiber optic,[25] which probes the reduction in transmitted beam intensity due to refractive index interfacial beam intensity coupling. Additionally, refractometric interference spectrometry shows promise for 'difficult' analytes and can be used to measure organic pollutants in water, as demonstrated by Gaugliz and co-workers.[17] These alternative approaches to RI sensing have limitations. For example, the concentration gradient detector[20,22] is insensitive to thermal noise, but most suitable for detection schemes in capillary isoelectric focusing or for separation systems where post-column detection is acceptable.

Bornhop and Dovichi[2] used a laser-based, off-axis, on-capillary technique to probe nanoliter volumes and detect nanogram amounts of sugars in micro-LC separations,[2] but this technique was found to be tedious to align and required the removal of the coating from the capillary. Bruno and co-workers[3] further developed the forward scatter, off-axis technique. They showed that improved performance is possible by using an RI-matching fluid to surround the capillary tube, a flow cell assembly with active thermal control and position sensitive detection. These improvements facilitated the use of the RI detector for capillary electrophoresis,[3] yet the device still requires removal of the polymer coating to aid in index matching and off-axis alignment.

Another technique for detecting changes in RI within small volumes uses a holographic grating to produce two-beam interference in a forward scatter configuration.[4] Krattinger and co-workers[4] were able to separate and detect metal ions by CE using the holographic grating, a capillary that is encapsulated in an index matching glue and a photodiode array wired to produce position sensitive detection. Bruggraf *et al.*[23] reported the application of the holographic forward scatter RI detector for CE on a chip showing that the holographic technique is a meritorious advance in universal detection in small volumes, eliminating the need for the capillary to serve as the optic. Yet as another arrangement of the grazing angle forward-scattering

refractive index technique it has an inherent pathlength dependence which ultimately hinders detection limits in ultra-small volumes.[2–4,21]

Deng and Li,[10] acknowledging the difficulties with forward scattering techniques, have recently shown that a retro-reflected beam interference technique similar to the use of a micro-interferometric backscatter detector (MIBD)[6–9] can be used for RI detection in CE. In their scheme, a focused laser beam impinges on a bare capillary surface causing interference of two retro-reflected beams originating from the outer surface of the capillary. By observing the retro-reflected interference pattern, RI measurements are possible. The main drawbacks of the retro-reflected beam interference RI detector are poorer detection limits than with the previously reported RI detectors[2–9] and enhanced complexity of the optical train.[6–9]

Work in our laboratory has shown that on-capillary RI analysis and capillary HPLC detection can be performed using interferometric backscatter.[6–9] The MIBD, previously termed laser interferometric backscatter (LIB) detector, employs a simple optical train, produces high sensitivity RI measurements in small diameter capillaries with minimal pathlength sensitivity[8] and requires no modification of the capillary tube. Success of the MIBD with flowing streams was shown with a separation of aromatic compounds using capillary HPLC.[9] The MIBD response proved to be linear over a dynamic range of about 2.5 decades. Picogram mass detection limits were achieved in nanoliter probe volumes using a massive aluminum flow-cell block on which the capillary was mounted for passive thermal control.

Here we describe the use of the MIBD for universal detection in CE. In the optical configuration for the MIBD,[8] an unfocused He–Ne laser beam impinges on a capillary tube producing a 360° fan of radiation, spatially contained and perpendicular to the long axis of the tube. When viewed on a flat surface placed coincident with but above or below the incident laser beam, the scattered light is seen as a series of high contrast interference fringes (light and dark spots). The central fringe pattern at a nearly 0° backscattering angle is similar in appearance to that produced by single slit diffraction. The positions of these maxima and minima can be employed in the sensitive measurement of fluid bulk properties. We show that the MIBD-CE system can be used to detect solutes at the ppm level, with mass detection limits in the picogram range. Such performance is illustrated with the separation of a mixture of organic dyes and a three component carbohydrate mixture.

Experimental

The basic optical configuration for the MIBD has been described in detail elsewhere[6–9] and is depicted in the generalized block diagram presented in Fig. 1. A low power, linearly polarized beam at 632.8 nm was provided by a 4 mW helium–neon laser (Melles Griot, Irvine, CA, USA). The laser output was directed on to the capillary, located 55 cm from the laser's aperture, creating an array of backscattered light or interference pattern. The capillary was mounted on a massive aluminum block acting as a flow cell assembly with no active thermal control. The complete flow cell assembly was tilted at an angle convenient for placement of the fringe on the photodetector and normally not exceeding 7°. The backscatter radiation was allowed to propagate *ca.* 30 cm to a small area (1.0 cm^2) photodiode (UDT Sensors, Hawthorn, NV, USA) housed in a fixture containing a 632.8 nm interference filter (Optical Coating Technology, Southampton, MA, USA) and a 150 μm precision air slit (Melles Griot). The photodetector assembly was mounted on a high precision translation stage. Translation of the detector allowed for easy selection of the central fringes. These fringes have been found to move significantly with an RI change caused by the analyte.[6]

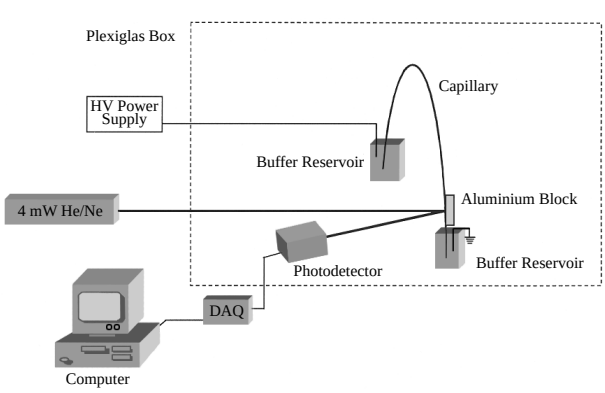

Fig. 1 Block diagram for the MIBD (RI) detector.

The output of the photodiode is conditioned with a current-to-voltage converter, consisting of a JFET operational amplifier, wired with a 10 MΩ feedback resistor in parallel with a 0.01 pF capacitor. No additional electronic filtering was applied to the voltage signal. The analog voltage signal from the RI detector was digitized with a DAQ board (National Instruments, Austin, TX, USA) and displayed on a PC running a digital strip-chart recorder (Labtech for Windows, Wilmington, MA, USA).

All optical components and detectors were rigidly mounted on a 4 × 3 ft optical bench (Newport Research, Irvine, CA, USA). Manual micrometer driven translation stages were used to provide reproducible translation of the photodetector, capillary tube and optical components. Excluding the laser, the entire experiment is enclosed in a Plexiglas box.

High sensitivity is easily achieved in MIBD by simply insuring that the laser beam illuminates the entire capillary in a region where the coating has not been scratched or marred. Upon obtaining a high contrast interference pattern, the flow cell assembly is tilted to direct the beam on to the photodetector assembly. Once a signal is obtained, the detector assembly is translated across two full fringes, allowing the identification of the minima. The detector is then placed so that a small voltage output is observed. This position corresponds to the edge of the sloping intensity gradient of the working fringe and is located at $I = 1/e^2$ of the essentially Gaussian intensity distribution.

A 100 μm id, 350 μm od capillary was used in all separations. The 17 μm polyimide coating was not modified or removed in the RI detection zone. For the dye separation (a) the RI detection window was located 34.5 cm from the injection end of a 42 cm long capillary and (b) injection was made by pressure driven (commercial instrument controlled) hydrodynamic injection at 1.5 psi for 1.0 s. For the carbohydrate separation (a) the detection window was 83.5 cm from the injection end of a 95 cm column and (b) injection was made by hydrodynamic siphoning at a height of 35 cm for a period of 5 s. The working buffer for the dye separation consisted of 10 mM anthraquinone-2-sulfonic acid, 4 mM sodium borate and 10% ethanol. A buffer consisting of 33 mM borate and 13.3 mM CAPS (3-cyclohexylanino-1-propane-sulfonic acid) was used for the carbohydrate separation. An applied voltage of 20 kV (56.7 μA) was administered to the capillary in order to elute the dyes (Bromothymol Blue, Thymol Blue and Bromocresol Green), while 12 kV (36 μA) were applied to the capillary to induce component separation in the carbohydrate analysis (maltose, lactose and D-ribose). All separations were conducted at room temperature (22–25 °C).

The dye mixture and individual dye standard solutions were prepared from analytical-reagent grade or better chemicals (Aldrich, Milwaukee, WI, USA) and dissolved in a solution of 4 mM sodium borate–10% ethanol. The carbohydrate standards and mixture were also prepared from analytical-reagent grade chemicals (Aldrich), but dissolved in de-ionized water. No other sample preparation or work-up was performed before injection. Calibration curves were generated in duplicate for each solute

using peak height and peak area, and the separation samples were analyzed in triplicate for each separation system.

Results and discussion

The principle of operation for the RI measurement using the MIBD has been described in detail elsewhere[6–9] and involves the detection of optical pathlength changes within the tube, as RI changes for materials contained in the tube. Upon solute introduction, the position of the backscattered fringes shift in response to a change in RI within the probe volume. We employ a simple approach to measure this fringe shift by placing the slit–photodetector assembly on the edge of a fringe. The positional movement is found to be proportional to analyte concentration and is generally linear over three decades.[14,15] This simple intensity-based detection method is inexpensive and easy to implement. However, such a configuration will ultimately limit the dynamic operation range of the MIBD to a refractive index shift equal to a distance corresponding to 2σ of the Gaussian-like profile of the backscattered fringe (*e.g.*, once the fringe maxima shifts to a position near or past the slit, non-linear operation ensues).

Previous investigations that have demonstrated CE with RI detection showed promise, but were constrained by pathlength sensitivity.[4,19] Here we performed analyses of several test mixtures to evaluate the performance of the MIBD (which has a many pass optical configuration) with CE. Fig. 2 shows a typical electropherogram for a three component mixture of dyes (in order of elution), Bromothymol Blue, Thymol Blue and Bromocresol Green. These solutes where chosen because they are known to be separable quickly and to provide a detection limit comparison with standard UV/VIS absorption (619 nm). Using the conventional configuration for CE shown in Fig. 1 (without *active* thermal control of either the capillary or the detection region), a high voltage was applied to a 42 cm × 100 μm diameter fused capillary, producing a current of 30 μA. A solution containing micromolar amounts of the dyes was injected by hydrodynamic introduction, producing an estimated sample volume of 22.9 nL.[26]

The detection limit, three standard deviations above the background, was estimated from the peak-to-peak noise in the electropherogram.[27] Calibration curves were generated for each solute using both peak height and peak area. The response was linear over three decades of concentration with correlation coefficients ranging from 0.993 to 0.999. As shown in Table 1, MIBD-CE can facilitate mass detection limits for the above three dyes, at the 3σ level, of 6.7, 11.9 and 11.2 pg or 10.7, 25.6 and 16.0 fmol, respectively. The concentration detection limit for Bromothymol Blue is 4.66×10^{-7} mol L^{-1} or about 0.29 ppm, and is in a region that facilitates analytical utility.

In order to compare the performance of a standard UV detector with the MIBD (RI) detector, a Linear Instruments (Reno, NV, USA) UV/VIS detector was affixed to the CE system. A typical electropherogram for the three component dye mixtures is shown in Fig. 3. Calibration was performed and the detection limits in CE-UV were determined (Table 2). It is noteworthy that for the dye solutes which have strong molar absorptivities at 619 nm (23 200 L mol^{-1} cm^{-1} for Thymol

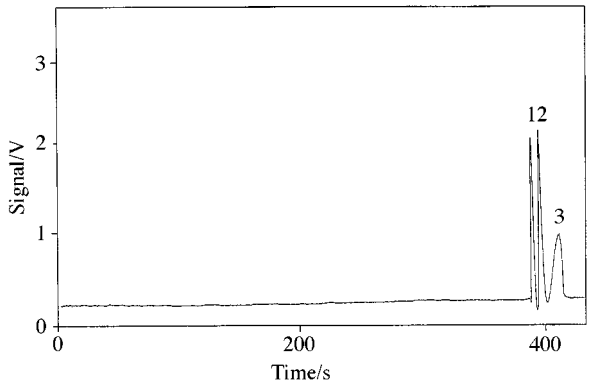

Fig. 2 Electropherogram of the dye mixture using the MIBD (RI) detector. Peaks: 1 = 30 μM Bromothymol Blue; 2 = 60 μM Thymol Blue, 3 = 30 μM Bromocresol Green.

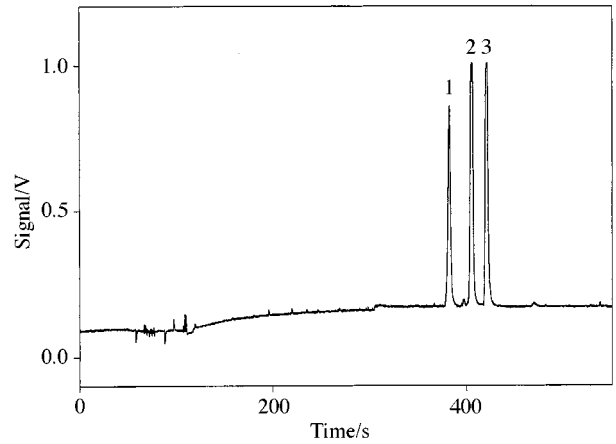

Fig. 3 Electropherogram of the dye mixture using UV/VIS detection (619 nm). Peaks: 1 = 30 μM Bromothymol Blue; 2 = 60 μM Thymol Blue, 3 = 30 μM Bromocresol Green.

Table1 Comparison of detection limits (DL) for the dye samples

Solute	M_r/g mol^{-1}	Concentration DL[a]/M (RI detector)	Injected mass DL[a] (RI detector)	Concentration DL[a]/M (UV detector)	Injected mass DL[a] (UV detector)
Bromothymol Blue	624.4	4.66×10^{-7}	6.7 pg or 10.7 fmol	1.16×10^{-6}	16.5 pg or 26.5 fmol
Thymol Blue	466.6	1.12×10^{-6}	11.9 pg or 25.6 fmol	2.23×10^{-6}	23.8 pg or 50.1 fmol
Bromocresol Green	698.0	7.23×10^{-7}	11.2 pg or 16.0 fmol	1.16×10^{-6}	18.5 pg or 26.5 fmol

[a] Calculated at 3σ.

Table 2 Detection limits (DL) and separation efficiency for the carbohydrate separation

Solute Identity	M_r/g mol^{-1}	Concentration DL[a]/M (RI detector)	Injected mass DL[a] (RI detector)	Number of theoretical plates (RI detector)	HETP (RI detector)
Maltose	360.32	1.28×10^{-5}	0.22 ng or 0.60 pmol	8.35×10^{4}	1.73×10^{-4}
Lactose	360.31	3.33×10^{-5}	0.57 ng or 1.57 pmol	1.21×10^{5}	1.22×10^{-4}
D-Ribose	150.13	6.53×10^{-5}	0.46 ng or 3.09 pmol	3.27×10^{4}	5.52×10^{-4}

[a] Calculated at 3σ.

Blue, 30 400 L mol^{-1} cm^{-1} for Bromocresol Green and 37 475 L mol^{-1} cm^{-1} for Bromothymol Blue),[28] the functional detection limits of the MIBD-RI detector are a factor of 1.6–2.5 times better than those reported for the standard UV/VIS absorption detector. Hence MIBD-RI can provide detection limits comparable to or even better than those with the conventional UV absorbance monitor under CE conditions using a 100 μm diameter capillary. If the capillary diameter was further reduced, as desired for many CE separations, the detection limit for the UV detector would be poorer, as predicted by Beer's law. However, no such performance reduction is expected for the relatively pathlength insensitive RI detector (MIBD has been applied to shorter pathlength sample cells without a decrease in sensitivity).[7] Consequently, for CE performed in capillaries of < 50 μm id, the detection limit for the RI detector is expected be at least five times better than that for the standard UV detector.

It is well known that applying a voltage to the CE capillary produces joule heat throughout the tube, and it is also known that dn/dT for H_2O corresponds to 1.7×10^{-3} RIU °C^{-1} (RIU = refractive index units).[8] This heating can perturb the RI detector and interfere with detection of solutes. We have developed a simple procedure to effectively eliminate this RI response to the rapid joule heating produced by applying a high voltage to the capillary on injection. Briefly, a voltage is applied for 15 s to a capillary filled with fresh buffer immediately before hydrodynamic injection of the sample, heating the capillary by about 10 °C. Then the injection is rapidly performed while the system is near the separation temperature and before the temperature can decay. We then quickly begin the separation. By implementing this procedure, the large temperature shock to the system which corresponds to $\Delta n = 1.7 \times 10^{-2}$ RIU for $\Delta T = 10$ °C are avoided, and therefore not observed by the RI detector upon reapplying the voltage to the capillary in order to drive the separation. Furthermore, it is noteworthy that the MIBD is relatively insensitive to short term thermal perturbations during the separation. Fig. 2 and 4 show typical baseline noise levels that are stable for the duration of the separation.

Glycoproteins, an important class of molecules, have been studied extensively, yet the rapid separation and detection of these analytes is still difficult because this class of molecules possess relatively low absorption coefficients. To demonstrate further the utility of MIBD as a 'universal' detector for CE, carbohydrate samples were studied. Fig. 4 shows the separation of (in order of elution) (1) maltose, (2) lactose and (4) D-ribose (the third peak is a solvent anomaly). Again, the simplest MIBD-CE system configuration was used, employing an 83.5 cm × 100 μm id capillary and no active thermal control for either the separation or detection system. Borate–CAPS buffer was used and 12 kV were applied to the capillary, producing 37 μA of current. Hydrodynamic injection for 5 s produces a volume of 47.3 nL[26] for a 5.0 mM solution of the sugars. Solute detection limits are in the range 196–38 μM (Table 2), which is an improvement over those previously reported[29] for RI-CE of underivatized species. These detection limits are 1–2 decades better than those obtained by standard UV absorbance at 195 nm[28] and are near those needed for glycoprotein analysis.[30] Ignoring dilution from the separation process, the RI change at the detection limit for maltose corresponds to $\Delta n = 1.39 \times 10^{-6}$. The system produces linear calibration plots ($r^2 > 0.995$) over three decades of concentration.

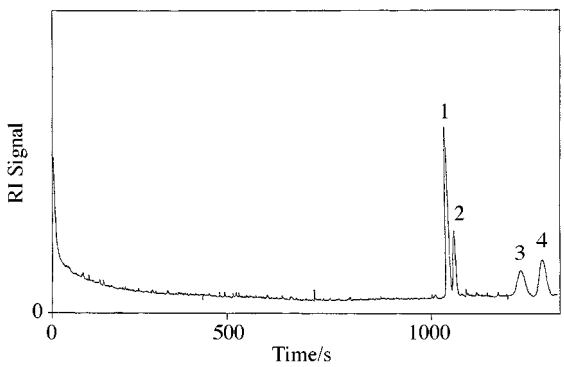

Fig. 4 Electropherogram of the carbohydrate mixture using the MIBD (RI) detector. Peaks: 1 = 5 mM maltose; 2 = 5 mM lactose; 3 = solvent anomaly; 4 = 5 mM D-ribose.

As noted before, dn/dT (thermal) perturbations ultimately limit the use of dc RI measurements in CE. MIBD is no exception, with the detection limits approaching those dictated by dT changes. Surprisingly, baseline stability for CE with MIBD is fairly good (particularly since no *active* control was employed) and the detection limits are in the region of analytical interest. The separation also shows that MIBD has the potential to be used for the determination of biological solutes, lacking good absorption, under CE conditions. We predict that with active thermal control and electronic filtering of the photodetector signal, nanomolar detection limits should be possible.

Band broadening induced by the detector dead volume can be estimated by calculating the number of theoretical plates and HETP from the electropherograms, as shown in Tables 2 and 3. As expected using an *unfocused* beam that produces a probe volume of approximately 4.7 nL, a slight reduction in separation efficiency is observed for MIBD compared with UV detection, which has a probe volume in the region of the 0.5 nL. This observation further illustrates the importance of the detector probe volume for separation performance in CE. In any event, the separation efficiency produced in the MIBD-CE system is comparable to that with conventional, on-column, UV detection. Although the electrophoretic efficiency could be improved, the performance is good, with about 6.80×10^5 theoretical plates for the dye separation and 1.21×10^5 plates for the carbohydrate separation. Even though the MIBD detection volume can be reduced by spatially masking the excitation source (currently under investigation), thus producing a smaller diameter probe beam, in these experiments the separation efficiency was primarily limited by other factors, including thermal instability in the column, during injection and separation. Reducing the diameter of the probe beam and applying active thermal control should improve the separation efficiency and the detection limits for MIBD significantly.

In summary, it has been shown that the MIBD can be used for universal detection in CE. The optical configuration is simple, facilitates the use of unmodified capillaries and can be constructed inexpensively (less the optical bench, the total cost is about US$ 1000). The MIBD is sensitive to small changes in refractive index (picogram amounts can be detected currently), allowing ppm quantities of poorly absorbing solutes to be quantified. Considering that the current trend toward 'system' miniaturization[23,31–33] is limited by the availability of an ultra-small universal detection system, a sensitive, low volume detector such as the MIBD represents a major advancement. Current investigations in our laboratories include the further application of the MIBD for RI detection in CE, for polarimetry measurements in capillary dimensions[33] and for RI detection with CEC.

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