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Postcolumn reactor in capillary electrophoresis for laser-induced fluorescence detection

Liling Zhang, Edward S. Yeung*

Department of Chemistry and Ames Laboratory-USDOE, Iowa State University, Ames, IA 50011, USA

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Abstract

The construction and utility of a postcolumn reactor in capillary electrophoresis using *o*-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME) as the fluorescent labeling reagent and laser-induced fluorescence detection are described. The reactor is based on a coaxial design consisting of two narrow-bore capillaries. The reagent is electroosmotically pumped into the reaction capillary and the flow is controlled independently by a separate high-voltage power supply. The response is linear over at least two orders of magnitude. Mass limits of detection are in the low attomole range for various amines, amino acids and proteins. The suitability of the system for single-cell studies is demonstrated with human erythrocytes.

Keywords: Postcolumn reactor; Laser-induced fluorescence detection; Erythrocytes; Derivatization, electrophoresis; Detection, electrophoresis; Amines; Amino acids; Proteins

1. Introduction

Capillary electrophoresis (CE), characterized by high separation efficiencies and short analysis times, has become an important analytical technique, with applications ranging from small inorganic ions [1,2] to large biomolecules [3–8]. Detection in CE usually is performed on-column in which the detector cell is a small section of the separation capillary. UV absorption [9,10], electrochemical [11,12], fluorescence [13,14] as well as mass spectrometric [15] detection schemes have been used in CE. Among all detection modes, laser-induced fluorescence (LIF), with mass limits of detection as low as 10^{-21} mol [16], represents the most promising method to meet the detection challenge posed by intrinsically small injection volumes (nl and pl). Laser-induced native fluorescence has been shown to be very sensitive for detecting tryptophan- and tyrosine-containing proteins and catecholamines [17–19]. A limit of detection of $1 \cdot 10^{-10}$ *M* for conalbumin has been achieved using the 275.4-nm line from an argon-ion laser [17], and subsequently, hemoglobin and carbonic anhydrase were quantified on a cell-by-cell basis in human erythrocytes [18]. Limits of detection of catecholamines are in the nanomolar range, and epinephrine along with norepinephrine were quantified in individual bovine adrenal medullary cells [19]. Chemical derivatization extends the use of LIF to non-fluorescent analytes through derivatization reactions with fluorescent tags. Various derivatization reagents have been developed in liquid chromatography [20], and some of these reagents are readily applicable to CE [21].

Precolumn derivatization is relatively straightforward since there are no additional hardware require-

*Corresponding author.

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ments. Also, excess interfering reagents can be removed before separation and even slow derivatization reactions may be useful. Naphthalene-2,3-dicarboxaldehyde (NAD) has been used to derivatize catecholamines with mass limits of detection in the low attomole range [14]. Precolumn derivatization, however, can be complicated by multiple labeling [22] or can compromise the separation process by making the analytes more similar.

Postcolumn derivatization, on the other hand, requires a reactor in which the separated analyte zones meet the derivatization reagent to form detectable derivatives. Fast reaction kinetics, efficient mixing, and minimal contribution to band broadening are essential in a postcolumn scheme. Several postcolumn reactors have been constructed in CE, including coaxial reactors [22–24], a cross-connector made from capillaries [25], gap junction reactors [26–28], and a free-solution reactor [29].

This paper describes a postcolumn reactor using two narrow-bore capillaries connected coaxially. This reactor differs from other coaxial reactors in terms of capillary dimensions, reagent flow control, ease of construction and most importantly, better limits of detection. The use of 15 μm I.D. vs. 25–50 μm I.D. capillaries reduces on-column dilution and makes the reactor amenable to single-cell analysis, which has been a very active research field in CE [30]. The derivatization reagent is electroosmotically driven into the reaction capillary and the reagent flow-rate is independently controlled by a high-voltage power supply. Amino acid, amines and proteins, derivatized with *o*-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME) using this postcolumn reactor coupled with LIF detection, show low attomole mass limits of detection. We also demonstrate here single-cell capability with this postcolumn derivatization scheme.

2. Experimental

2.1. Postcolumn reactor

Fig. 1 shows a cross-section of the postcolumn reactor consisting of two capillaries (Polymicro Technologies, Phoenix, AZ, USA). A 2-cm section of polyimide coating was burned off from one end of a 15 μm I.D.×150 μm O.D. capillary, which functioned as the separation capillary, and this end of the capillary was etched in a concentrated hydrofluoric acid solution (Aldrich Chemical, Milwaukee, WI, USA) until the outer diameter was smaller than 30 μm. The etched section was then dipped briefly in a saturated sodium carbonate solution and rinsed with deionized water. A 1-cm section from the etched tip was cut off because the inner walls near the tip were also etched by hydrofluoric acid drawn into the capillary by capillary action, and the rest of the etched section was carefully inserted into a 30 μm I.D.×360 μm O.D. capillary, which functioned as the reaction column. The junction was carefully slid into a microcentrifuge vial (volume of 1.5 ml) (Midwest Scientific, St. Louis, MO, USA) through two small holes drilled through the cap and the bottom of the vial in which the derivatization reagent (about 1.2 ml) was held during experiments. Capillaries were secured onto the vial with epoxy (True Value Hardware Stores, Chicago, IL, USA).

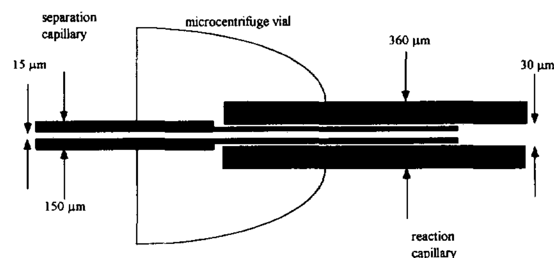

Fig. 1. Schematic of postcolumn reactor.

Some preliminary tests were performed with reactors made from 50 μm I.D.×150 μm I.D. and 150 μm I.D.×360 μm O.D. capillaries. These reactors were put together by removal of a 1 cm length of polyimide coating of a 50 μm I.D. capillary and insertion of this section into a 150 μm I.D. capillary without any chemical etching.

2.2. Instrumentation

The CE apparatus coupled with a LIF detection system, shown in Fig. 2, is home built. A 30-kV d.c. power supply (Glassman High Voltage, Whitehorse Station, NJ, USA) was connected between the inlet

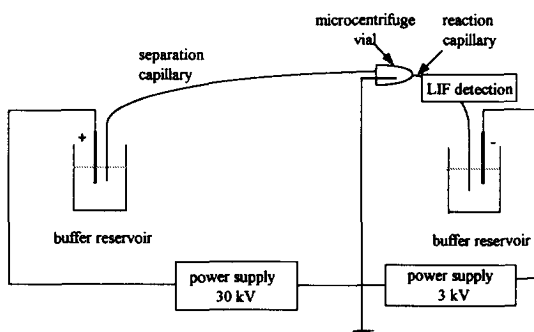

Fig. 2. Schematic of CE system with postcolumn reactor and LIF detection.

of the separation capillary and the microcentrifuge vial, which was grounded, while a 3-kV d.c. power supply (Bertan, Hicksville, NY, USA) was connected between the ground and the outlet of the reaction capillary. A glass microscope slide, onto which the postcolumn assembly was glued with epoxy, was mounted to a two-dimensional stage (Edmund Scientific, Barrington, NJ, USA) with an angle of about 110° relative to the 325-nm incident laser beam from a He–Cd laser (Model 4240PS; Liconix, Sunnyvale, CA, USA). The incident beam was focused by a 1-cm focal length glass lens (Edmund Scientific) and the fluorescence emission was focused by a 20× microscope objective (Edmund Scientific) onto a R928 photomultiplier tube (PMT) (Hammamatsu, Bridgewater, NJ, USA). A 456-nm interference filter was placed in front of the PMT which was biased at −950 V. Fluorescence signals were converted into voltages through a 10-kW resistor. Data was collected via a 24-bit A/D interface (Justice Innovation, Palo Alto, CA, USA) at 5 Hz and stored in an IBM PC/AT 286 computer (IBM, Boca Raton, FL, USA). The entire electrophoresis and detection system was enclosed in a sheet-metal box with a high-voltage interlock.

Samples were introduced by electrokinetic injection, and derivatives were detected at about 5 mm from the outlet of the separation capillary. New reactors were pressure flushed with 1 *M* NaOH for 30 min followed by a 5-min rinse with deionized water, and then equilibrated under low applied field overnight.

2.3. Reagents and samples

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Fluorescein was from Molecular Probes (Eugene, OR, USA). Water used for solution preparation was from a Milli-Q system (Millipore, Bedford, MA, USA). Borate buffer, used for all experiments, was made by adjusting a sodium tetraborate solution (20 m*M*) to pH 9.5 with NaOH. Samples were prepared in the borate buffer.

The OPA reagent was made by dissolving 2 mg of *o*-phthaldialdehyde in a mixture of 3.9 ml borate buffer, 80 μl ethanol and 20 μl 2-mercaptoethanol. The reagent was aged overnight at 4°C and discarded after 48 h. Prior to use, buffer, sample and OPA solutions were filtered through 0.2-μm filters (Alltech, Deerfield, IL, USA).

Human erythrocytes were isolated by washing whole blood samples with a phosphate buffer saline solution (135 m*M* NaCl and 20 m*M* NaH_2PO_4 at pH 7.4) as described before [18]. The washed cells were suspended in an 8% glucose solution before injection and stored at 4°C when not used. Cells were introduced into the separation capillary by gentle suction from the outlet end of the reaction capillary placed in an airtight vial [18].

3. Results and discussion

3.1. Effect of control voltages

In a coaxial postcolumn reactor, analyte zones emerging from the separation capillary mix with the reagent by means of diffusion, convection and migration [23]. Although thorough mixing is the key for a maximum yield of fluorescent derivatives, turbulent mixing itself broadens the analyte zones. Since the electric field strength applied to the separation capillary here is independent of that applied to the reaction capillary, minimum band broadening due to mixing can be achieved by using the appropriate combination of voltages. A series of measurements were carried out using fluorescein as a test compound and borate buffer as the "reagent". The electric field strength across the reaction capillary was kept at 80 V/cm, while different electric

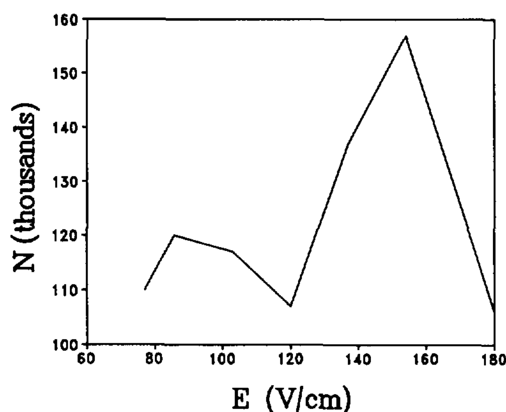

Fig. 3. Plot of theoretical plate number (N) as a function of electric field strength (E) applied to the separation capillary when the electric field strength applied to the reaction capillary was kept at 80 V/cm. Separation capillary: 50 μm I.D., 32 cm long; reaction capillary: 150 μm I.D., 12 cm long.

field strengths were applied to the separation capillary. Fig. 3 shows a plot of the theoretical plate number versus the field strength applied to the separation capillary. The theoretical plate number normally increases as a consequence of increased field strength in the separation capillary in CE [31]. However, axial diffusion is not the limiting factor here. Fig. 3 depicts the influence of various degrees of turbulent mixing due to unequal linear flow velocities between the two inlet streams. The abrupt degradation in efficiency when the field strength for the separation capillary exceeds 154 V/cm suggests the onset of excessive turbulence.

It is interesting to note that the optimum field strength applied to the separation capillary is different from that of the reaction capillary, resulting in unequal linear flow velocities in the two sections. This is likely due to a combination of non-uniform geometry [32], unequal ionic strengths in the two capillary sections, and unequal surface charge (electroosmotic flow coefficient) at the two capillary surfaces. This implies that optimization is needed for each junction if maximum efficiency is required.

The relative and absolute electric field strengths also affect the signal size for post-column derivatization. This is because adequate mixing and reaction time are necessary for derivatization. In our case, the peak areas did not vary significantly over the range of voltage combinations used. The peak heights thus increase as the separation efficiency increases. So, fortuitously, Fig. 3 also serves to optimize the signal-to-noise ratio.

3.2. Linearity and limits of detection

From $1\cdot10^{-7}$ to $5\cdot10^{-5}$ M of glycine, the reactor showed a linear response with a slope of 1.09 from a log–log plot of peak area vs. molar concentration. For $2\cdot10^{-7}$ to $2\cdot10^{-9}$ M of human hemoglobin A_0, the slope is 0.971. Fig. 4 shows an electropherogram of $3\cdot10^{-8}$ M human carbonic anhydrase I with a peak efficiency of 190 000. The peak corresponds to 30 amol of carbonic anhydrase I injected and a 3.8 amol mass limit of detection, estimated by the criterion of three times the RMS noise. The mass limit of detection of carbonic anhydrase I from another reactor is 3.3 amol. This shows the very good reproducibility of these reactors. Limits of detection ($S/N=3$) of some biologically important compounds are summarized in Table 1. All compounds were tested with junctions made from 15 μm I.D.×150 μm O.D. separation and 30 μm I.D.×360 μm O.D. reaction capillaries.

When compared with the best concentration limits of detection reported in Ref. [19], metanephrine and norepinephrine show lower limits of detection with

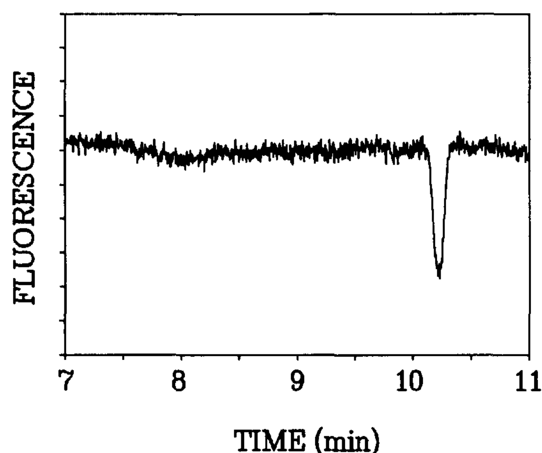

Fig. 4. Electropherogram of human carbonic anhydrase I. Peak corresponds to 30 amol carbonic anhydrase I injected. Separation capillary: 15 μm I.D., 70 cm long; reaction capillary: 30 μm I.D., 12 cm long. Electric field strengths: 300 V/cm and 150 V/cm for separation and reaction capillaries, respectively. Injection: 5 s at operating voltages.

Table 1
Detection limits ($S/N=3$) of amines, amino acids, proteins and peptides derivatized by OPA/2-ME with LIF detection

	Concentration limit of detection (10^{-8} M)	Mass limit of detection (amol)
Amines		
Serotonin	11	153
Metanephrine	2.2	23
Dopamine	3.7	38
Norepinephrine	2.3	20
Dopa	18	100
Amino acids		
Glycine	2.7	17
Arginine	2.6	32
Tryptophan	3.9	35
Phenylalanine	10	84
Threonine	3.5	26
Serine	2.2	17
Proteins		
Human hemoglobin A_0	1.1	9.7
Human carbonic anhydrase I	0.38	3.8
Peptides		
Glutathione	24	310
Leucine enkephalin	600	4400
β-Casomorphin fragment (1–5)	890	6700
Gly–Gly–Phe–Met	720	5700
Gly–Gly–Phe–Leu	1300	10000

the postcolumn reactor, while serotonin, dopamine, and dopa have better limits of detection with the laser-induced native fluorescence scheme. Differences in concentration limits of detection between these two methods do not exceed 2.3 times except that for serotonin, which is 85 times worse with the postcolumn reactor. This highlights the inherent limit of derivatization reactions at low analyte concentrations. Mass limits of detection for all amines tested fall into the low attomole range.

For glycine, the best reported mass limit of detection is 130 amol from an open gap reactor constructed with 10 μm I.D. capillaries [27], while the best reported concentration limit of detection is $1.4 \cdot 10^{-7}$ M from a coaxial reactor [23]. Values in Table 1 for glycine show 7.6 times and 5.2 times improvements in mass and concentration limits of detection, respectively. Mass limits of detection for amino acids tested are all fairly close to each other, i.e. in the low attomole range.

OPA/2-ME has been known as a good fluorogenic reagent for proteins [33,34]. Mass limits of detection listed in Table 1 for human hemoglobin A_0 and carbonic anhydrase I are better than those for amines and amino acids. The 9.7 amol mass limit of detection for hemoglobin A_0 is similar to that obtained from the laser-induced native fluorescence [18] and is sufficient for hemoglobin assay in individual human erythrocytes.

OPA/2-ME labeled peptides have been found to have low fluorescence quantum yields [35]. Although mass limits of detection for peptides are poor compared with those for proteins, they still fall in low femtomole range except for that for glutathione, which is at 310 amol.

3.3. Single-cell analysis

Fig. 5 depicts typical electropherograms from single human erythrocytes. The blood sample in (A) was from a healthy female adult. Between consecutive cell injections, capillaries were pressure flushed for 5 min with borate buffer followed by a 3-min re-equilibration under operating voltages. This treat-

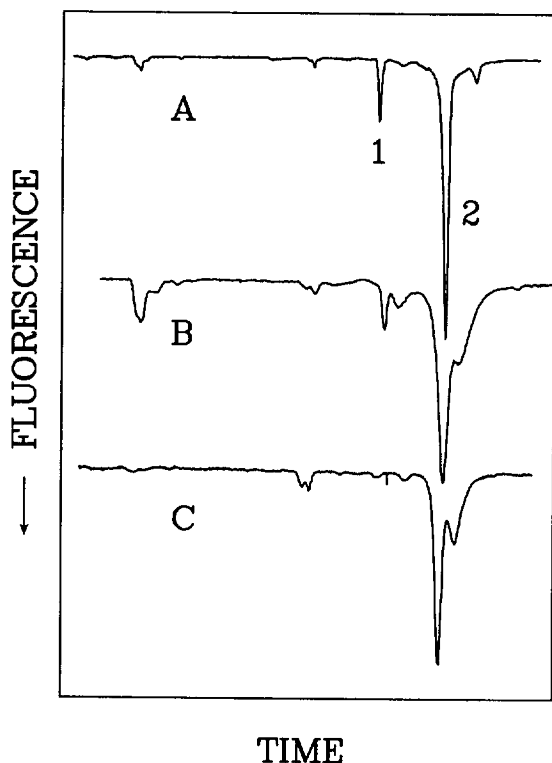

Fig. 5. Electropherograms of single human erythrocytes from (A) normal adult, (B) diabetic adult, and (C) newborn. Separation capillary: 15 μm I.D., 70 cm long; reaction capillary: 30 μm I.D., 12 cm long. Electric field strengths: 300 V/cm and 150 V/cm for separation and reaction capillaries, respectively. Injection: see text.

ment turned out to be crucial because the efficiency and sensitivity were greatly degraded after one run.

The common features in these electropherograms are peaks 1 and 2. By spiking a hemolysate of the same blood sample, peak 2 was identified as hemoglobin, the most abundant protein in erythrocytes (existing at about 450 amol per human erythrocyte) [36]. Peak 1 was identified as carbonic anhydrase I. Detailed discussion on variations in hemoglobin quantity along with migration time variations from cell to cell can be found in Ref. [18]. At first glimpse, carbonic anhydrase I, contained in human erythrocytes at a mean level of about 10 amol per erythrocyte [37], should not give a peak of the size shown in these electropherograms based on Fig. 4. The concentration of carbonic anhydrase, however, varies significantly from race to race [38,39], person to person [40] and also from cell to cell [18]. Other less prominent peaks are believed to be from minor species in erythrocytes containing primary amine groups.

Fig. 5B is an electropherogram from single human erythrocyte experiments with a blood sample from an adult diabetic patient. Compared with Fig. 5A, this electropherogram shows a very different composition from that of normal erythrocytes. The fact that this type of difference is not detected by laser-induced native fluorescence [41] indicates the postcolumn derivatization reactor opens up a new way to assay non-fluorescent components in individual cells.

Fig. 5C depicts a set of electropherograms of single-cell experiments with a human fetal blood sample. An earlier survey concluded that the mean concentration of carbonic anhydrase I (the main carbonic anhydrase isoenzyme found in human erythrocytes accounting for 83% of total carbonic anhydrase concentration [37]) in one-year old children reaches only about 40% of the mean concentration of normal adults [40]. The absence of peak 1 in these electropherograms, showing that the concentration of carbonic anhydrase I in fetal erythrocytes is much lower than that in adults, is in good agreement with the earlier findings.

In summary, we have described a new postcolumn reactor for CE. Mass limits of detection in the low attomole range provided by this postcolumn reactor facilitate sensitive detection of a variety of biologically important amines, amino acids and proteins simultaneously with a single optical arrangement and a relatively inexpensive laser. Single-cell capability demonstrated here shows that this reactor could find other applications in assaying non-fluorescent or electrochemically inactive components in individual cells in the future.

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