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Review

Preconcentration and microreaction technology on-line with capillary electrophoresis

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Abstract

The techniques of capillary electrophoresis (CE) are often the methods of choice for the analysis of complex mixtures of chemically similar molecules. Their applicability to studies across many diverse disciplines is quite remarkable. However, poor concentration limits of detection of CE methods often preclude their use for the analysis of dilute analyte mixtures. This limitation has been addressed by the development of analyte concentrator and membrane preconcentration cartridges. These devices are used to insert an appropriate adsorptive phase into the inlet of the CE capillary, to enable the analysis of sample amounts that are in vast excess of the total capillary volume. An ability to perform on-line microreactions is a further advantage afforded by these devices. In this report, we present all attempted approaches at on-line preconcentration and microreaction with analyte separation by CE. We discuss the potential merits of each technique and describe our views of the future developments of analyte concentrator and membrane preconcentration technologies.

Keywords: Reviews; Sample preparation; Preconcentration; Membranes; Derivatization, electrophoresis; Peptides; Enzymes

Contents

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1. Introduction

The techniques of capillary electrophoresis (CE) are widely accepted for the separation of structurally related compounds that originate from many chemically diverse sources. This remarkable versatility coupled with a mechanism of analyte resolution that is complimentary to more traditional techniques, such as high-performance liquid chromatography (HPLC), has led to widespread application of this technology throughout many research and industrial laboratories. Furthermore, recently validated CE methods for analyte quantification will aid the acceptance of these techniques for development of applications pertinent to both clinical chemistry and process control environments [1].

The techniques of CE are characterized by the use of narrow bore, small volume capillaries, typically 10–100 μm I.D. and 20–100 cm in length. Analyte mixtures are injected directly into the separation capillary. This dramatically reduces system dead volumes since there is no requirement for injector valves or column connectors. Consequently, analyte losses to surfaces and/or dead volumes are substantially reduced. Application of a high voltage (5–30 kV) across the CE capillary, which contains a suitable electrolyte or buffer solution, causes analytes to migrate and effects analyte separation. Characteristic of CE is the high separation efficiencies that can be achieved and theoretical plate values of $1–1.5 \times 10^6$ are not uncommon [2].

It is paradoxical that one of the noted advantages of CE, namely the small volume of a conventional CE capillary, also leads to a significant drawback of the technique. The total volume of the capillary is typically only ~1–2 μl, and this results in a very limited loading capacity of analyte solutions. Optimal analyte resolution and separation efficiency are usually obtained when the sample injection is <2% of the total capillary volume [3,4]. Ultimately, this results in poor concentration limits of detection (CLOD) and leads to a major problem when attempting to analyze relatively dilute analyte mixtures, particularly those derived from biological or environmental sources. In attempts to overcome the poor CLOD of CE, a number of workers have developed a series of injection techniques, including analyte stacking, field amplification and transient isotachophoresis (tITP) that facilitate the analysis of

larger sample volumes [5–7]. All of these techniques are implemented as voltage is applied across the CE capillary. This causes analyte zones to be stacked or focused due to variation of ion mobilities in various field strengths or chemical microenvironments [5,8]. As a result, relatively large sample volumes can be analyzed with minimal loss of analyte resolution or separation efficiency. However, since these techniques are carried out within the conventional CE capillary, the maximum sample volume that can be analyzed is predetermined by the total capillary volume. Hence, even in most favorable cases such optimized injection techniques can normally only tolerate the introduction of <1 μl of sample without loss of CE performance. Therefore, while such electrophoretic introduction techniques are a partial solution for enhancing CE CLOD, they have not completely overcome this major limitation. Coupled capillary isotachophoresis (cITP) has been shown to enhance loading capacity of the CE capillary [9]. However, this method appears to be significantly more complicated than conventional CE or tITP methods and may not be well suited to routine operation.

Another approach to circumvent poor CE CLOD is to undertake off-line sample pretreatment and analyte concentration. However, if possible, this should be avoided for dilute analyte solutions since losses to exposed surfaces (e.g., walls of Eppendorf tubes, pipette tips, solid extraction phases, etc.) can be substantial. Furthermore, excessive handing of a concentrated solution of biopolymers can lead to denaturation, aggregation, precipitation, and, ultimately, poor analyte recovery. Therefore, minimal sample handling is advisable. This can be achieved using an analyte concentrator [3,4,9–33] or membrane preconcentration cartridge [34–40] on-line with the CE capillary. These devices usually consist of an adsorptive phase at the inlet of the CE capillary and serve to enrich trace levels of analytes, as well as allow on-line sample cleanup prior to component separation by CE. Injection of sample volumes in excess of 100 μl into a CE capillary of <300 nl total volume have been recently reported [40].

The concept of on-line preconcentration with CE was originally conceived by Guzman [10–12]. In preliminary studies, the coupling of an antibody to a solid support for the on-line extraction of analytes from urine and subsequent on-line separation by CE

Table 1
Summary of on-line preconcentration and microreaction chambers with capillary electrophoresis

Method of analyte preconcentration or microreaction	Detector	Class of analyte	Reference
A. Nonspecific			
1. ODS coated open tubular capillary	UV	herbicides	[3]
2. HPLC solid-phase packing	UV	small molecules peptides/proteins	[14–21,24] [25,29,31]
3. HPLC solid-phase packing	On-line MS	small molecules peptides/proteins	[21,23,35] [46]
4. Meltable agarose	UV	macromolecules	[28]
5. Impregnated membrane	UV	small molecules peptides	[36]
6. Impregnated membrane	On-line MS	small molecules peptides/proteins	[35,37–40]
B. Specific			
1. cITP	UV	protein	[9]
2. Capillary bundles	UV/off-line MS	metabolites in human urine	[10]
3. Analyte concentrator with antibodies	UV/offline MS	metabolites in human urine	[10]
4. Protein G immunoaffinity	UV	protein	[30,33]
5. Affinity open tubular capillary	UV	protein	[4]
C. Enzyme Digestion and Chemical Modification			
1. Trypsin-open tubular capillary	LIF	protein–peptide digestion	[55,56]
2. S. aureus V8-analyte concentration plus on-line FITC derivatization	UV	protein–peptide digestion	[32]
3. Solid-phase reaction	UV/LIF	amino acids	[25]
4. Trypsin-open tubular microreactor	On-line MS	protein-peptides	[57]

was described [10]. Guzman also described the more general concept of non-specific adsorption of analytes onto an appropriate solid-phase [11–13], as well as on-line enzymatic protein digestion [41–44]. Following these studies, a number of variations, improvements and novel ideas have been described (see [45] for a recent review). All of these approaches, including the development of the membrane preconcentration cartridge by Tomlinson and Naylor [34–40], are summarized in Table 1 and discussed in detail below.

2. On-line non-specific analyte preconcentration–CE

2.1. Solid-phase preconcentration–CE (spPC–CE)

Subsequent to the demonstration of the concept of analyte preconcentration on-line with CE by Guzman et al. [10] and with cITP by Kasicka and Prusík [9],

Cai and El Rassi developed an open tubular preconcentrator for use on-line with free solution CE (CZE) [3]. However, while this approach enabled the analysis of larger sample volumes than conventional CZE, the precapillary was easily saturated. This was demonstrated by the fact that analyte signal was not significantly increased as sample introduction was extended beyond a critical injection time. Therefore, a subsequent and logical progression of this work was the insertion of a small bed of an appropriate adsorptive solid-phase at the inlet of the CE capillary [14,16,18–23,29,31,46] (see Table 1).

Several groups have demonstrated the introduction of increased sample volumes into the CE capillary for a variety of analyte types by using spPC–CE [16,18,20,29,31]. However, while this technique significantly enhances CLOD, it is our experience that CE performance is also frequently compromised [19,23,35,38,39]. The use of spPC–CE often results in reduced analyte resolution, broader peaks and substantial component tailing. These observations

have been attributed, at least in part, to increased analyte–analyte and analyte–wall interactions that can occur in the CE capillary when analyzing concentrated analyte solutions. Furthermore, analysis time are typically longer for spPC–CE applications than are observed for CE [47]. The increased back pressure induced in the CE capillary by the solid-phase and frit material (used to prevent solid particles from entering and blocking the CE capillary) leads to a reduced hydrodynamic flow and the potential for impaired ion mobility during electrophoresis. This causes an anomalous endoosmotic flow (EOF) and irreproducible analyte migration times. In addition, our studies of spPC–CE led us to conclude that the relatively large volume of organic phase required to efficiently remove analytes from the adsorptive material also tends to reduce EOF and compromise CE performance [48,49]. We have also reported [35,38] that the latter phenomenon can be overcome by reducing the volume of organic elution buffer. However, this is counter balanced by a need to use an adequate volume of this solvent for complete recovery of adsorbed analytes from the relatively large bed of solid-phase.

Various attempts have been made to overcome these limitations. Reduction of the size of the solid-phase bed or completely removing the adsorptive phase from the CE capillary during electrophoresis by means of a switching valve have both been reported [14,35]. However, each of these approaches are not without limitations. The former is difficult to achieve due to problems associated with reproducible construction of small bed volume spPC–CE capillaries. Equally, the latter approach may require substantial modification of commercially available CE instrumentation, thereby prohibiting its use by many investigators for analyte preconcentration on-line with CE. A recent and novel approach to on-line spPC–CE was the use of a meltable solid-phase (e.g. agarose gel) [28]. In this method, the solid-phase is effectively removed from the system after analyte isolation by raising the temperature of the CE capillary above its melting point. Analytes are resolved from the molten adsorptive phase during electrophoretic separation. This latter approach is appealing since removal of the solid-phase from the capillary prior to electrophoresis overcomes all of the limitations of spPC–CE. However, it is currently

unclear whether a vast excess of a molten solid-phase will adversely affect the CE separation of complex analyte mixtures.

2.2. Membrane preconcentration–CE (mPC–CE)

The development of mPC–CE was undertaken to decrease or remove all of the limitations observed in studies of spPC–CE [34–39]. Using a suitably coated/impregnated membrane it is possible to minimize the bed volume of adsorptive phase at the inlet of the preconcentration capillary. Ultimately, this modification leads to a more efficient removal of analytes from the adsorptive phase in a reduced volume of organic elution solvent. This, coupled with an increased hydrodynamic flow through the mPC–CE capillary and reduced ion impedance during electrophoresis, ensures a more reproducible EOF. Furthermore, the high adsorptive capacity of impregnated membranes permits the analysis of large volumes (>100 μl) of dilute sample solutions without compromising either analyte resolution or separation efficiency afforded by conventional CE methods [38,40].

The membrane is installed in a cartridge that is usually prepared from Teflon tubing as shown in Fig. 1. Fused-silica and metal (stainless steel or titanium) tubing has also been used to prepare mPC–CE cartridges. This design conveniently permits easy disassembly of the mPC–CE capillary to allow cleaning/conditioning of the CE capillary, as well as rapid off-line activation of the adsorptive membrane. Furthermore, the mPC–CE cartridge allows sample to be loaded prior to final assembly of the mPC–CE capillary. This is advantageous when using capillaries of small internal diameter, typical of those used for obtaining high sensitivity on-line CE-mass spectrometry (MS) applications [50]. For example, the flow-rate of an aqueous separation buffer can be as low as ~100 nl/min in a 25 μm I.D. mPC–CE capillary. This is a result of the back pressure induced by the CE capillary and also a limited injection pressure supply on some commercially available CE instruments. Consequently on-line loading of microliter volumes of sample in such capillaries can often take several hours. Clearly, this is a sizable inconvenience and an obvious limitation of mPC–CE. However, mPC cartridges can withstand
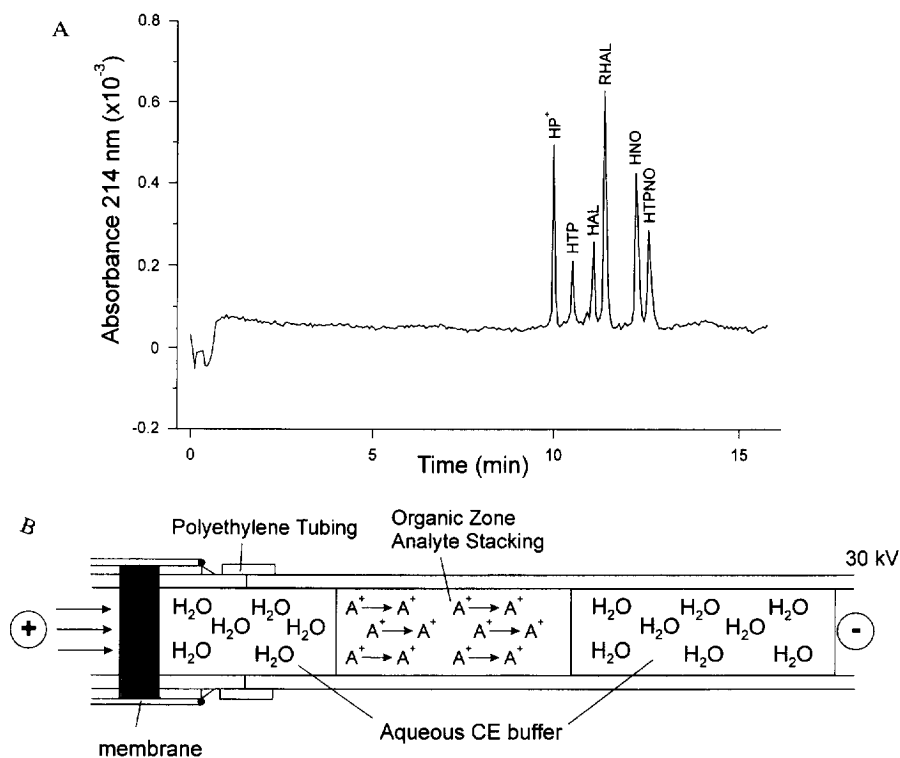
Fig. 1. Schematic of on-line mPC–CE–MS using a Finnigan MAT 900 mass spectrometer. Insert shows details of the mPC–CE cartridge.

relatively high pressures (~60 psi). In addition, since system back pressure is reduced when the cartridge is removed from the CE capillary, higher solvent/sample flow-rates through the cartridge can be achieved. As a result, up to 100 μl of an analyte solution can often be loaded on to a mPC cartridge in <5 minutes prior to final assembly of the mPC–CE capillary, and this significantly reduces analysis times [40].

In addition to analyte preconcentration, mPC–CE technology can also be used to effect sample cleanup. This is particularly important for physiologically derived samples such as blood, bile, urine, etc., where the presence of high salt concentrations can dramatically effect analyte separations by CE. Furthermore, these matrix components can complicate, and even degrade electrophoretic stacking and focusing procedures [51], often precluding the use of these methods for preconcentration of biologically derived samples within the CE capillary. In contrast, mPC–CE technology is relatively unaffected by such contaminants. Indeed, this approach ensures that these compounds are removed from the CE capillary

prior to electrophoresis. Furthermore, when using an off-line sample loading strategy, the bidirectional flow through a mPC cartridge allows samples to be loaded with either reverse or forward flow. We utilize a back flow to load sample followed by subsequent sample cleanup with a forward flow of a suitable solvent (typically an aqueous medium) leading to flushing of sample-derived particulates from the mPC cartridge prior to its installation onto the CE capillary. This improves the reproducibility of mPC–CE performance by reducing the tendency for clogging the system and adversely affecting EOF.

While the use of a coated/impregnated membrane effectively minimizes the bed volume of adsorptive phase, the high loading capacity of these materials ensures efficient trapping of analytes from dilute solutions. This is demonstrated in Fig. 2A by the mPC–CE analysis of a series of five analogs of the neuroleptic agent Haloperidol (HAL). In this example, all analytes were dissolved in methanol, then diluted with CE separation buffer (50 mM NH_4OAc–1% acetic acid–10% methanol) to yield a final concentration of 3.3 ng/ml of each compound.

Fig. 2. (A) mPC–CE electropherogram with analyte stacking of a mixture of HAL and five analogs (see [47] for structures). Separation was performed on a Beckman P/ACE 2100 instrument using a purpose built mPC–CE capillary (50 μm I.D.×67 cm, 60 cm to a UV detector). A 55 μl volume of a sample containing ~3.3 ng/ml of each compound was loaded in separation buffer by pressure $13.7 \cdot 10^8$ Pa (20 psi) injection. Analytes were eluted from the SDB membrane using ~50 nl of a solution consisting of acetonitrile–methanol (50:50 v/v). Separation was effected in 50 mM NH$_4$OAc–10% methanol–1% acetic acid in water at a voltage of 30 kV (25 μA). The capillary was at 25°C and analyte detection was by UV at 214 nm. (B) A schematic representation of analyte stacking as used in conjunction with mPC–CE for the analysis of small organic molecules.

A 55 μl aliquot of this solution was applied to a mPC–CE cartridge on-line with a 50 μm I.D.×67 cm uncoated fused-silica CE capillary. The mPC–CE capillary was next washed with CE separation buffer prior to elution of the analytes from the membrane with a solution containing acetonitrile–methanol (50:50 v/v). Analyte separation was effected by electrophoresis at 30 kV with component detection by UV at 214 nm. In this example, all components were baseline resolved. Furthermore, the sample volume analyzed was at least three orders of magnitude greater than could have been injected and separated by conventional CZE.

This example demonstrates the enhanced sample volume that can be loaded into a CE capillary by mPC–CE technology. However, significant volumes

(typically 50–200 nl) of elution solvent are often required to ensure complete recovery of analytes from the membrane. Such large volumes can lead to some compromised CE performance. This is avoided by the selection of post elution conditions that induce electrophoretic stacking/focusing as voltage is applied across the capillary. In the HAL example, elution of the synthetic HAL analogs in a mixture of acetonitrile–methanol (50:50 v/v), as shown schematically in Fig. 2B, causes analyte stacking to occur upon electrophoresis. This ensures optimal analyte resolution and conventional CE performance.

The technique of mPC–CE has also been effectively applied to the analysis of dilute mixtures of peptides [35–40]. However, unlike the strategy developed for small organic molecules, a require-

ment of the elution solvent used for this application is that it contains at least some water [e.g. methanol–water (80:20 v/v)] to ensure the efficient removal of peptides from the membrane. Furthermore, optimal peptide recovery is achieved by use of a significant volume (>50 nl) of this solvent [35,38]. The relatively large volumes of such solvents and the inefficient analyte stacking that it induces can result in loss of analyte resolution and a reduced separation efficiency [35]. However, we have shown that mPC–CE performance can be enhanced by the inducing moving boundary tITP conditions after peptides are eluted from the adsorptive membrane [35,37–39]. For this application, tITP conditions are used to focus analyte zones and aid the dispersion of the elution solvent within the mPC–CE capillary. These conditions are effected by elution of the peptides between zones of a leading stacking buffer (LSB), typically 0.1–5% NH_4OH in water and a trailing stacking buffer (TSB), typically 1% acetic acid in water or more commonly CE separation buffer. This is shown schematically in Fig. 3B. The effectiveness of this approach is dependent on the volume and concentration of LSB and TSB and the volume of elution solvent used to remove peptides from the mPC cartridge, and this is described in detail elsewhere [40].

The use of mPC–CE in conjunction with tITP for the analysis of peptides is shown in Fig. 3. In this example, the outlet of the mPC–CE capillary was inserted into an electrospray source of a MS as shown schematically in Fig. 1B. Additional details of such interfacing of CE with MS are reported elsewhere [46]. Here, 5 μl of a nine component peptide mixture containing angiotensin II, bombesin, bradykinin, luteinizing hormone-releasing hormone, α-melanocyte-stimulating hormone, leucine-enkephalin, methionine-enkephalin, oxytocin and thyrotropin-releasing hormone (TRH), all at a concentration of 50 pg/μl, was loaded onto a styrene divinylbenzene (SDB) mPC–CE cartridge prior to assembly of the mPC–CE capillary and subjected to analysis by mPC–tITP–CE–MS. This method used an LSB of 1% NH_4OH in water (~60 nl), an elution solvent of (80:20 v/v) MeOH–H_2O (~60 nl). TSB was the CE separation buffer, 2 mM NH_4OAc–1% acetic acid in water (~90 nl). Baseline separation of all detected peptides resulted and demonstrated the

minimal effect of the mPC–CE cartridge on CE–MS performance. The tripeptide TRH was not detected in this analysis. This has been attributed, at least in part, to its loss to the wall of the uncoated mPC–CE capillary. Subsequent analysis of the same nine component peptide mixture using a polybrene coated mPC–CE capillary facilitated the detection of all nine peptides (results not shown).

The previous example demonstrates that in addition to its compatibility with UV detection, mPC–CE is readily coupled to a MS. Furthermore, Tomlinson and Naylor have demonstrated that use of the mPC–CE in conjunction with analyte stacking or tITP does not compromise either CE or CE–MS performance [37–39]. These investigators have also demonstrated the wide applicability of this technology, including the analysis of small organic molecules (e.g. drug metabolites) [35], peptides [36–40] and proteins [35]. Furthermore, preliminary reports from this group describing the application of mPC–CE–MS to clinically relevant studies clearly demonstrates the future potential of this technology for the analysis of widely diverse analyte mixtures. For example, endogenous components in several body fluids, including aqueous humor [52], cerebrospinal fluid [53], blood and brain dialysates [53], tears [52] and urine [35], have been analyzed by mPC–CE–MS.

3. On-line analyte specific preconcentration–CE

Analyte concentrators that contain covalently bound antibodies are appropriate for use with CE for those applications that warrant detection of a specific analyte. The concept for such devices was first demonstrated by Kasicka and Prusík [9] in conjunction with cITP and subsequently described for CZE by Guzman [10–13] and Kennedy [28]. In these studies, specific antibodies were covalently bound to either a solid-phase [9], glass beads [11,12], multiple capillary bundles or, more recently, a piece of solid glass predrilled with a laser beam [33].

A typical immunoaffinity analyte concentrator constructed from multiple capillary bundles is shown schematically in Fig. 4. The performance of such a device of this construction was recently compared to that of a similar concentrator made from a solid piece of glass with through holes. In these studies,
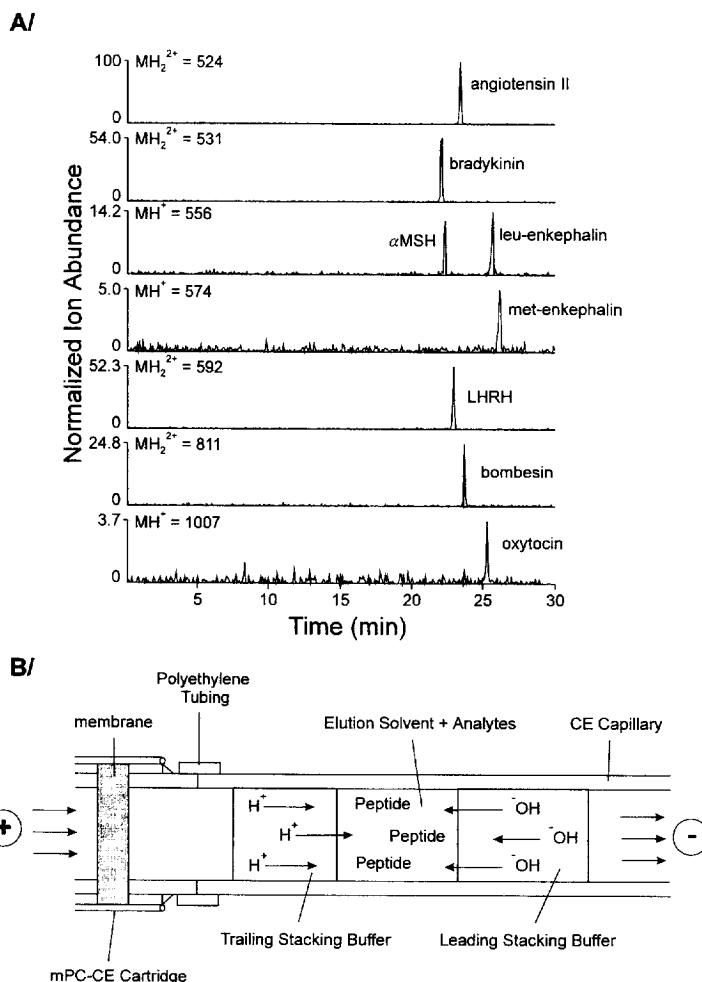
Fig. 3. (A) Ion electropherograms of a mPC–CE–MS analysis of a nine component peptide mixture using a Beckman P/ACE 2100 CE connected to a Finnigan MAT 95Q mass spectrometer. Separation buffer was 2 mM ammonium acetate–1% acetic acid (pH 2.9). Capillary dimensions were 25 μm I.D.×74 cm uncoated fused-silica. A 5 μl volume of sample (50 pg/μl) was loaded onto a mPC-cartridge off-line and washed off-line with ~10 μl of separation buffer. CE separation voltage was 25 kV (~1.3 μA). LSB was 1% ammonium hydroxide (~60 nl), elution solvent was methanol–water (80:20 v/v, 60 nl) and TSB was separation buffer (90 nl). A sheath liquid of isopropanol–water–acetic acid (60:40:1 v/v/v) was used at a flow-rate of 3 μl/min. Electrospray voltage was 3.6 kV referenced against an accelerating voltage of 4.7 kV. Scan range was 300–1300 ml. mol. mass at 2 s/d and instrument resolution was ~1000. (B) A schematic representation of the moving boundary tITP conditions as used for peptide analysis by mPC–CE–tITP–MS.

both immunoaffinity concentrators were used to determine immunoglobulin E (IgE) in serum by CE [33]. Results of these investigations indicated a broadness of the peak response for IgE when analyzed using the concentrator made from multiple capillary bundles on-line with CE (Fig. 5A). Furthermore, a second minor response was detected using this approach. A significant variability of analyte migration time was also observed. It was concluded

that such variability of performance was due, at least in part, to a reduction of EOF. This was observed to be progressive and suggested to be caused by partial blocking of the cartridge through sequential analysis of serum samples. In contrast, the immunoaffinity analyte concentrator made from a single piece of glass with through holes yielded only a single peak (see Fig. 5B). In addition, the migration of IgE in this system was substantially faster than was ob-
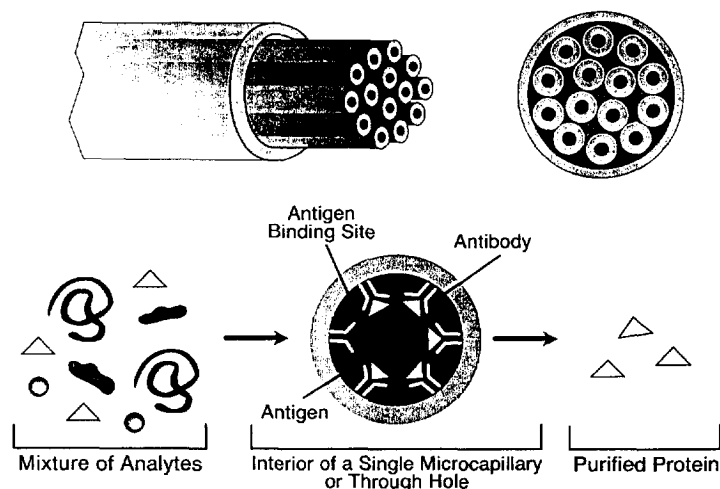
Fig. 4. Schematic of an analyte concentrator containing multiple capillaries. (A) A plan view of a portion of the analyte concentrator fabricated with 25 μm I.D., 150 μm O.D. capillaries inserted into a sleeve connector for coupling to a capillary of 75 μm I.D., 365 μm O.D., 65 cm to the cathode, 7 cm to the anode. (B) A sectional view of the same analyte concentrator cartridge depicted on (A) and (C) a representation of the immunochemistry that is conducted within such an analyte concentrator. (Reprinted from [33] by courtesy of Marcel Dekker).

served using the analyte concentrator made from multiple capillary bundles (Fig. 5A). Furthermore, peak profile was improved and IgE migration was more consistent using the single piece immunoaffinity analyte concentrator.

The major response from both of these studies was collected from the CE capillary, using a purpose built fraction collector [13,54]. The fractions collected from several consecutive injections were pooled and shown to be IgE by biological assay.
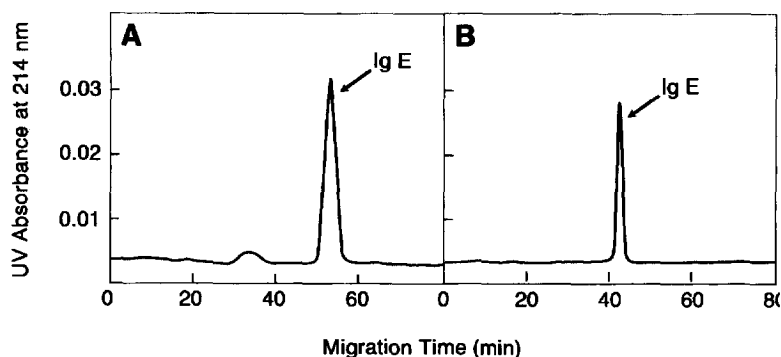

Fig. 5. Immunoaffinity microreactor electropherograms of IgE. Purified anti-IgE antibodies were covalently bound to the walls of several capillaries of 27 μm I.D. and 150 μm O.D. assembled in bundles, or to a solid glass rod containing several small diameter passages of comparable surface area (approximately 25 μm I.D.). A microreactor chamber (5 mm in length) was fabricated using one of these two assemblies or cartridges. Every independent cartridge was connected, through sleeve connectors sealed with epoxy resin, to the capillary column for affinity capillary electrophoresis. Approximately 20 μl of serum containing high titers of IgE were injected by pressure into the capillary column followed by a cleanup procedure consisting of a wash buffer to remove salts and other serum constituents. The bound IgE were then eluted by pressure using an optimized buffer system, and finally capillary electrophoresis was performed. A typical electropherogram was obtained using the cartridge fabricated of multiple capillaries associated in bundles (panel A), or using the cartridge fabricated from a solid glass rod having a plurality of small diameter rod passages or through holes (panel B).

These examples demonstrate the high specificity of the immunoaffinity analyte concentrator, as it is remarkable that IgE was isolated from serum with no detectable presence of human serum albumin or other immunoglobulins such as IgA, IgG or IgM.

Affinity interactions of proteins with a variety of substrates (other than antigen–antibody complexation) have yet to be fully exploited for on-line analyte preconcentration-CE. An early demonstration of such process was reported by Cai and El Rassi [4]. In these studies, an open tubular precapillary was modified with a surface-bound metal chelating group (e.g., iminodiacetic acid Zn II complexes) and coupled on-line with a CE capillary. This tandem capillary arrangement was used to isolate, preconcentrate, and analyze proteins that exhibited affinity for the chelated metal. Therefore, while it was found in this and previous studies [3] that the open tubular precapillary was easily saturated, these results provide motivation for further investigation of on-line preconcentration–CE using a variety of analyte–substrate affinity interactions.

4. On-line analyte microreactor concentrators with CE

An attractive feature of microreactions (either chemical or enzymatic) prepared from a solid support on-line with CE is the potential for enhanced efficiency of these processes. This is often accompanied by shorter reaction times, consumption of smaller amounts of reagents and, perhaps most importantly, the ability to react, derivatize, or digest lower analyte concentrations than possible by conventional solution chemistries. Amankwa and Kuhr [55,56] have described the use of a trypsin modified open tubular precapillary for enzymatic digestion of proteins. These investigators have demonstrated that the use of a 50 cm length of this enzyme-modified capillary (off-line from the CE) affords complete digestion of ~100 pmols of β-casein (flowing at a rate of ~40 nl/min) in about 25 min at room temperature. Subsequently, such precapillaries have been coupled on-line with CE via a fluid joint connector [56] and have been used with UV [56] and MS [57] detection to demonstrate the efficacy of this

approach for enzymatic protein digestion on-line with CE and CE–MS.

Recently, an improvement to on-line protein digestion methodology was the construction of an enzyme modified analyte concentrator as described by Guzman [32]. The analyte microreactor concentrator used in these studies was constructed by covalently linking Staphylococcus aureus V8 protease to porous glass solid support enclosed in a chamber defined by glass frits. Specific digestion of the α-subunit of prolyl-4-hydroxylase was demonstrated by comparison of the electropherograms generated by interacting the α-subunit in analyte microreactor concentrators containing covalently linked cytochrome C, bovine serum albumin or S. aureus V8. Proteolytic digestion was only observed when the subunit was reacted in the microreactor containing the covalently bound S. aureus V8 protease (Fig. 6A). From these results, peak area measurements were used to determine the efficiency of this digestion process, which was found to be >70% in 10 min at 30°C.

A further refinement of the on-line proteolytic digestion microreactor was also recently described by Guzman [32]. In this strategy, a second analyte concentrator microreactor is coupled on-line with the first proteolytic microreactor and the CE capillary. This second reactor contains glass beads modified with fluorescein isothiocyanate (FITC), linked to immobilized anti-FITC antibodies. The purpose of this refined approach is to chemically derivatize the peptides produced by on-line protein digestion to increase their UV and fluorescence absorbance characteristics. This further alleviates the poor CLOD of conventional peptide analysis by CE through enhanced peptide detection capabilities. The two reactor system is demonstrated by the on-line digestion of the α-subunit of prolyl-4-hydroxylase followed by consecutive FITC derivatization and ultimately CE separation of generated peptides (see Fig. 6B). In this example, the higher UV absorbance of FITC derivatized peptides was clearly observed. Furthermore, the on-line generation of FITC-labeled peptides has aided component resolution when compared to the electropherogram obtained from the analysis of the on-line generated but underivatized peptides (Fig. 6A).
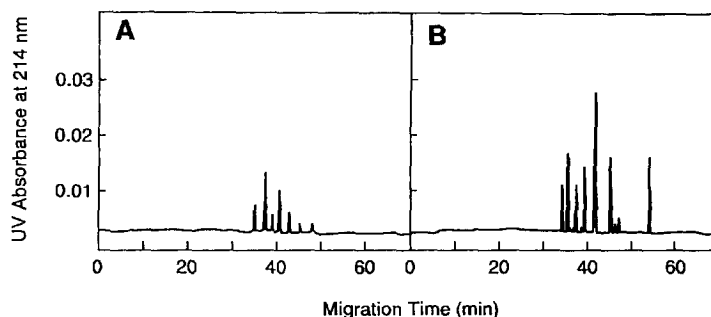
The latter example is one attempt at trying to

Fig. 6. Tandem enzyme digestion and fluorescent tagging microreactor electropherograms of peptide mapping of prolyl 4-hydroxylase-subunit and derivatization of the resulting peptides. Comparison of the electropherograms of the two peptide mappings generated on-column: (A) without derivatization and (B) with FITC derivatization. For this experiment, two microreactors placed in tandem were used. The first microreactor contained *S. aureus* V8 protease immobilized to controlled-porous glass, where protein digestion occurs. The second microreactor contained antibodies (directed against FITC) immobilized to controlled-porous glass, and holding specifically FITC molecules through antigen–antibody bondings. In this microreactor derivatization of the generated peptides of the α-subunits of prolyl 4-hydroxylase to form FITC–peptides occurs.

overcome the inherently poor sensitivity of CE–UV detectors. The on-line generation of fluorescently tagged peptides enhances UV absorbance and, if appropriate, enables analyte detection by a more sensitive LIF detector.

5. Conclusions

In summary, while the techniques of analyte concentration and membrane preconcentration continue to be refined [45], it is clear that these techniques can overcome the current limitations of CE CLOD. Furthermore, the demonstrated coupling of mPC–CE to MS is directly applicable to studies that require the structural characterization of analytes that are extracted from sample matrices in low concentration. In addition, preliminary results from the analysis of several biologically derived fluids (including cerebral spinal fluid, blood and brain dialysates, aqueous humor and tears) have indicated a promising potential for the development of mPC–CE(MS) assays of direct relevance to clinical chemistry. Ultimately, validated, quantitative mPC–CE(MS) methods will also aid the acceptance of this technology for use in the clinical environment or other industrial applications (e.g., the registration of pharmaceuticals or other products/foodstuffs). Additionally, the technologies of analyte concentration and membrane preconcentration afford practitioners

of CE and CE–MS a means of specific analyte extraction and cleanup prior to detection/study of analytes of interest. This reduces the need for exhaustive off-line sample preparation prior to analysis by CE or CE–MS, which tends to increase the efficiency of analyte recovery and also reduce human exposure to previously unidentified materials of unknown toxicity. Finally, the use of these devices as microreactors affords enhanced chemical derivatization or enzymatic reactions at lower analyte concentrations than is currently possible by conventional solution chemistries.

6. List of abbreviations

CE	Capillary electrophoresis
CLOD	Concentration limits of detection
tITP	Transient isotachophoresis
cITP	Coupled capillary transient isotachophoresis
spPC–CE	Solid-phase preconcentration–CE
CZE	Free solution-CE
EOF	Endoosmotic flow
FITC	Fluorescein isothiocyanate
HAL	Haloperidol
HPLC	High-performance liquid chromatography
LIF	Laser induced fluorescence
LSB	Leading stacking buffer

mPC–CE   Membrane preconcentration–CE
MS   Mass spectrometry
SDB   Styrene divinylbenzene
TSB   Trailing stacking buffer

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