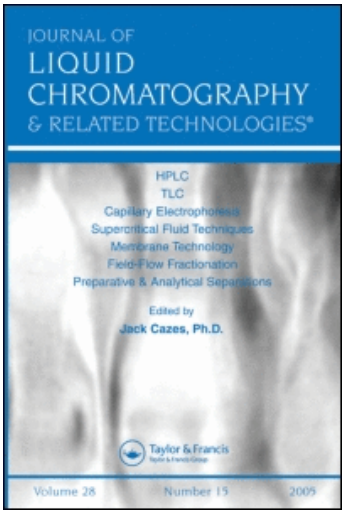

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On-Column and Post-Column Derivatization for Capillary Electrophoresis with Laser-Induced Fluorescence for the Analysis of Single Cells

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ON-COLUMN AND POST-COLUMN DERIVATIZATION FOR CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED FLUORESCENCE FOR THE ANALYSIS OF SINGLE CELLS

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ABSTRACT

Derivatization methods for analysis of single cell components by capillary electrophoresis with laser-induced fluorescence detection are reviewed. Recent advances have lead to the use of this technique for the identification of amino acids and proteins in many different cell lines ranging in size from 8 to 140 μm. Pre-column, on-column, and post-column methods of derivatization are examined in order to determine which results in the least amount of sample handling and dilution leading to better detection limits for the analytes of interest.

INTRODUCTION

Biological systems are extremely complex. In many parts of the body, the cellular environment is highly heterogeneous. In specific tissues, many different cells coexist each with their own purpose and chemical processes. This is especially true of the brain where

a large number of nerve cell types is present. In these cases analysis at the single cell level is necessary for an understanding of their biological significance.

Analyzing samples on the order of a single cell is very challenging. Not only are the cell volumes small, but also the chemical species present are typically found in femtomole to zeptomole amounts. Techniques such as enzyme activity measurements (1), immunoassay (1,2), microgel electrophoresis (3), fluorescence imaging (4), voltammetric microelectrodes (5-7), microcolumn separations (8-27), optical and electron microscopy (13-15), and secondary ion mass spectrometry (16) have all been used to examine single cells; however, each has limitations. They either require an excess of sample, extensive sample preparation, or lack sensitivity or selectivity.

In the late 1980's, Jorgenson and co-workers used the microcolumn separation technique, open-tubular liquid chromatography (OTLC) to analyze single cells (8-11). The microbore columns were 1 to 3 meters in length and had inner diameters (i.d.) ranging from 15 to 20 μm. This resulted in total column volumes between 180 and 940 nL. These investigators studied the large neurons of *Helix aspersa* (about 120-140 μm in diameter). Neurons were dissected and transferred to a 500 nL vial where they were homogenized. A portion of the supernatant was injected onto the OTLC column. Injection volumes were on the order of 5 nL with total analyte amount on the femtomole to attomole level (9-11). Detection of such low analyte levels requires very sensitive detection methods. One detection method with the sensitivity and selectivity for the analysis of single cells is electrochemical (EC) detection.

OTLC-EC was used to determine the easily oxidizable species tyrosine, tryptophan, dopamine and serotonin at femtomole levels (10). In another study, a cell

homogenate was derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) prior to analysis (11). NDA tags primary amines causing them to become electroactive. Seventeen different amino acids were identified with attomole detection limits (11). Smaller mammalian cells have also been examined with microcolumn techniques. Packed LC columns were used to separate components of adrenomedullary cells which are about 16 μm in diameter. This analysis indicated that norepinephrine and epinephrine are often present in the same cell (12).

In this article, we review the recently developed procedures developed to derivatize amine-containing solutes for fluorescence detection following capillary electrophoretic separation of single cell components. Capillary electrophoresis (CE) offers many of the same advantages as microbore OTLC without the need for a bonded or coated phase. CE separations are very fast, on the order of 5 to 30 minutes, due to the high separation potentials used. The capillaries typically range from 10 to 100 cm in length and 2 to 100 μm i.d. The ability of CE to sample and analyze extremely small volumes with good selectivity, high efficiency and resolving power has lead to its use for cells as small as 8 μm in diameter (17-31). Although many detection schemes have been demonstrated, the three with the low mass detection limits necessary to identify the femtomole to zeptomole levels of analytes found in single cells are electrochemical (6, 18-21), immunoassay (29), and laser induced fluorescence (LIF) detection (22-26, 28, 31).

Detection methods for CE analysis of singe cells have initially relied on the native properties of analytes. Species which were easily oxidized were detected using electrochemical methods whereby an electrode was positioned at or in the elution end of the capillary. Small easily oxidized species from whole snail cells (21, 27), snail cytoplasm

(18-21, 27), and whole human lymphocytes (30) have been examined with CE-EC. Analytes which are naturally fluorescent have been identified with laser induced fluorescence (32).

Generally, LIF detection is done on-column through a window in the capillary. A laser is focused on the capillary and analytes are detected as they migrate through this window. CE-LIF has been used for separation and detection of hemoglobin and carbonic anhydrase native fluorescence from single human erythrocytes (red blood cells) (23). In these experiments, the 274-nm line of an Argon ion laser was used to excite these proteins for fluorescent detection without derivatization. In addition, CE-LIF has been used to determine the activities of several lactate dehydrogenase isoenzymes in single erythrocytes by monitoring the enzyme-catalyzed production of NADH (28). Materials which have no easily detected native properties have been examined with indirect detection, which is based on the property of displacement. The background buffer is filled with a fluorescent or electrochemical ion which is continuously detected as it elutes past the detector. If a non-fluorescent molecule is present in a high enough concentration, it will displace the background ions and a negative peak will result. Sodium, potassium, lactate and pyruvate have all been identified in single red blood cells with indirect LIF detection (22, 26).

Unfortunately, most species are not easily identified in their natural state and must be modified to be detected. One such method is to derivatize the analyte and label it with a fluorophore. There are a wide variety of fluorophores available to label amino acids, proteins, peptides, and carbohydrates. Some of the most common derivatizing agents used for CE-LIF are NDA (33, 34), o-phthaldialdehyde (OPA) (33, 35, 36), fluorescein isothiocyanate (FITC) (37, 38), and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde

(CBQCA) (30, 39, 40). These fluorophores are readily excited using the lines from the helium-cadmium or argon ion lasers typically used. The first two also have the advantage of being non-fluorescent until the derivatization adduct is formed causing very little background interference. LIF detection boasts very low detection limits, typically around 10^{-18}-10^{-21} moles (33, 38, 41, 42). Derivatization of analytes, however, is not always an easy task. Some of the problems with derivatization procedures include extensive sample handling which can cause loss of analyte and/or contamination, dilution of the reaction products which may cause diminished sensitivity, slow reaction kinetics which may cause incomplete reactions and lead to irreproducibility, and multiple labeling of solutes with several functional groups. Methods for pre-column, on-column, and post-column derivatization have been developed to address these problems and to determine the optimum time to perform these derivatization labeling reactions.

PRE-COLUMN DERIVATIZATION

Pre-column derivatization is the most straight-forward method to carry out derivatization as it requires no changes to the capillary electrophoresis system. The derivatization reaction is carried out prior to sample injection and separation and often offers the advantage of complete reaction. Pre-column derivatization of single cells has been demonstrated in several ways. Initial experiments included isolation of large invertebrate cells in 200 nL vials. The cells were homogenized and derivatized with NDA in the presence of cyanide (CN^-). A portion of this homogenate was then injected into the capillary and analyzed (8, 25). Individual snail neurons (8) and bovine adrenal medullary cells (25) have been handled in this manner, and several amine containing analytes have

been identified (8, 25). However, dilution was extensive and only 20% of the cell contents were analyzed in any one separation. A similar method has been used for the analysis of human cerebrospinal fluid using CBQCA derivatization (30). The reaction time for this analysis was 2 hours, necessitating the use of pre-column techniques. Ten different amino acids have been identified and quantitated in the cerebrospinal fluid samples (30). Using a different approach, red blood cells were incubated with monobromobimane (mBBr) which was transported through the cell membrane where it reacted with thiols, particularly glutathione, directly inside the cell (22). This method is only useful when the cell membrane is permeable to the derivatization agent and not the derivatization product and for derivatization reactions which occur rapidly at physiological pH (22).

A recent development in our laboratory is aimed at solving the sample handling and dilution problem associated with pre-column derivatization. Reducing the volume of the reaction vessels for derivatization while maintaining the ability to manipulate such small volumes of solution is necessary to alleviate these problems. Arrays of square pyramidal reaction vials ranging in size from 10 to 500 pL have been fabricated in silicon or polystyrene using standard photolithographic techniques. These vials are similar to the 118 nL vials created by Janssen et. al (43), but are several orders of magnitude smaller. An SEM of a 50 pL vial is shown in Figure 1. The vials are evenly spaced 1 mm apart to allow for eventual automation of sample transfer. Methods for filling these small volume chambers have been developed. Micropipettes are constructed from 5 to 10 μm i.d. fused silica capillaries etched in hydrofluoric acid to an outer diameter around 50 μm. This tip is then epoxied into a glass capillary and the pipette is filled with solution. An Eppendorf pressure injector is used to reproducibly deliver solutions into the picoliter vials. Evaporation from these vials is controlled using 4 to 15% glycerol in all of the solutions.

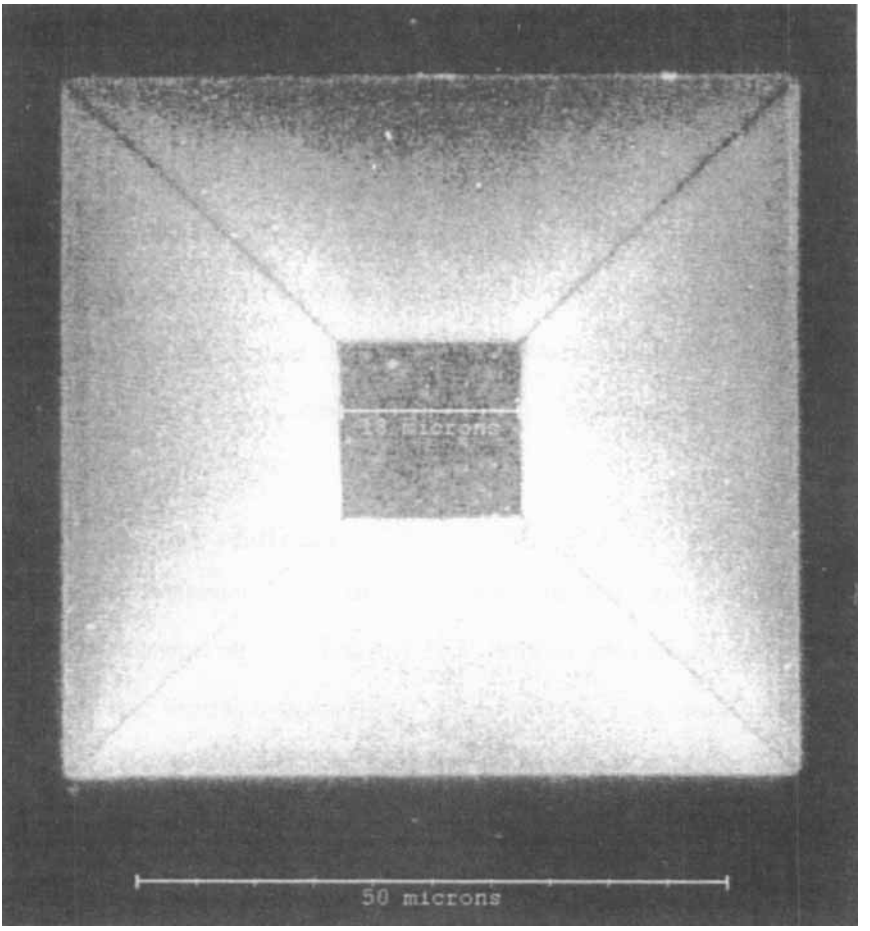

Figure 1 SEM of a 100 pL vial. Dimensions: top, 75 μm; bottom, 18 μm; depth 40 μm.

Preliminary experiments have demonstrated the feasibility of performing NDA/CN$^-$ derivatization reactions in these vials. Amino acid solutions (10^{-5} M) were injected into 1 nL and 100 pL vials, followed by an equal volume of NDA/CN- solution. The solutions were allowed to react for 10 min. The entire surface of the array of vials has been coated

with a thin metallic layer to serve as an electrophoresis anode for injection. Using this anode, the derivatized amino acid solution was electrokinetically injected into a CE capillary and the peaks for arginine, norepinephrine, dopamine, and gycine are shown in Figure 2. Preliminary detection limits for the vials appear higher than from standard solutions. This may be due in part to adsorption of analytes to the metallic layer in the vials. For this reason, polystyrene vials have been fabricated for future experiments. The next step in these experiments will be to transfer a single cell into a vial followed by derivatization, sampling and separation of cell components.

ON-COLUMN DERIVATIZATION

Another procedure whereby derivatization has been performed prior to separation is known as on-column derivatization. In this type of reaction, the introduction portion of the capillary is used as the reaction chamber (34). For analysis of single cells, the inside of the capillary tip is etched to a cone in HF. This facilitates easier visualization for electrokinetic injection of the cells into the capillary tip. Following cell injection, a derivatization and cell lysing solution is drawn in over the cell. A schematic diagram of this process is shown in Figure 3. As an example of this methodology, individual rat pheochromocytoma (PC12) cells have been analyzed (34).

Individual PC12 cells can be selected and drawn into the capillary at a low potential by electroosmotic flow. Injection times range from 15 to 90 s. In order to determine the amount of cell media and associated solutes injected into the capillary along with the cell, an internal standard of γ-glu-gly is added to the media just prior to injection. After cell injection, the capillary is moved to a solution containing the NDA derivatization

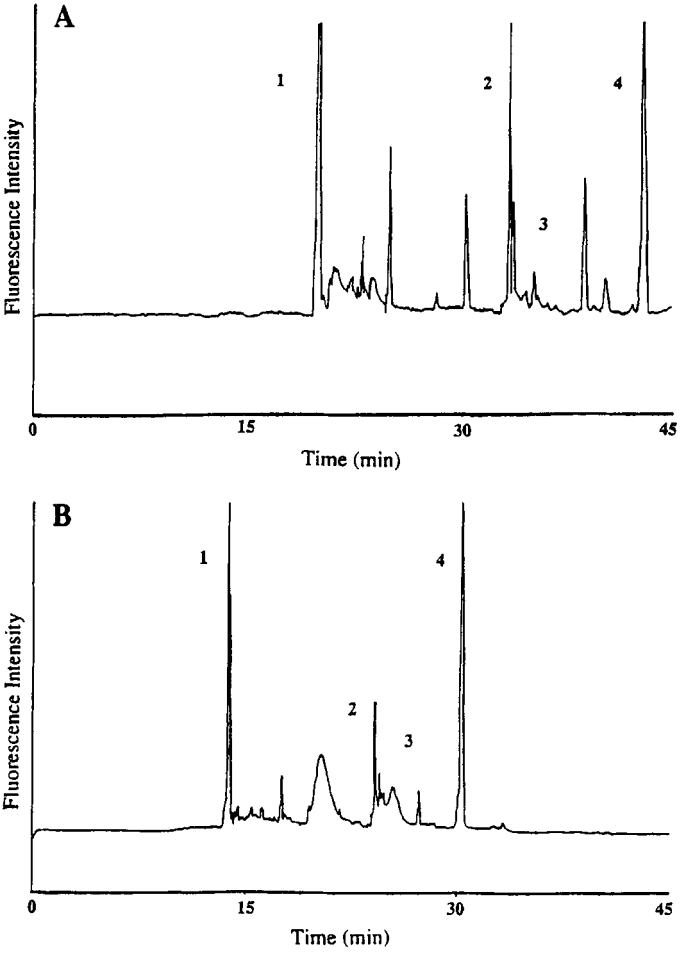

Figure 2 A. Electropherogram of NDA-derivatized solution from a 1 nL vial. Peaks are 1, Arg; 2, Norepinephrine; 3, Dopamine; 4, Gly. Conditions: capillary 50 μm i.d., separation buffer, 100 mM borate, pH 9.5; injection, 10 s at 5 kV; separation potential, 20 kV. B. Electropherogram of NDA-derivatized solution from a 100 pL vial. Peaks identities are the same as in A. Conditions: capillary 22 μm i.d., separation buffer, 100 mM borate, pH 9.5; injection 2 s at 5 kV; separation potential, 30 kV.

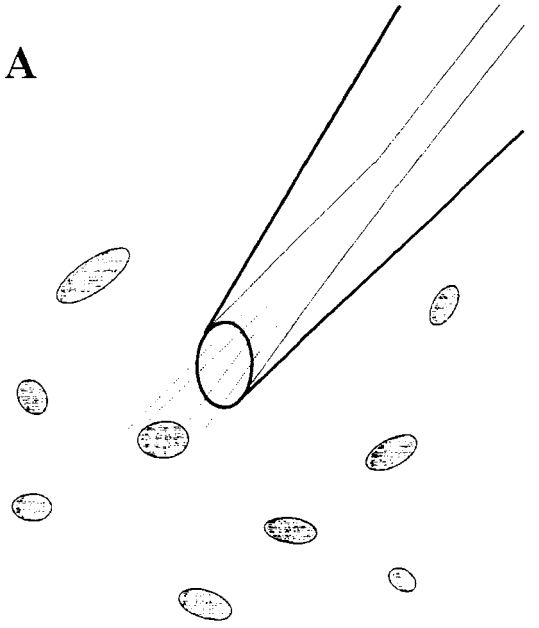

Figure 3 Schematic representation of the on-column derivatization procedure for analysis of single cells. A. A cell is dislodged from the culture dish with the tip of the capillary, and a potential is applied to draw the cell into the front of the capillary by electroosmotic flow. B. The capillary is placed in a reservoir containing derivatizing/lysing buffer, and a potential is applied to draw the reagent over the cell. C. The cell is allowed to lyse in the reagent buffer, and cell contents are derivatized. After sufficient time is allowed for lysing and derivatization, the separation potential is applied, and the NDA-labeled cell contents are detected using LIF after electrophoresis.

(continued)

reagent and a cell lysing agent (digitonin). A second standard (val-tyr-val) is added to determine the extent of derivatization. A 30 s injection of this solution is made, and a 10 min incubation time allowed. After the reaction, the capillary is returned to the separation buffer, and a separation voltage of 30 kV applied (34).

An electropherogram of PC12 cell components and of the cell medium is shown in Figure 4. The analytes have been identified based on electrophoretic mobilities or by

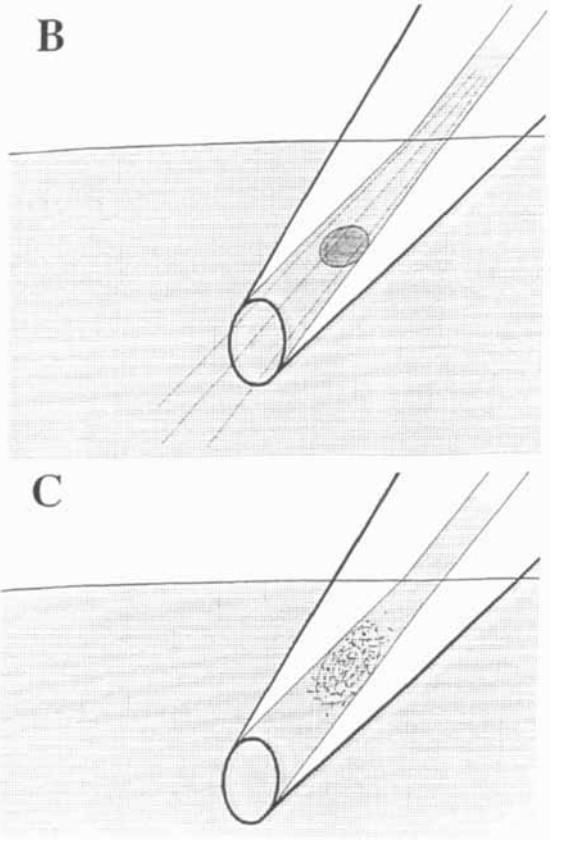

Figure 3 (continued)

spiking the reagent buffer with standard solutions of analytes. Six compounds were quantitatively identified in the PC12 cell experiments. Levels of dopamine, alanine, taurine, glycine, glutamic acid and aspartic acid were in agreement with previous estimates (34).

Dilution in on-column derivatization experiments is limited by the size of the capillary bore and the reaction time. The only source of dilution is diffusion of the

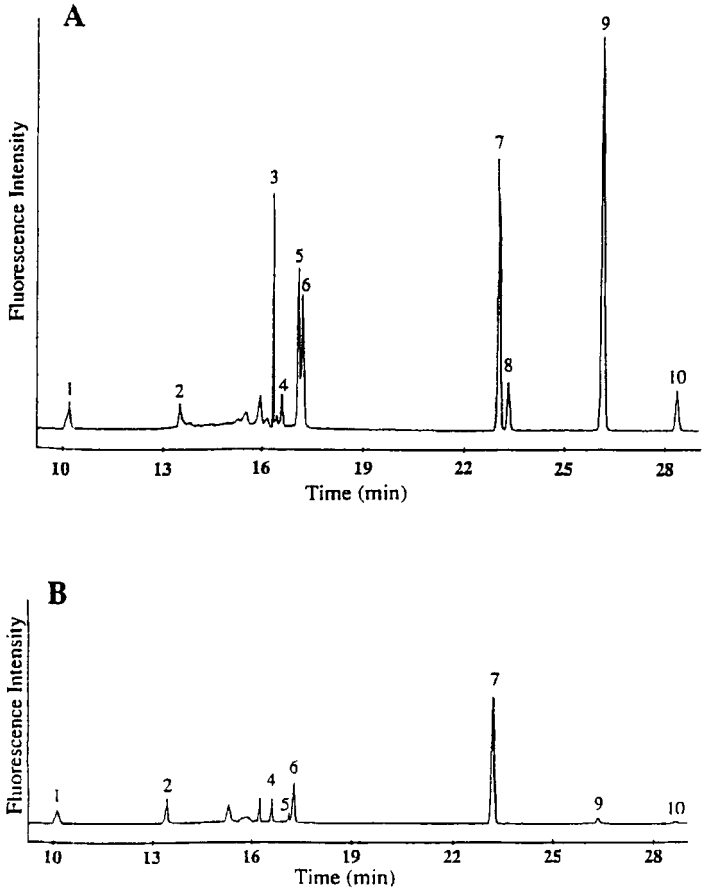

Figure 4 A. Electropherogram of the NDA-derivatized contents from a single PC12 cell. Peaks are 1, neutral peak; 2, Val-Tyr-Val (internal standard); 3, unidentified peak; 4, Ala; 5, taurine; 6, Gly; 7, γ-Glu-Gly (internal standard); 8, unidentified peak; 9, Glu; 10, Asp. B. Electropherogram of a control injection of the medium from the cell culture dish from which the PC12 cell in Figure 4A was injected. The vertical scale is identical to that in Figure 4A, and peak identities are as in A. Reproduced with permission from Ref. 34.

analytes along the capillary bore. The dilution factor in these experiments is around 100, greatly reduced from pre-column derivatization schemes, and sample handling is also reduced (34). There is no need to transfer cells from one vial to another, since the separation capillary acts as the transferring mechanism as well as the reaction chamber.

POST-COLUMN DERIVATIZATION

It is not always optimal to perform derivatization prior to separation. One difficulty with this is multiple labeling of solutes (35). Components such as proteins which may contain more than one amino acid group can result in multiple peaks for the same analyte. In addition, loss of sample can result from the multiple sample handling and transfer steps involved in pre-column derivatization schemes. These difficulties provide the rationale for post-column derivatization work. Three derivatization reagents act rapidly enough to be used in post-column derivation. These are OPA (35, 44-47), fluorescamine (47, 48), and NDA with 3-mercaptoethanol (38). Several post-column reactor designs have also been demonstrated.

In most cases, the separation capillary must be modified to accommodate the introduction of the derivatization reagent. However, one method involves derivatization of the analytes in free solution between the elution end of the capillary and the detector (46). Several post-column designs employ pressure driven introduction of the derivatization agent through coaxially coupled capillaries (35, 44, 48) or cross connectors built directly into the capillary (45). Electroosmotic flow has also been used to introduce reagents and initiate mixing by way of a free-solution reactor (46) or a fluid gap reactor (47). The fluid gap reactor has been designed with the reaction capillary larger than the

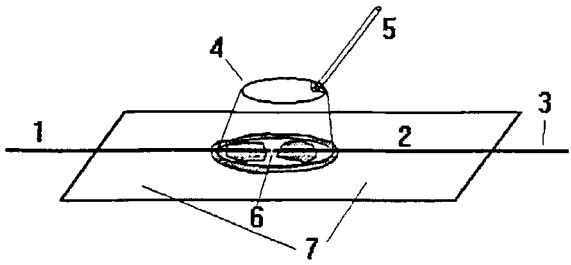

Figure 5 Schematic representation of a gap reactor: 1, separation capillary (applied positive high voltage); 2, reaction capillary (grounded); 3, detection window; 4, plastic gap reservoir (floated); 5, plastic syringe port; 6, capillary gap; 7, glass microscope slide. Lightly shaded areas represent epoxy. Reproduced with permission from Ref. 36.

separation capillary and derivatization agent was pulled into the fluid flow as the analytes traversed the gap. The best detection limits for these post-column reactors has been 41 attomoles with theoretical plates ranging from 175,000 to 600,000 (47). These methods have the capability to detect the levels of material found in single cells, however, they use 25-50 μm i.d. capillaries which are a little too large to conveniently handle single cells.

Another post-column detector based on the fluid gap reactor design uses a break in a single 10 μm i.d. capillary as the introduction port and therefore uses the same i.d. capillary for both the separation and detection ends. This allows for easy alignment of the two ends. A schematic diagram of this detector is shown in Figure 5. Reagent introduction in this system is due to reagent diffusion into the gap and an optimum gap distance of about 4 μm has been determined where peak tailing and band broadening are minimized. A sample electropherogram is shown in figure 6. Reagent introduction through this gap has been demonstrated using amino acid and protein separations with OPA derivatization, and detection limits of 130 amol and 5.2 amol for glycine and transferrin have been determined, respectively (36).

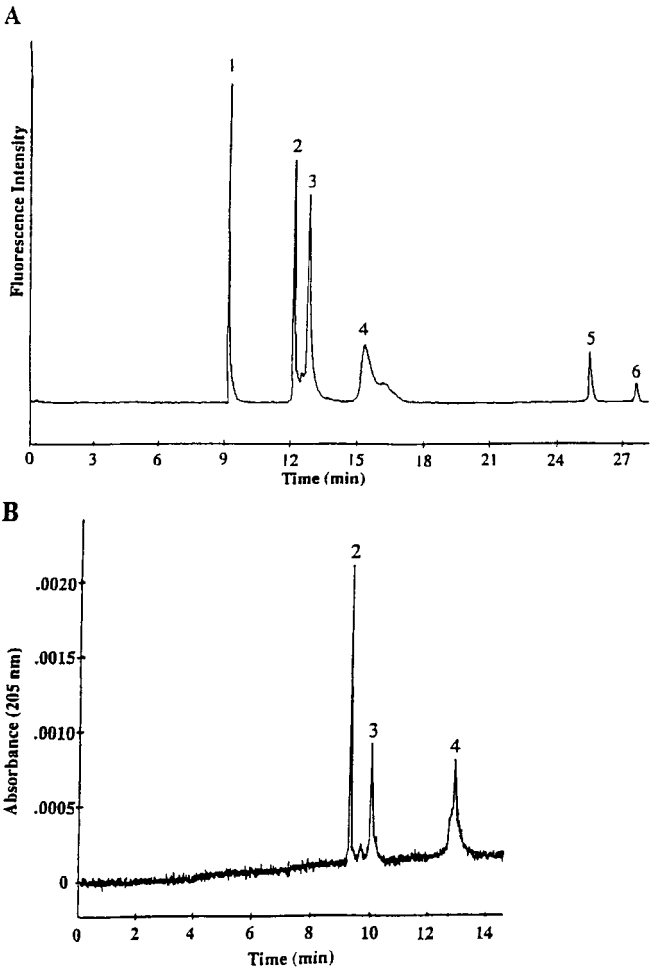

Figure 6 Separation of three proteins and three amino acids using post-column derivatization and LIF detection. Peak identities and sample concentrations are: 1, DL-arginine hydrochloride (5.0×10^{-5} M); 2, horse heart myoglobin (0.5 mg/mL); 3, iron-free human transferrin (0.25 mg/mL); 4, bovine serum albumin (0.5 mg/mL);, 5, (L+)-glutamic acid (5.0×10^{-5} M); 6, DL-aspartic acid, (5.0×10^{-5} M). Conditions: capillary, 10 μm i.d. ; 100 cm; 4 μm gap; injection, 5 s at 10 kV; separation potential, 30 kV; separation buffer, 100 mM borate, pH 9.5. B. Separation of the same protein and amino acid mixture as in Figure 6A with UV absorbance detection at 205 nm. Peak identities, sample concentrations, and separation buffer are the same as in Figure 6A. Conditions: capillary, 25 μm i.d., 73.3-cm length, 57.6 cm to detector; separation potential, 20 kV; injection, 5 s at 7.0 kV. The three amino acids can not be detected at the concentrations used here. Injection amounts of proteins are: horse heart myoglobin, 25.4 fmol; human transferrin, 2.7 fmol; bovine serum albumin, 5.0 fmol. Reproduced with permission from Ref 36

This same design has also been used with NDA/2-mercaptoethanol derivatization. The use of NDA as a derivatization reagent usually involves reaction with cyanide to for a stable fluorescent product. When reacted with mercaptoethanol the reaction produces a product which is relatively unstable and is not suitable for pre-column techniques; however, the reaction is sufficiently rapid for post-column work. Post-column derivatization with NDA has been used to separate and detect components from homogenates of *Planorbis corneus* neurons and single red blood cells (49).

CONCLUSIONS

CE-LIF is a very sensitive method for the analysis of single cells. It has been used on many different cell lines to examine amino acids and proteins. Pre-column, on-column and post-column methods of derivatization have been examined in order to determine which results in the least amount of sample handling and dilution leading to better detection limits for the analytes of interest. Pre-column derivatization methods have always been limited by dilution. The use of the picoliter reaction vials described here may be a tremendous advance in overcoming that limitation. With these vials it is conceivable that more extensive derivatization procedures which tag otherwise unidentifiable analytes may be used without the dilution problem. On-column derivatization provides an alternative scheme to pre-column work where the sample is confined to the entrance of the capillary as a reaction chamber. This method is highly effective at minimizing sample dilution; however, solutes that can be multiply labeled can still present a problem. In post-column work, multiple labeling of solutes is not problematic as the solutes have already been separated and dilution has been reduced because the volume is limited by the size of

the migrating zone of analyte. However, the derivatization reaction must be complete in a few seconds. Future advances in these analyses will likely focus on new reaction chemistry for rapid and quantitative derivatization as well as instrumental advances to immobilize cells in microvials, and to minimize dilution during derivatization while maintaining the integrity of the separation.

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