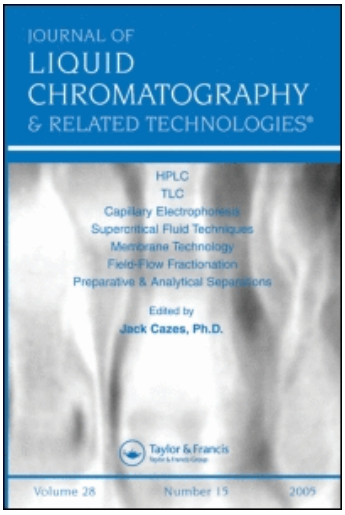

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Biomedical Applications of On-Line Preconcentration-Capillary Electrophoresis Using an Analyte Concentrator: Investigation of Design Options

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BIOMEDICAL APPLICATIONS OF ON-LINE PRECONCENTRATION-CAPILLARY ELECTROPHORESIS USING AN ANALYTE CONCENTRATOR: INVESTIGATION OF DESIGN OPTIONS

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ABSTRACT

A method to perform on-line sample preconcentration of serum immunoglobulin E by affinity capture is described. Purified anti-IgE antibodies were covalently bound to an analyte concentrator-reaction chamber or cartridge. The immunoglobulins (IgE) were bound to and eluted from the cartridge by the optimum dissociating buffer system, and the eluent(s) were then subjected to capillary electrophoresis.

The first design used was a 5 mm solid-phase cartridge fabricated by assembling a bundle of multiple microcapillaries in which a monoclonal antibody directed against IgE was covalently bound to the surface of every microcapillary. The whole assembly was connected, through sleeve connectors, to the capillary column for affinity capillary electrophoresis.

The second design used consisted of an analyte concentrator-reaction chamber that was fabricated from a solid rod of glass.

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Several small diameter passages or through holes containing a similar surface area was tested for the same experiments and performance as described above.

A major advantage of these designs, over previously described designs, is the absence of frits and beads. The previously reported designs consisted of derivatized beads confined to the concentrator cartridge by a frit at each end. After limited usage of the cartridge, the beads tends to pack at the outer frit. This lead to restricted flow through the concentrator chamber and ultimately clogging of the system. The designs reported here allows for a constant electroosmotic flow, superior reproducibility of the electropherograms, a reduced possibility of blocking the microcapillaries, and increase number of usages of the cartridge.

The use of this novel analyte concentrator design for the determination of immunoglobulins in biological fluids is demonstrated by capillary electrophoresis of IgE in serum. The general utility of this technique for a variety of biomedical applications is discussed.

INTRODUCTION

Since its discovery in 1967 (1,2) immunoglobulin E and its receptors have been extensively studied to provide a better understanding for their role in host defense against parasites and in the etiology of allergic diseases (3). Biochemical characterization studies were hampered for some time since IgE[1] constitutes a minuscule fraction of the total human immunoglobulin content in serum of non-atopic individuals. Normal levels of IgE in serum of non-atopic individuals range between 50-300 ng/mL versus 7-12 mg/mL of IgG. The discovery of two patients exhibiting a myeloma protein of the Immunoglobulin E class (1,4) and the availability of human myeloma cell line SK007/U266 (5) which secretes human IgE (6) have made possible the purification of this immunoglobulin and its subsequent biochemical and biological characterization. At the present time, a variety of immunoassays are available for the determination of IgE in serum (7,8). In general, enzyme immunoassays offer a number of advantages when compared to radioimmunoassays. Primarily, they eliminate handling of radioactive isotopes and permit the storage of labeled-reagents for prolonged periods. More recently, capillary electrophoresis has been examined as a useful separation method for rapid and efficient immunoassays (9-14).

Capillary electrophoresis has found widespread applications in analytical and biomedical research and can perform analytical sepa-

rations that are often more efficient, faster and with other capabilities far superior to those of traditional separations techniques (15-18). However, the small volume of the capillary limits the amount of sample that can be loaded. This is a major limitation of CE and compromises its use for analysis of very dilute samples. The volume injected into the capillary (without analyte concentrator) normally range between one to twenty nanoliters. In many instances analysis of several microliters of sample solution is often required to enable detection of the analytes of interest. In this regard, several approaches has been developed to preconcentrate samples, including analyte stacking (19-24), field amplification (25-28), and transient isotachophoresis (29-37). However, despite of the unique features of these techniques for preconcentration and separation, they still can only afford the introduction of approximately 1 μL of total volume into the CE capillary, and the presence of high salt concentration affect the efficiency of stacking processes.

In many instances, however, biologically active compounds are present in tissue biopsies, cells, and biological fluids at extremelly low concentrations. In order to detect minute amounts of solutes in these complex matrices, it is necessary to introduce sample volumes that vastly exceed the total volume of the CE capillary (> 2 μL). The availability of on-line analyte preconcentration, using the analyte concentrator technique (38-66), is a major advance in overcoming the problem of poor limits of detection for dilute samples. For example, enhancing the loading capacity of CE result in the detection of analytes found in very low concentrations (< 50 ng/mL) in biological fluids, as is the case of IgE in serum.

An analyte concentrator-reaction chamber (AC-RC) consists of a solid support, inserted near the inlet of the CE capillary. Various chemistries have been covalently bound to the AC-RC in order to concentrate solutes (38-66), or to cleave, on-line, biopolymers into more fundamental components such as peptides or nucleotides (67-75). The dimension of the cartridge normally ranges between 1 and 5 mm in length, and 25 to 400 μm in diameter. The only exceptions to these dimensions, have been the reports in which pieces of entire capillaries of several centimeters have been used to couple enzymes directly to the inner surface (instead of binding to beads or membranes) for macromolecule cleavage (69,73,74).

In this report, we describe an analyte concentrator design made of multiple capillaries associated in bundles, and another having a plurality of small diameter rod passages or through holes. Each with sufficient surface area to covalently link monoclonal antibodies directed against IgE to extract IgE molecules present in serum. IgE molecules were bound to and eluted from the cartridge,

with a very small amount of high salt dissociating buffer, and finally subjected to capillary electrophoresis.

The second design, made of glass and containing several through holes, had several advantages when compared to the cartridges fabricated with the bundle design and those fabricated by other methods (38-66). For example: (a) easier fabrication of the cartridge; (b) better consistency of the electroosmotic flow enabling a high reproducibility of the peak area and migration time; (c) reduced possibility of clogging the system; (d) increased number of uses; (e) greater stability of the chemistries, since no heat was involved in the production of the cartridge; and (f) increased length (up to 5 mm) without changing significantly the electroosmotic flow. The fabrication of a cartridge containing microbeads or membranes, greater than 3 mm in length, can yield a product having consistently low reproducibility of the electroosmotic flow after several uses, an increased tendency for blocking the system, and a short life span. This is due primarily to: (a) use of irregularly-made frits; (b) compacting of the microbeads due to increasing pressure; (c) increase tendency of the membranes to pack yielding an increase back pressure; and (d) difficulty in reproducing the fabrication of the cartridge. Analyte concentrators made longer than 3 mm have a greater tendency to diminish electroosmotic flow and ultimately blocking the system. This is especially true when using several microliters of complex matrices such as serum.

In the present report, we demonstrate the use of both analyte concentrator-reaction chamber designs described above for the determination of the IgE content in serum by capillary electrophoresis. The use of these novel designs enabled a superior affinity capture of IgE. This procedure enhanced the detection of IgE in serum samples where IgE is present as a minute fraction of the total immunoglobulin content. The routine use of this technique for a variety of other biomedical applications is possible. Especially for molecules compatible with affinity interactions and present at concentrations of fg/mL in complex matrices such as tissue biopsies, cells, and biological fluids.

EXPERIMENTAL

Reagents

All chemicals were obtained at the highest purity level available from the manufacturer and were used without additional purification. Sodium hydroxide, Hepes, boric acid, bovine serum albumin,

fetal calf serum, Tween-20, immunoglobulin G, immunoglobulin A, immunoglobulin M, o-phenylenediamine dihydrochloride, horseradish peroxidase, human serum albumin, and ethylene glycol were purchased from Sigma Chemical Co. (St. Louis, MO). IgE was purified by electrophoretic and immunochromatographic procedures (76), and the monoclonal antibodies directed against IgE were generated and purified according to the method described elsewhere (77). All other inorganic chemicals were reagent grade or better and obtained from Mallinckrodt (St. Louis, MO). Reagent solutions and buffers were prepared using triply distilled and deionized water and were routinely degassed and sonicated under vacuum after filtration. Disposable filter units (0.22 μm) were purchased from Scientific Resources, Inc. (Eatontown, NJ), and fused-silica capillary columns were obtained from Polymicro Technologies (Phoenix, AZ).

Fabrication of an analyte concentrator-reaction chamber

Design 1: Bundles of Microcapillaries

Five to 14 polyimide coated capillaries of 25 μm I.D. and 150 μm O.D. were tightly hand-fitted into a rigid plastic tubing of 5 cm in length and about 400-800 μm I.D. as depicted in Figure 1. Portions of approximately 5 mm were cut, first with a stainless steel blade and then with a tip-diamond glass cutter, to produce an analyte concentrator. Monoclonal antibodies directed against IgE were covalently bound to the surface of every microcapillary of the analyte concentrator by a minor modification of a previously described method (43). The whole assembly was connected, through sleeve connectors sealed with epoxy resin, to the capillary column for affinity capillary electrophoresis. (Normally, 5 to 14 microcapillaries can be arranged into bundles with a total length of 2 to 5 mm without significantly affecting performance after a few injections of serum.)

Design 2: Multiple Channels Bored Through a Single Glass Rod

Several small diameter passages or through holes of approximately 25 μm I.D., containing a surface area comparable to design 1, was fabricated from a solid glass rod by laser drilling with a laser beam. Anti-IgE antibodies were covalently bound to the surface of every microcapillary, or through holes, of the analyte concentrator by the same method used above. The whole assembly was also connected, through sleeve connectors sealed with epoxy resin, to the capillary column for affinity capillary electrophoresis.

In order to apply the appropriate chemistry to the inner walls of the microcapillaries, or through holes, the analyte concentrator

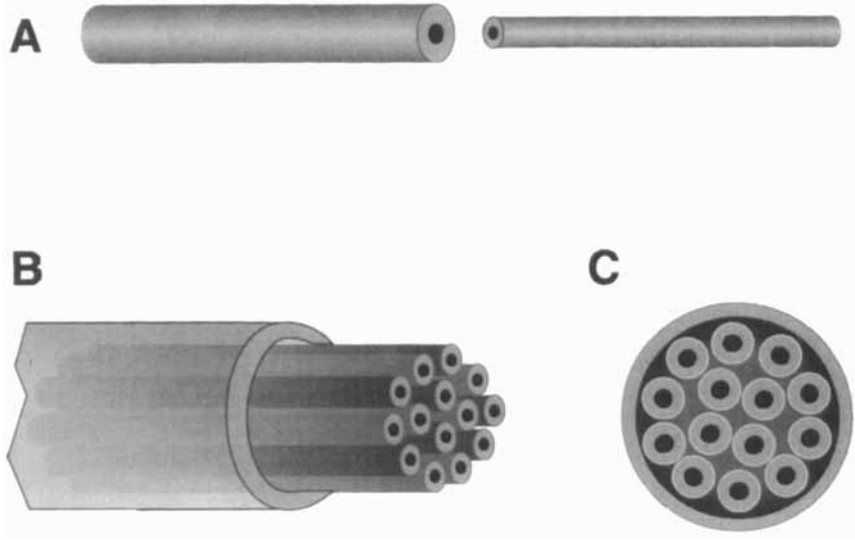

Figure 1. A Schematic Diagram of an Analyte Concentrator Fabricated of Multiple Capillaries. A. Comparison between a 75 μm I.D., 365 μm O.D., and a 25 μm I.D., 150 μm O.D. **B.** A plan view of a portion of the analyte concentrator fabricated with 25 μm I.D., 150 μm O.D. capillaries, and inserted into a sleeve connector for coupling to a separation capillary of 75 μm I.D., 365 μm O.D., 65 cm to the cathode and 7 cm to the anode. **C.** A sectional view of the same analyte concentrator cartridge depicted on B.

cartridge was connected to a syringe, through luer lock-like connector, as described in Figure 2. The various chemistries were sequentially applied slowly at a controllable speed using a syringe pump. The last step of the procedure, was the covalent coupling of the purified monoclonal antibody directed against IgE to form a stable bridge with the column. At this stage, the newly-created multi holes anti-IgE affinity analyte concentrator was removed from the syringe set and connected, and sealed with epoxy resin, to two longer underivatized fused-silica capillary columns (75 μm x 65 cm) for CE analysis. The location of the AC-RC was at 7 cm from the injection side of the capillary.

The entire process of fabrication, coupling and testing of the analyte concentrator was monitored under a stereomicroscope.

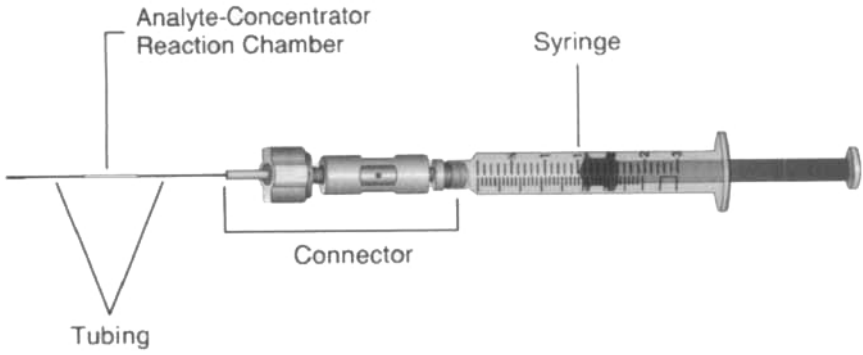

Figure 2. A Schematic Representation of a Syringe Attachment to the Analyte Concentrator. In order to apply the various chemical reagents onto the analyte concentrator, to link IgE to the surface of every microcapillary, it was necessary to fabricate an adaptor system. This system consists of a syringe linked to a commercially available connector to press the capillary in order to avoid any leakage of fluid. The analyte concentrator cartridge was, in turn, connected to two pieces of capillary to enable binding and washing of the AC-RC with appropriate reagents. These capillaries were removed after the AC-RC processing was complete. The 5 cm cartridge-containing bundles of microcapillaries, to which anti-IgE antibody was bound to their inner surfaces, was cut into a working analyte concentrator of a length of approximately 5 mm. This cartridge, was in turn, coupled to the separation capillary. For details see Experimental Section.

Normally, the finished product was allowed to rest for 24 hr at 25°C before use to permit a complete seal of the system. When the AC-RC was not in use, it was stored at 4°C in buffer containing 0.1 M sodium azide.

On-line affinity capillary electrophoresis of IgE

Twenty microliters of serum containing high titers of IgE were injected by pressure into the capillary column followed by a clean-up procedure consisting of a wash buffer to remove salts and other

serum constituents. Once the column was washed with separation buffer, and then equilibrated with 10 column volumes of the same buffer, a plug of 100 nanoliters of an optimized buffer system was applied to elute the antibody. The elution buffer consisted of 75 mM Hepes/NaOH buffer, pH 7.2, containing 3 M $MgCl_2$, and 25% ethylene glycol (78). After elution of IgE from the AC-RC by the sequencial use of elution buffer followed by separation buffer under hydrodynamic pressure, the power was turn on and capillary electrophoresis was performed at 300 Volts/cm. The separation buffer consisted of 50 mM sodium tetraborate buffer, pH 8.3, and the separated component(s) were monitored at 214 nm.

Instrumentation

A laboratory-made apparatus was used similar to the one previously described (77). Briefly, the apparatus contained an on-column UV detection system. Electropherograms were obtained with a strip chart recorder model L-6512 (Linseis Inc., Princeton Junction, NJ) at 20 cm/hr.

Fraction Collection of Samples

Separated samples were fraction collected for further analysis. The fraction collection system used was previously described (79). This system couples two fused-silica capillaries (75 μm I.D. each) together with a third fused-silica capillary of smaller I.D. (10 μm x 10 cm) through a tee asembly system (79). This tee assembly forms a joint that is electrically conductive through the thinner capillary. The location of the tee assembly was after the detection system, five cm before the end tip of the collecting capillary.

Monitoring of the Electroosmotic Flow

The rate of electroosmotic flow was calculated by the method we previously described (80). Using the fraction collection system mentioned above, one measures how long it takes for a drop to form at the tip of the detection capillary. Under a microscope the drop is aspirated by capillarity into a piece of capillary column. Because one can measure with a caliper the distance between the two menisci formed, the total volume that is loaded into the piece of capillary column can be calcultated using the formula of a cylinder [$V = \pi (d^2/4)$]. By knowing the time needed for the drop to form, the volume of the drop, and the size of the detection capillary, one can calculate the rate of electroosmotic flow in nL/mm/sec.

Enzyme-Linked Immunosorbent Assay

Fraction collected samples using capillary electrophoresis were quantitated by ELISA (81,82) as follows: flat bottom 96-well microtiter plates were coated for 16 hr at 25°C with 50 μL per well of purified anti-IgE monoclonal antibody (at a concentration of 1 μg/mL in sodium tetraborate buffer, pH 9.6, containing 0.6 M NaCl). After incubation, the microtiter plates were washed three times with phosphate-buffered saline (PBS), pH 7.5, containing 0.05% Tween-20. Nonspecific binding sites were blocked with 2% BSA in PBS and incubated at 90 min at 37°C. Plates were rinsed four times with PBS, pH 7.5. Fifty μL of fraction collected samples using capillary electrophoresis (or purified IgE, or commercially available IgA, or IgG, or Ig M, or human serum albumin) were added to the wells of the microtiter plate by duplicate, followed by incubation for 1 hr at 37°C. Plates were washed four times with PBS, followed by addition of 50 μL of purified monoclonal IgE conjugated to horseradish peroxidase (at a concentration of 2 μg/mL in PBS, pH 7.5, containing 0.05% Tween-20, and 2% fetal calf serum). Plates were incubated for 45 min at 37°C and then were washed again four times with PBS, pH 7.5. The final reaction was visualized by incubation for 30 min at 25°C in the dark with the chromogen substrate o-phenylenediamine (OPD) dihydrochloride. The reaction was stopped with 3N HCl The resulting absorbance was measured at 492 nm in an ELISA reader.

RESULTS

The efficacy of using on-line preconcentration capillary electrophoresis for determination of IgE in serum is demonstrated. In this study, approximately twenty microliters of serum containing high titers of IgE (900 ng/mL) was analyzed using CE columns containing two kinds of analyte concentrators.

The first design, consisted of a bundle of microcapillaries of 25 μm I.D., 150 μm O.D., which were tightly hand-fitted into a rigid plastic tubing of 5 cm in length and about 400-800 μm of total I.D. The working analyte concentrator consisted of approximately 5 mm in length. As shown in Figure 3A, the eluted material consisted of two main peaks ranging in migration times between 31 to 34 min for the minor peak, and 42 to 54 min for the major peak. Contrary to this observation, the second design of analyte concentrator, made of through holes, shows a single peak ranging in migration times between 42 and 43 min (Figure 3B). The significant variability in mi-

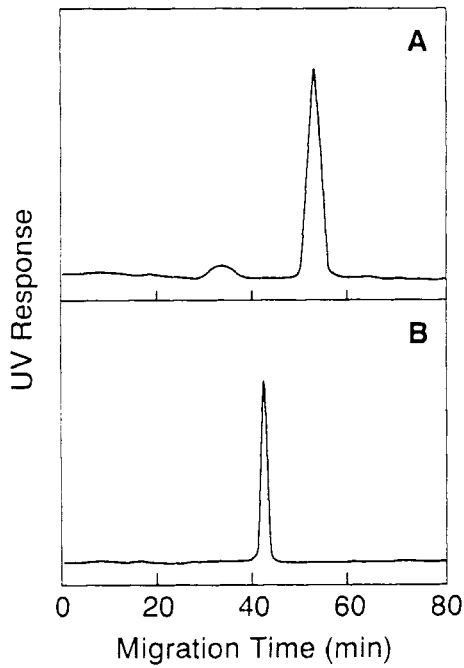

Figure 3. Electropherogram of IgE. Using the analyte concentrator fabricated of multiple capillaries associated in bundles **(panel A)**, or using the analyte concentrator fabricated from a solid glass rod having a plurality of small diameter rod passages or through holes **(panel B)**. For details see Experimental Section.

gration times (see Table 1), for the analyte concentrator fabricated of multiple capillaries, was due in part to a progressive clogging of the system as reflected by a reduction in the electroosmotic flow (Table 2).

The major peak in Figure 3A, and the single peak in Figure 3B, were collected after several injections by a method described elsewhere (49,79). Further analysis of these collected peaks by ELISA (Table 3) demonstrated that the substances present in the major peaks corresponded to IgE, as indicated by the higher O.D. values for IgE and the major peaks collected versus the near basement O.D. values for the control samples. This observation, strongly suggests

Table 1. IgE Migration Time (in minutes) for Six Consecutive Serum Analyses Using the Same AC-CE Capillary

Analyte concentrator made of bundles of microcapillaries		Analyte concentrator made of rod passages or through holes	
1	42	1	42
2	44	2	42
3	46	3	42
4	49	4	42
5	51	5	43
6	54	6	43

Table 2. Electroosmotic Flow (nL/mm/sec x 10^3) for Six Consecutive Injections of Serum into the Same AC-CE Capillary

Analyte concentrator made of bundles of microcapillaries		Analyte concentrator made of rod passages or through holes	
1	54	1	56
2	51	2	55
3	48	3	55
4	45	4	54
5	42	5	53
6	39	6	53

Table 3. Confirmation of IgE in Collected Analyte Zones Using an IgE Specific ELISA Method

Substance	O.D. 492 nm
Peak 1 (Figure 3A)	0.085
Peak 2 (Figure 3A)	1.200
Peak 1 (Figure 3B)	1.300
Purified IgE	1.500
Commercial IgA	0.080
Commercial IgG	0.095
Commercial IgM	0.060
Commercial HSA	0.055
Background	0.050

that this analyte concentrator design functions with a high specificity since human serum albumin, IgA, IgG, IgM and other constituents present at high concentrations in serum were not identified in the electropherograms or the ELISA method.

DISCUSSION

From the early reports on capillary electrophoresis, it has been stressed that this technique is quite unique in many features. Some of the most important of these features are: the small sample volumes used, and the high resolution achieved for the separation of components. However, limitations were also frequently reported. The reactive nature of the silica capillary was cited to adsorb some analytes, particular peptides and proteins. As a consequence of the interaction of a protein with the surface of the CE capillary, reproducibility becomes difficult. Sensitivity was also an issue frequently cited. The limited total volume of the capillary restricts

injection sample volume to less than 50 nL, which for low concentration samples can result in no detection of the analyte unless derivatization or concentration of sample is performed.

Since those early reports, considerable progress has been made in reducing the effects of the charged capillary wall. These innovatives strategies have been able to minimize the problems of analyte adsorption and lack of reproducibility. However, the remaining of the problem related to the limitation in sample volume that can be injected into the CE column, and still be able to achieve analyte detection sensitivity, has experienced less progress.

In general, components of interest in biological fluids are often found in extremely low concentrations. This fact combined with only being able to sample nanoliters has restricted the use of CE in biomedical applications. Recent reports addressing this issue have focused on derivatization, off-line concentration, as well as low volume on-line preconcentration techniques in order to increase the detection of the material being analyzed (for reviews see ref. 15-18,61,65,75). Derivatization techniques are often difficult to be carried out stoichiometrically for complex molecules. Most preconcentration techniques for CE, although have improved detection limits, still limit the volume sampled to less than 1 μL.

The present report outlines a major advance in the detection of molecules at ultra-low concentrations (e.g., fg/mL) in biological fluids. It is feasible to increase detectability of samples present in small concentration in biological fluids by affinity concentration using an analyte concentrator-reaction chamber. In this case, it is demonstrated that IgE was bound to and eluted from the analyte concentrator-containing anti-IgE. However, technical problems were encountered with the fabrication of the analyte concentrator made of bundle of microcapillaries. The microcapillaries were hand fitted within another larger tube. This method of fabrication produced empty spaces in between the microcapillaries which made account for the non-specific binding of serum substances, and a progressive reduction in the electroosmotic flow. In turn, the electropherogram of IgE was not reproducible. On the other hand, the multi holes analyte concentrator fabricated without these interspaces, produced a system with a superior performance. The addition of several chemicals to link antibodies directed against IgE to the inner surface of the capillary, may have produced a different kind of chemical reaction(s) in the interspace of the capillaries, since they were coated with polyimide. The resulting chemistries may have cause the accumulation of excess amount of material producing a dysfunctional capillary.

The analyte concentrator-reaction chamber offers the potential of solving some of the problems still unresolved in the CE technology. Especially those problems in which limitations exist in reaching practical concentration limits of detection of biologically active analytes present in tissue biopsies, cells, and biological fluids. Nevertheless, some practical considerations in the fabrication of the AC-RC for routine use is still under investigation. For example, the compactness of beads in the small space within the frits, and the high packing tendency of membranes, normally increases back pressure that reduces hydrodynamic flow within the CE capillary. In fact, it is almost impossible to fabricate a cartridge (containing beads) greater than 3 mm in length without affecting reproducibility of separation and increasing the tendency of blocking the system after limited usage. In the case of commercially available membranes coated/impregnated with an appropriate chemistry, a 1 mm in length solid support is tolerated for an optimum AC-RC, higher capacity preconcentrators-containing membranes using a larger bed volume can produce irreproducible data, the possibility of progressive clogging of the system, and a very short life span. However, it is possible that larger surface areas may be unnecessary when using membranes, since suitably impregnated membranes normally have a high binding capacity for analytes.

In conclusion, a new way to pre-concentrate samples found in minute quantities in biological fluids using a novel design of analyte concentrator is presented. We are currently comparing the efficiency and practicality of fabrication of various kinds of analyte concentrators-reaction chambers described in the literature. We are also improving the methodology of fabrication for massive production, facile of use and performance, and repetitive usages of the cartridges. However, the fabrication of an analyte concentrator-reaction chamber for commercial use faces many improvements in the years to come.

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