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End-Column Chemiluminescence Detector for Capillary Electrophoresis

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A chemiluminescence detector is designed for capillary electrophoresis in which the signal is generated at the column outlet. As the analytes emerge from the column, they react with chemiluminescent reagents to produce visible light that is transported by a fiber optic to a photomultiplier tube. The luminol chemiluminescence and the firefly luciferase bioluminescence reactions are adapted for use with this type of detection scheme. The reactions yield concentration detection limits of 2×10^{-8} M for luminol and 5×10^{-9} M for ATP, which are approximately 3 orders of magnitude lower than those obtained with absorbance.

Although capillary electrophoresis (CE) as a separation method has the virtues of high resolution and rapid separation and the ability to analyze small samples, its weakness is thought to be its detection capabilities.[1,2] Most commonly, UV/visible absorption is used as the detection method, with sensitivities typically of 10^{-5}–10^{-6} M. Significantly better limits of detection can be realized when the analytes allow the use of laser-induced fluorescence (LIF),[3,4] amperometric,[5] or radiometric[6] detection.

An alternative detection scheme is the use of chemiluminescence (CL), which has been applied to liquid and gas chromatography and to immunoassays.[7–12] Because of its low-background nature, CL detection can be anticipated to provide excellent sensitivity. Several chemiluminescent reactions, such as the peroxyoxalate,[7,8] luminol (5-amino-2,3-dihydro-1,4-phthalazinedione),[9] lucigenin,[10] and firefly luciferase,[11] have been utilized with liquid chromatography (LC) or flow injection analysis. Though the peroxyoxalate reaction is the most often used, it generally requires the use of organic solvents because of the poor solubility and chemiluminescence efficiency of the commonly available oxalates in aqueous solutions.

Recently, the use of CL for detection purposes has also been applied to CE.[13–17] Dadoo, Colón, and Zare[13] showed preliminary results with the luminol reaction, Ruberto and Grayeski[14] utilized the acridinium reaction, and Hara et al.[15] and Wu and Huie[16] have demonstrated the use of peroxyoxalate CL with CE. Zhao, Labbe, and Dovichi[17] also have used the luminol reaction to detect derivatized amino acids. Detection sensitivities have ranged from the low-nanomolar range for the luminol work to approximately ~100 nM for the peroxyoxalate work. With the exception of the work done by Dovichi and co-workers,[17] all of the other CE-CL detection schemes have involved a variation of a postcapillary reactor (to mix reagents) originally developed by Rose and Jorgenson[18] to carry out derivatization reactions before LIF detection. The reactors all require the insertion of one capillary (separation column) into another larger capillary (reaction/detection column). The separation capillary also has to be etched with hydrofluoric acid when relatively small inner diameter reaction columns are used. The procedures for etching and insertion of one capillary into another are manually intensive and cumbersome, and the construction of reproducible reactors is difficult. The CL detector constructed by Dovichi and co-workers[17] uses a postcolumn sheath flow cuvette as the mixing chamber and a sheath stream from a syringe pump to carry the analytes away from the detection zone once they emerge from the column. For easy, reproducible alignment, the outer diameter of the capillary must match the width of the flow chamber in the cuvette.[17]

Here, we describe a new and simpler design for a chemiluminescence detector interface that is easily implemented with CE. The luminol reaction is used for detection to facilitate comparison with prior CE-CL detector designs. In addition, a bioluminescent reaction (firefly luciferase) is adapted to CE for the measurement of adenosine 5′-triphosphate (ATP). Results for the sensitivity and linearity using this design are also presented.

EXPERIMENTAL SECTION

Capillary Electrophoresis Apparatus. A CE system similar to that described previously[13] was used for the CL experiments. In brief, a 0–30-kV power supply (Spellman High Voltage Electronics, Bronx, NY) provided the separation voltage. The capillary used for all the runs was 65-μm inner diameter, 375-μm outer diameter, and 50 cm long (Polymicro Technologies, Phoenix, AZ). It was flushed with 100 mM sodium

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(1) Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem.* **1981**, *53*, 1298–1302.
(2) Gordon, M. J.; Huang, X.; Pentoney, S. L., Jr.; Zare, R. N. *Science* **1988**, *242*, 224–228.
(3) Sweedler, J. V.; Shear, J. B.; Fishman, H. A.; Zare, R. N.; Scheller, R. H. *Anal. Chem.* **1991**, *63*, 496–502.
(4) Cheng, Y. F.; Dovichi, N. J. *Science* **1988**, *242*, 562–564.
(5) Wallingford, R. A.; Ewing, A. G. *Anal. Chem.* **1989**, *61*, 98–100.
(6) Pentoney, S. L., Jr.; Zare, R. N.; Quint, J. *Anal. Chem.* **1989**, *61*, 1642-1647.
(7) Kobayashi, S.-I.; Imai, K. *Anal. Chem.* **1980**, *52*, 424–427.
(8) Sigvardson, K. W.; Birks, J. W. *Anal. Chem.* **1983**, *55*, 432–435.
(9) Kawasaki, T.; Maeda, M.; Tsuji, A. *J. Chromatogr.* **1985**, *328*, 121–126.
(10) Veazey, R. L.; Nieman, T. A. *J. Chromatogr.* **1980**, *200*, 153–162.
(11) Worsfold, P. J.; Nabi, A. *Anal. Chim. Acta* **1985**, *171*, 333–336.
(12) Bronstein, I.; McGrath, P. *Nature* **1989**, *338*, 599–600.
(13) Dadoo, R.; Colón, L. A.; Zare, R. N. *J. High Resolut. Chromatogr.* **1992**, *15*, 133–135.
(14) Ruberto, M. A.; Grayeski, M. L. *Anal. Chem.* **1992**, *64*, 2758–2762.
(15) Hara, T.; Yokogi, J.; Okamura, S.; Kato, S.; Nakajima, R. Presented at the Fifth International Symposium on High Performance Capillary Electrophoresis, HPCE '93, Orlando, FL, 1993; Poster T105.
(16) Wu, N.; Huie, C. W. *J. Chromatogr.* **1993**, *634*, 309–315.
(17) Zhao, J.-Y.; Labbe, J.; Dovichi, N. A. *J. Microcolumn Sep.*, in press.
(18) Rose, D. J., Jr.; Jorgenson, J. W. *J. Chromatogr.* **1988**, *447*, 117–131.

0003-2700/94/0366-0303$04.50/0

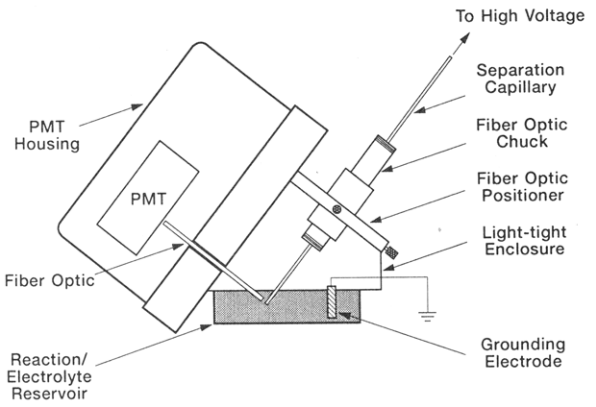

Figure 1. Schematic of the end-column chemiluminescence detector for capillary electrophoresis.

hydroxide for 10 min before use. The high-voltage end of the capillary was enclosed in a plexiglass box for safety reasons. A 1–3-mm section of the polyimide at the outlet end of the capillary was removed by burning to facilitate detection.

Chemiluminescence Detection Apparatus. Figure 1 shows the interface used for CE-CL detection in these experiments. A light-tight enclosure machined out of Delrin was attached to a photomultiplier tube (PMT) housing (Products for Research, Danvers, MA) on one side, a fiber optic positioner on another side, and a reagent reservoir on the third. The fiber optic positioner (Newport, Irvine, CA) was used to hold the capillary rigidly in place with a chuck. The positioner allowed the chuck (and, hence, the capillary) to move up to 2 mm in all three dimensions. The outlet end of the capillary was immersed in the reservoir that contained the separation electrolyte and the reagents for the CL reaction. The reservoir was also made of Delrin and, hence, was light-tight. As the analytes emerged from the column, they mixed with the CL reagents in the reservoir to initiate light production. Perpendicular to the capillary with an end also immersed in the solution was a fiber optic (Fiberguide Industries, Stirling, NJ) ~5 cm long with a 1-mm core diameter that carried the light produced to a PMT (Model R4632, Hamamatsu, Inc., San Jose, CA) in the housing. The ends of the capillary and the fiber optic were aligned visually by moving the capillary with the use of the positioner. A platinum wire for the grounding electrode was immersed in the reservoir to complete the CE electrical circuit.

The output from the PMT (operated at 800 V) fed a photon counter (EG&G Princeton Applied Research, Princeton, NJ) connected to a chart recorder (Linear Instruments, Reno, NV) to produce electropherograms.

Procedure. Stock solutions of the analytes (1–10 mM) were prepared in appropriate electrolyte solutions and diluted as needed. Luminol and *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) were prepared in a 10 mM sodium phosphate (pH 11.7) solution. ATP was prepared in 10 mM sodium phosphate (pH 7.8). Sample injections were done either by gravity or electrokinetically, as mentioned in the figure captions.

A reaction described by Kawasaki, Maeda, and Tsuji[9] to label primary and secondary amines with ABEI was modified for our purposes to label amino acids. First, a 5 mM solution of ABEI in methanol was added to an equal volume of a 5 mM solution of *N,N'*-disuccinimidyl carbonate (DSC) in acetonitrile. The resulting solution was sonicated for 2 h. Then, the desired amino acids were added (in excess) to aliquots of the ABEI/DSC solution, and the aliquots were also sonicated for 1–2 h. The labeled amino acid solutions were diluted by a factor of 10 or more in the separation buffer before injection.

The chemiluminescent reagents required for the reaction were generally added to the outlet reservoir. For the luminol reaction, 25 mM potassium ferricyanide was added to the outlet, and 100 mM hydrogen peroxide was added to the separation electrolyte (10 mM sodium phosphate, pH 10.8) in the inlet. For the luciferase reaction, the outlet reservoir contained a commercially available reaction mixture (ATP Assay Mix, Sigma Chemical Co., St. Louis, MO), while the inlet contained 10 mM sodium phosphate (pH 7.8) as the separation electrolyte. The capillary was initially flushed with the solution in the inlet reservoir before the samples were injected. For both reactions, the solution containing the reagents in the outlet reservoir showed no deterioration during 50 or more runs and, hence, did not require frequent replenishments.

Chemicals. Sodium phosphates (mono-, di-, and tribasic) were purchased from J. T. Baker (Phillipsburg, NJ). Luminol, ABEI, and DSC were obtained from Aldrich (Milwaukee, WI). The potassium ferricyanide was bought from J. T. Baker, and a 30% hydrogen peroxide solution from Fisher Scientific (Fair Lawn, NJ). ATP was purchased from Sigma.

RESULTS AND DISCUSSION

In our previous work with CE-CL detection that utilized a postcolumn reactor,[13] the luminol reaction was used for demonstration because of its compatibility with aqueous solutions. Although limits of detection in the nanomolar range were obtained for luminol and ABEI, the separation efficiencies achieved were poor (5000–10000 theoretical plates), possibly because of the increased band broadening caused by turbulence created at the end of the separation capillary. For comparison purposes, we decided to use the luminol reaction with the end-column CL detector. An electropherogram of a sample containing ABEI and luminol is shown in Figure 2. The separation efficiencies achieved are between 10 000 and 20 000 theoretical plates, which is a slight improvement over those obtained with the previous design, and the variance in the peak heights (for the same amount injected) was less than 5% (relative standard deviation). The limit of detection (defined as the amount of analyte injected to give a peak height that is 3 times the standard deviation of the baseline noise) measured for luminol was found to be 500 amol (2×10^{-8} M), which is lower than, but within 1 order of magnitude of, that reported before. In this case, the light collection efficiency obtained with the fiber optic (less than 10%) is worse than that with the parabolic mirror that was used in the apparatus with the postcolumn reactor. Also, the PMT was not cooled to –20 °C as in the previous experiment, thus leading to a higher dark current. Both of these factors could account for the 3–5-fold decrease in sensitivity obtained with the end-column design.

As a demonstration, Figure 3 shows an electropherogram of a sample containing two amino acids (arginine and glycine) derivatized with ABEI and detected by CL. The peaks labeled B in the electropherogram correspond to unreacted ABEI and ABEI/DSC and are present in the "blank" runs. Isoluminol isothiocyanate can also be used to derivatize amino

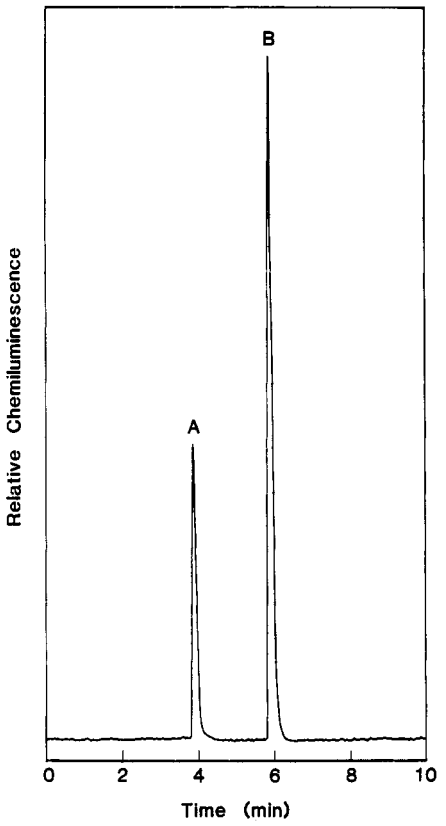

Figure 2. Electropherogram of ABEI (A) and luminol (B). The column dimensions are 65-μm inner diameter and 50-cm length. Both the inlet and the outlet reservoirs contain 10 mM sodium phosphate (pH 10.8). In addition, 100 mM hydrogen peroxide is added to the inlet and 25 mM potassium ferricyanide to the outlet. The separation voltage is 12 kV. Injection is performed by gravity for 10 s (at a height of 10 cm). The concentration of ABEI is 1×10^{-4} M and that of luminol is 2×10^{-5} M.

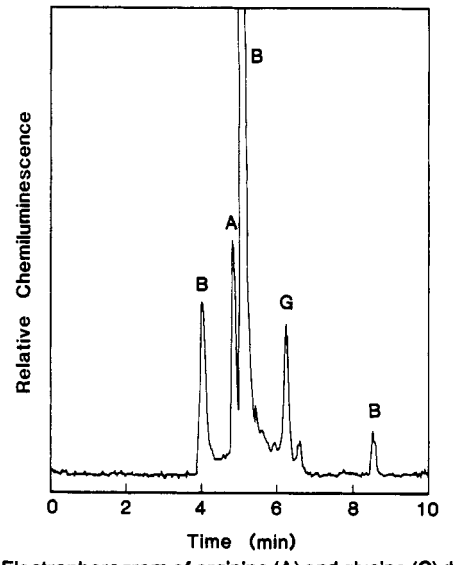

Figure 3. Electropherogram of arginine (A) and glycine (G) derivatized with ABEI. The peaks labeled B are from unreacted derivatizing reagents. The separation and detection conditions are the same as in Figure 2.

acids for CL detection, as shown by Spurlin and Cooper[19] for LC and Dovichi and co-workers[17] for CE.

The possibility of adapting a bioluminescent reaction for detection in CE was also investigated. The firefly luciferase reaction was chosen because of its high CL quantum

(19) Spurlin, S. R.; Cooper, M. M. *Anal. Lett.* **1986**, *19*, 2277–2283.

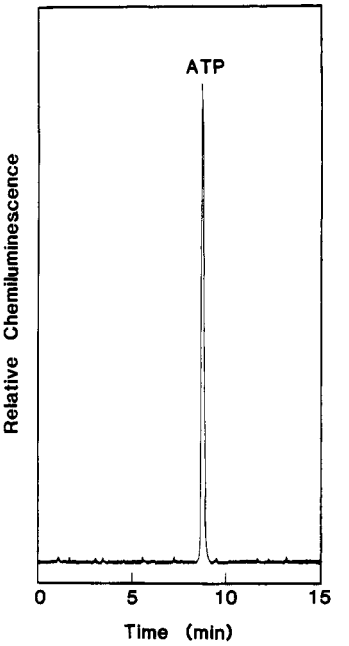

Figure 4. Electropherogram of ATP injected at 10^{-6} M. The separation electrolyte is 10 mM sodium phosphate (pH 7.8). The outlet reservoir contains a commercially available ATP assaying reagent (see text for details). The separation voltage is 15 kV, and the injection is performed electrokinetically at 10 kV for 10 s.

efficiency[20] (which can reach 90%) and its relatively fast reaction kinetics compared with other bioluminescent reactions. Though the reaction is highly selective for ATP and has been used for assays, interfering compounds exist.[21,22] For example, some anions (SCN^-, I^-, etc.) inhibit the reaction[21] and thereby decrease the light emission. Also, nucleotides other than ATP generate light in this reaction,[22] although with much lower efficiencies. By use of CE to separate the species within a sample, the effect of the interfering compounds should be minimized, thereby yielding more accurate results, especially when the sample matrix is unknown. Figure 4 shows an electropherogram of ATP injected into the column (at a concentration of 10^{-6} M) and measured with the end-column CL detector. The peak elutes in under 10 min with an efficiency of 20 000 plates. The detection limit for ATP was measured to be 5×10^{-9} M (100 amol injected, S/N = 3), which is a factor of 1000–10000 better than that typically obtained by UV absorption. The response was linear for greater than 3 orders of magnitude (up to 5×10^{-5} M), and the correlation coefficient was $R = 0.996$. Hence, ATP may be quantitated in a sensitive and selective manner with our end-column CL detector. This capability has obvious applications to biological systems because of the involvement of ATP in metabolism and energy conversion, membrane transport, and neurotransmission.

Although the end-column CL detector provides relatively high detection sensitivity, the separation efficiencies obtained are lower than those typically found in CE. Band broadening as a result of the mixing at the column outlet, slow CL reaction kinetics, and the relatively large detection zone (1 mm) may explain the relatively low number of theoretical plates. More studies are needed to characterize and elucidate the reaction

(20) Seliger, H. H.; McElroy, W. D. *Arch. Biochem. Biophys.* **1960**, *88*, 136-141.
(21) Denburg, J. L.; McElroy, W. D. *Arch. Biochem. Biophys.* **1970**, *141*, 668–675.
(22) Kiebling, M.; Winsel, K.; Freytag, B. *Isotopenpraxis* **1990**, *26*, 330-331.

mixing and the kinetics of light emission at the end of the column. If CL reactions with slow kinetics are utilized, some form of stirring may be needed in the outlet reservoir to help mixing and carry the reaction products away from the detection zone. Also, if required, the detection sensitivity of the current detector may be improved with some minor design changes, such as lowering the dark current of the PMT (by cooling) and using additional fiber optics to increase light collection. Thus, when CL detection in CE is applicable, concentration detection limits in the nanomolar range should be routinely possible using this end-column detector.

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