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Review

Sample enrichment techniques in capillary electrophoresis: Focus on peptides and proteins☆

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Abstract

Compared to chromatography-based techniques, the concentration limits of detection (CLOD) associated with capillary electrophoresis are worse, and these have largely precluded their use in many practical applications. To overcome this limitation, researchers from various disciplines have exerted tremendous efforts toward developing strategies for increasing the concentration sensitivities of capillary electrophoresis (CE) systems, via the so-called sample enrichment techniques. This review highlights selected developments and advances in this area as applied to the analyses of proteins and peptides in the last 5 years.

Keywords: Sample enrichment; Focusing; Concentration sensitivity; Proteins; Peptides

Contents

1. Introduction

Capillary electrophoresis (CE), in the conventional capillary or microdevice format, is widely considered as one of the most important tools of separation science in the last two decades. Because it operates using a distinct separation mechanism (based on differences in the analytes' charge-to-size ratios), it can serve as an alternative method for samples that are not easily resolved by traditional chromatographic techniques. Several separation modes (capillary zone electrophoresis [CZE], micellar electrokinetic chromatography [MEKC], capillary isoelectric focusing [CIEF], and capillary gel electrophoresis [CGE]) are available, enabling fast and highly efficient analyses of both charged and neutral species.

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Although excellent mass limits of detection have been reported, the concentration limits of detection (CLODs) in CE, under nonstacking or continuous buffer conditions, are generally acknowledged to be worse (by two orders of magnitude) than those obtained with chromatography-based techniques, thereby limiting its use for many practical applications. To overcome this limitation, improvements in the detector side, including the use of more sensitive detection schemes (mass spectrometry [MS], laser-induced fluorescence, conductivity, etc.) and modifications in capillary geometry and design (e.g., use of bubble-cell capillary) have been explored. To date, however, more emphasis has been given to increasing sample loading. The so-called sample enrichment (on-line preconcentration, focusing) techniques enable introduction of volumes of sample into the capillary beyond the usual 1–2% of the total capillary volume. By some electrophoretic or chromatographic mechanism, a large volume of dilute sample is reduced and becomes concentrated; hence, resolution and efficiency are preserved.

The present review highlights selected advances in sample enrichment techniques as applied to CE analyses of proteins and peptides in the last 5 years. Some comprehensive reviews on related topics published recently are listed as Refs. [1–20]. Although we have attempted to provide a brief description of the mechanism involved in each technique, mechanistic details are reviewed elsewhere [1–3,5,8,9,13].

2. Electrophoretic preconcentration

Generally, in electrophoretic preconcentration techniques, the capillary is partitioned into discrete sections delineated by a discrepancy in some property (e.g., conductivity of solution, pH), or by a physical boundary (e.g., a porous membrane). By appropriate choice of analytical conditions, the analyte can be made to speed up or slow down in the different sections of the capillary, enabling compression of long sample bands into narrow, concentrated zones. They are very practical to use, as no modification in existing instrument configuration is required; however, they are largely limited by the volume of the separation capillary used.

2.1. Stacking

The term "stacking" in CE refers to a broad range of techniques which effect the concentration of a relatively large plug of dilute sample into a smaller volume prior to detection. The decrease in peak width of an analyte translates to an increase in its peak height, thereby increasing the signal-to-noise ratio and improving detectability. Generally, stacking exploits a discontinuity in electrolyte systems, i.e., a difference between the sample matrix and the background solution (BGS) in terms of some physico-chemical property (e.g., conductivity [21–23], pH [24–27], additive [28]), in order to bring about a change in the migration velocity of an analyte as it transits the boundary between these two regions. The sample band is narrowed because either the front end of the plug migrates more slowly than the rear, or the rear migrates more quickly than the front. The increase in signal output may vary from a modest few folds to astounding million-fold [29] through the combination of techniques.

2.1.1. Conductivity difference-dependent stacking

Mikkers et al. [30] originally advanced the idea of injecting samples in a low-conductivity matrix. This was later taken up by Chien and Burgi [21,22] who developed several stacking strategies based on the use of low-conductivity sample matrix and high-conductivity BGS, which they referred to as "field-amplified sample stacking" (FASS). Over the years, several other techniques (normal stacking, large-volume sample stacking [LVSS], field-enhanced sample injection [FESI], head column sample stacking) utilizing the same concept were developed, these varying in terms of injection and electrode polarity configuration schemes. These strategies are based on the principle that the velocity of an ion is the product of its mobility and the electric field strength. Upon application of voltage, an enhanced electric field is established in the low-conductivity sample zone, while a large drop is experienced in the BGS zone across the rest of the capillary. This causes the ions to migrate quickly in the sample zone, then slow down drastically and stack upon reaching the sample—BGS boundary, resulting in band narrowing. The sample may be injected into the capillary hydrodynamically (e.g., in normal stacking, LVSS), in which case the zone sharpening effect takes place when the separation voltage is applied; or electrokinetically (e.g., in FESI, head column sample stacking), in which case zone sharpening occurs during the injection process itself. While this kind of stacking remains one of the most popular because of the ease with which it can be implemented (only dilution of the sample is required), it is seldom used for analytes present in biological matrices because of its incompatibility with salts.

Chun et al. [31] reported an enhancement factor of more than 100-fold using LVSS. A coated capillary was almost completely filled with dilute protein solutions, and then the electroosmotic pump was used to back out the sample plug toward the injection side, while stacking proceeded at the interface between the sample plug and the BGS. Similarly, Siri et al. [32] used LVSS, together with laser-induced fluorescence detection, for the analysis of peptides at picomolar concentration. Locke and Figeys [33] employed FESI for optimization of proteomic processes and evaluation of protocols used for protein analyses. Monton and Terabe [34] used the same, in this case coupling CE with MS for detection, to amplify the signals of peptides in low-concentration tryptic digests, and then used MS or MS/MS information on these peptides for identification of the original protein. More recently, Law et al. [35] combined FESI with the use of a bubble-cell capillary. With this additional boost via extension of path length, as much as 26,000-fold enhancement in signal for some proteins was obtained.

2.1.2. pH-mediated stacking

pH has been used to modify migration behavior of analytes within the capillary both indirectly and directly. To trigger FASS-like stacking in high salt-containing samples, an interesting technique was developed by Schwer and Lottspeich [25], who sandwiched a solution of peptides between plugs of highly

acidic and highly basic solutions. When an electrical potential was applied, the H^+ and OH^- ions migrated toward each, converting the original sample zone into a low-conductivity zone through which the analytes could migrate quickly.

In CZE format, the concept of stacking as a consequence of the changes in the proteins' and peptides' ionization states as a function of pH was advanced by Aebersold and Morrison [24], and as much as 200-fold improvement in concentration sensitivity was shown. Focusing was initiated at the junction between the sample and the BGS zones when anionic peptides in the basic sample zone entered the acidic milieu of the BGS; hence, they experienced a reversal of charge, and consequently, migration direction. Neusüß et al. [36] employed the technique for the analysis of peptides in the low femtomole range by CE–MS. Despite the use of a sheathflow interface which tended to degrade sensitivity because of dilution, the calculated CLODs were comparable to published values using sheathless couplings. Wang et al. [37] reported a modification, whereby moderation of migration velocities of a few proteins as they entered a relatively more basic BGS was utilized for stacking, in lieu of complete charge reversal. More recently, Monton et al. [38] reported an analogous strategy under reversed conditions, i.e., an acidic sample matrix and a basic BGS (Fig. 1). More than 100-fold improvement in detector response for some peptides was obtained, and the efficiency of focusing was shown to be related to the net change in the peptides' effective charges between the sample and BGS zones, the greater the change the better the focusing. Nesbitt et al. [39] isoelectrically trapped proteins at the interface of two buffers with pH values that were higher or lower than the p*I* of the protein. By appropriate choice of buffers (i.e., those that provide significant buffering capacities in the acidic and basic regions, but little or no buffering capacity at the protein's p*I*), the pH junction could be sustained for a longer time, enabling larger sample loading and an increase in signal output of more than 1700-fold.

The success of pH-mediated techniques depends largely on the proper combination of buffers, which can prove to be complicated in some cases. To preclude this, Wei et al. [40] developed an interesting strategy to generate a pH gradient in a single-buffer system, which involved the insertion of a platinum wire into the capillary. When voltage was applied, electrolysis of water resulted in a sharp pH gradient which propagated across the capillary, and resulted in concentration of large volumes of injected analytes due to change in their charges.

2.1.3. Isotachophoresis

In isotachophoretic (ITP) stacking, the sample is sandwiched in between BGSs of higher (leading electrolyte, LE) and lower (terminating electrolyte, TE) electrophoretic mobilities. It is particularly attractive for salt-containing samples because the components of the matrix itself can be used as leaders (e.g., naturally occurring chloride and sodium in biological samples can act as leaders for anionic and cationic analyses, respectively). On application of voltage, a potential gradient is established over the sample and electrolyte zones, with each zone experiencing a field strength that is inversely proportional to the mobility of the ion in that zone, so they migrate with the same velocity (iso-tacho). At steady state, each analyte moves as a discrete band, with high-mobility bands migrating ahead of low-mobility bands.

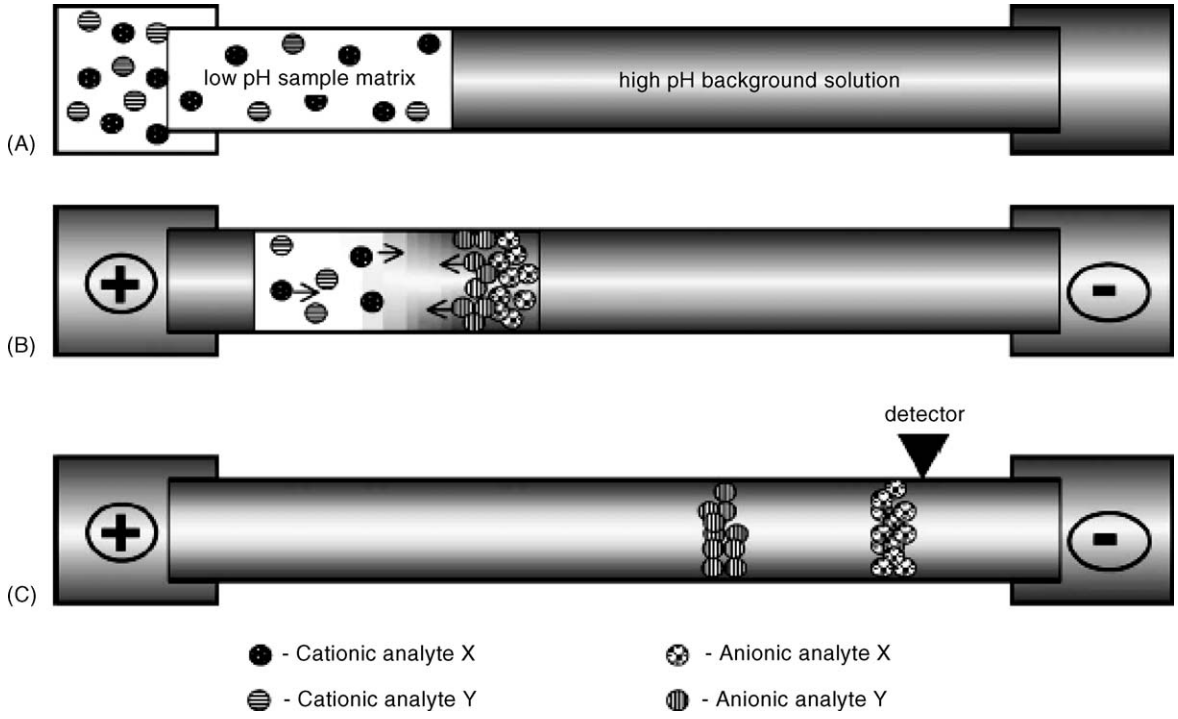

Fig. 1. A dynamic pH junction model for peptides and proteins. (A) A long plug of sample in a low pH matrix is injected into an uncoated capillary filled with a high pH BGS. (B) A steep pH boundary develops at the front end of the plug and sweeps throughout the sample zone during electrophoresis, converting the cationic analyte into anionic and significantly retarding its migration velocity. (C) The focused peak migrates to the detector. Reprinted from [38] with permission from Elsevier.

ITP may be performed in one of two modes. The first is a two-capillary approach known as coupled-capillary ITP (CITP). ITP preconcentration takes place in the first, large-internal diameter capillary permitting introduction of large volumes of sample. Then, the focused zones are directed into the second capillary for separation. Kvasnička [41] developed a simple, CITP-based method for determining lysozyme and for detecting undeclared egg residues in food products. Ölvecká et al. [42] exported this column-coupling configuration to the microchip format, resulting in sub-μg/mL detection limits for some proteins using conductivity detection. Huang et al. [43] integrated ITP with gel electrophoretic (GE) separation on a glass microchip. The CLODs of some sodium dodecyl sulfate (SDS)-proteins were improved by approximately 40-fold compared to GE mode only.

Aside from high stacking effect and tolerance to complex matrices, CITP offers the potential for selective enrichment, since it is possible to transfer only a selected part of the original sample to the second capillary. Its widespread use, however, has been largely limited by the dedicated instrumentation that it requires. For relatively simpler samples, the single-capillary technique is more popular since both preconcentration and separation steps take place within the same capillary. The ITP process continues to persist for some time, affecting the separation step [1]; hence, it is often referred to as transient ITP (tITP). Larsson and Lutz [44] and Stutz et al. [45] used tITP for sensitivity enhancement prior to CZE separation and MS detection of endogenous peptides and metal-binding proteins, respectively, with limits of detection in the femtomole level. Hirokawa et al. [46] developed electrokinetic supercharging, a variant of tITP in which sample injection was carried out electrokinetically. They subsequently adapted it for combination with GE on a single-channel chip [47]. A 30-fold reduction in CLOD of some SDS-protein complexes compared to chip GE was obtained.

Shihabi [48,49] described a unique type of stacking in the presence of acetonitrile and high concentrations of salts, which has been shown to be suitable in the analyses of peptides released from proteolytic enzymes [50], enkephalins and angiotensin [51], insulin in pancreatic tissue [52], and oxidized and reduced forms of glutathione in erythrocytes and myocardial tissue [53] and in plasma [54]. The stacking effect was thought to be due to a tITP-like mechanism (hence, the name pseudo-tITP for the technique), in which salts acted as leading ions while acetonitrile acted as a pseudo-terminating ion [55]. Stacking in ITP occurs as diluted zones adjust their concentrations to yield the appropriate field strength necessary for them to move at the fixed velocity determined by the leading ion. The terminating ion, because of its high field strength, "pushes" the zone of intermediate mobility and keeps it moving at the required pace; hence, the edge of the zone is sharpened. Acetonitrile and other water-miscible organic solvent, like acetone or small alcohols, because of their low conductivity, exhibit a high field strength that can speed up the velocity of ions in that region. This type of stacking is particularly attractive for biological samples because it enables deproteinization (in the case of acetonitrile), counteracts the deleterious effects of high concentrations of inorganic ions present in the sample, and stops the enzymatic reaction [51]. Sample loading can be increased up to one-third of the total capillary volume, giving about a 20-fold increase in detection signal [51–54].

2.1.4. Isoelectric focusing

Capillary isoelectric focusing separates amphoteric compounds according to their p*I*s, with difference as small as 0.004 [56]. The whole separation capillary is filled with the sample mixed with carrier ampholytes, which help to generate a pH gradient under the influence of an applied field. Every component of the sample is focused at a position in the pH gradient where its net charge is zero.

At the completion of focusing, the analyte bands remain inside the capillary; hence, CIEF requires a different detection scheme compared to other CE modes. This can be accomplished by mobilizing the bands (chemically [57,58], hydraulically (pressure [59,60], gravity [61,62]), or by combined means [63,64]) or transporting the entire capillary past a single, fixed detection point [65]; or by whole column imaging detection (WCID) [66–73]. The use of a stationary detector results in longer analysis time and might cause distortion of the focused bands, two difficulties that are overcome when WCID is employed. In WCID, by using a short separation column and a charge-coupled device (CCD) camera, the focused sample bands within the whole column are perpendicularly imaged [66]. CIEF–WCID has been used to study a broad range of analytes, ranging from small proteins to viruses and bacterial cells [67,68]. Additionally, it has permitted direct observation of the dynamics of IEF [69] and facilitated monitoring of a number of protein reactions such as conjugation [69], reduction, carbamylation and denaturation [70], protein–drug [71], and protein–phospholipid [72].

Because of its self-sharpening effect on analyte bands, CIEF is used as a preconcentration strategy as well. Whereas most CE applications for proteins and peptides dealt with simple model mixtures or proteolytic digests, the high-resolution capability of CIEF, frequently in conjunction with a powerful MS detector, has been harnessed for complex samples, such as cell lysates, for proteome characterization. Jensen et al. [63] reported 400–1000 putative proteins in the analyses of cell lysates of *Escherichia coli* and *Deinococcus radiodurans*. Storms et al. [59] used CIEF-ion trap MS combination to analyze the periplasmic proteins of *E. coli*, identifying 159 proteins in a single run.

To further enhance sample loading and analyte concentration, Chen et al. [62] developed dynamic sample introduction by electrokinetic injection (Fig. 2). The usefulness of this approach was demonstrated using tryptic peptides from the yeast *Saccharomyces cerevisiae*. Depending on the mobilities of the peptides, loading capacity could be increased by as much as a factor of 45, and the concentration sensitivity by as much as a factor of 7700.

For highly complex samples, two- (or higher) dimensional systems are particularly attractive. The separation mechanisms in these dimensions should be orthogonal, and the separation in the first dimension should be preserved in the second. By increasing overall peak capacities (i.e., the product of the peak capacities in each dimension), high-resolution separations can be achieved. CIEF, because of its larger loading capacity relative

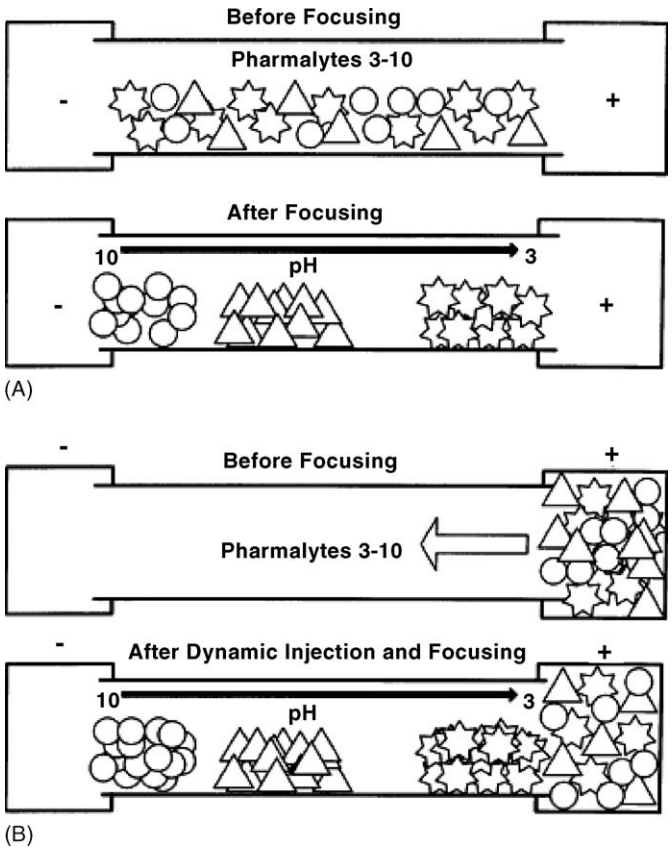

Fig. 2. Schematics of (A) conventional CIEF and (B) dynamic injection/focusing CIEF. Reprinted from [62] with permission. Copyright 2003 American Chemical Society.

to other capillary electrokinetic-based techniques, is frequently used as the first dimension.

Mohan and Lee [61] combined CIEF with tITP. With UV detection, the maximum peak capacity was estimated to be ~1600 and could be significantly increased by simply using a longer capillary and manipulating the range of pH gradient in the IEF stage. In a subsequent work, the same platform was coupled with an MS detector [74], enabling identification of 1174 unique proteins from the cytosolic fraction of *Shewanella oneidensis* with less than 500 ng of its proteolytic digest loaded into the capillary.

In a number of reports [60,75–78], CIEF has been integrated with reversed-phase liquid chromatography (RPLC). Using UV detection, Chen et al. [75] demonstrated the resolving power of such system using the soluble fraction of *Drosophila* salivary glands, with the overall peak capacity estimated to be ~1800. With MS detection [76], a total of 1132 unique proteins and 1894 unique peptides were identified from the soluble fraction of the cell lysate of the yeast *S. cerevisiae*.

Zhang et al. used a dialysis interface to couple CIEF with capillary gel electrophoresis [57], and capillary nongel sieving electrophoresis (CNGSE) [79]. These 2D separation strategies provided much improved resolving power, enabling more detailed studies on hemoglobin (Hb) variants.

CIEF systems in the microdevice format have also been reported, with studies ranging from characterization of focusing behavior in microchannels (e.g., generation of natural pH gradients [80], multistage chip IEF [81]) to feasibility of hyphenation with a second separation scheme (chip CE [82], GE [83,84]).

2.1.5. Miscellaneous techniques

Analogous to the sweeping method developed by Quirino and Terabe [28] in the MEKC mode in which the migration velocity of an analyte is altered by its interaction with micelles in the BGS, strategies specifically designed for proteins and peptides in which the concentration effect depends on their interaction with a buffer component have been developed. Chang and co-workers [85–87] used poly(ethylene oxide) PEO to cause proteins to stack due to retardation by the PEO matrices. Up to one-third of the effective capillary length could be filled with the sample solution, resulting in CLODs in the subnanomolar level for some proteins. Recently, Jing et al. [88] described a stacking method which took advantage of the interaction between protein molecules and SDS monomers. When a potential was applied, proteins from an SDS-free solution interacted with the SDS monomers in the BGS to form complexes that migrated more slowly than the corresponding uncomplexed molecules. This method enabled detection of fluorescent-labeled protein at trace levels.

2.2. Filtration-based techniques and electrocapture

When BGS and sample solution constraints do not justify the use of stacking techniques, a straightforward alternative is to concentrate the analytes by porous filtering at semipermeable interfaces. The basic principle in such scheme is that, on application of a potential, small buffer ions are allowed to pass through the interface while analytes are not; hence, they collect and become concentrated at that section. It is applicable to almost any analyte, but its efficiency is obviously limited by the size of the target. Wu et al. [89] connected a short, semipermeable hollow fiber to the inlet end of the capillary. An injection electric field was applied across the hollow fiber, causing proteins to electromigrate into the hollow fiber and to collect there since they could not pass through its walls. As much as 1000-fold increase in concentration sensitivity was obtained. In a follow-up work [90], they etched the inlet end of the capillary with HF prior to insertion into the hollow fiber for closer and more reliable contact. To dispense with the use of a fiber membrane for concentration, Wei and Yeung [91] etched a short section of the capillary to make it a porous joint. When voltage was applied, large peptides and proteins concentrated at the etched section. The technique was shown to be effective even for protein digests with high ionic strengths. Recently, Wu and Umeda [92] improved the mechanical strength of the etched section by coating it with a cellulose acetate membrane.

In the chip format, Song et al. [93], laser-patterned a membrane at the junction of a cross channel in a microchip. By implementing electrokinetic pinched injection, proteins were trapped at the surface of the membrane, enabling as much as four orders of magnitude increase in concentration. In a recent work, Foote et al. [94] positioned a porous silica membrane between

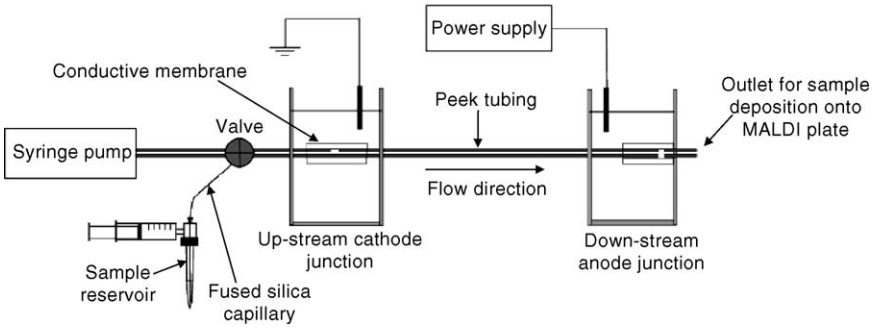

Fig. 3. Schematic representation of the microfluidic electrocapture device. Reprinted from [97] with permission. Copyright 2003 American Chemical Society.

adjacent microchannels, and obtained approximately 600-fold improvement in signal for fluorescently labeled ovalbumin.

A number of reports dealt with the concentration of DNA [95], proteins [96,97] peptides [98] by an electrocapture technique using a small device consisting of two electrified junction zones (Fig. 3). An electric field of appropriate strength and direction is applied between these two zones and counter to the direction of the flow stream, enabling charged analytes to resist the hydrodynamic sweeping force and be trapped in a sharp zone within the flow stream. Aside from the preconcentration effect, this method has the advantage of sample desalting and cleanup.

3. Chromatographic preconcentration

Significant enhancements in concentration sensitivity can be obtained using electrophoretic preconcentration schemes. In many cases, however, the increase in signal output is largely limited by the volume of the capillary itself, since a good length must remain for separation of the sample components. In contrast, multiple capillary volumes can be loaded onto chromatographic preconcentration devices positioned at the inlet end of the capillary; hence, higher signal enhancements are feasible. In addition, they afford the benefits of sample cleanup and matrix removal, and provide higher selectivity. Traditionally, their direct integration with CE systems was not very straightforward, but with recent advances in technology and instrumentation, this can be accomplished more easily.

Most reported techniques are predicated on solid adsorptive phase chromatography, including solid-phase extraction (SPE) and membrane preconcentration. In SPE, the sample is loaded onto the column or cartridge, filled with porous solid particles (e.g., poly(styrenedivinylbenzene) by pressure or suction, extracted, washed, and finally, eluted using a small volume of an appropriate solvent. SPE is fairly simple and can be automated easily; however, subsequent CE performance is often compromised.

The use of a preconcentrator device at the head of the capillary has been explored in a number of works [73,99–103]. Waterval et al. [85] developed a preconcentrator using commercially available extraction disks containing poly(styrenedivinylbenzene) adsorbent particles in a matrix of inert Teflon to create a mechanically stable sorbent. Instead of nanoliter volumes typically used in CE, at least 25 μL of the sample could be loaded, resulting in three to four orders of magnitude improvement in detections limits. Vizioli et al. [100] packed a short length of fused silica capillary with silica-based C_{18} reversed-phase chromatographic

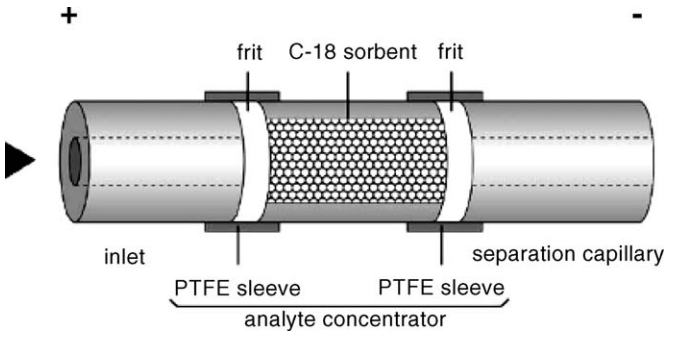

Fig. 4. Schematic representation of the analyte concentrator cartridge. Reprinted from [100] with permission from Elsevier.

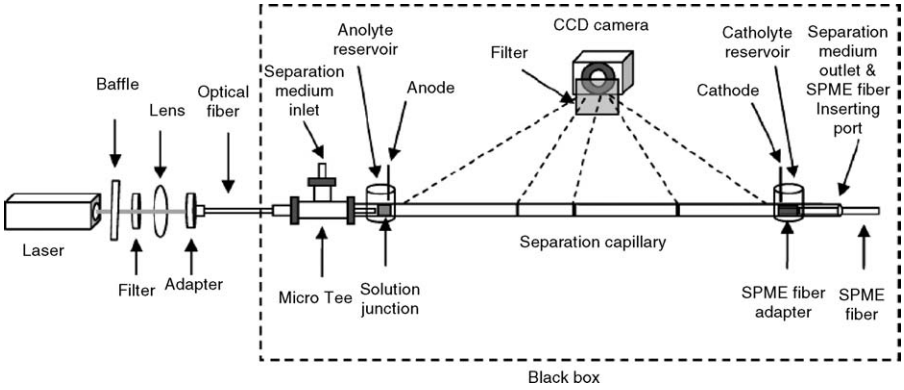

Fig. 5. Schematic diagram of the experimental setup for SPME–CIEF–LIFWCID. Reprinted from [73] with permission. Copyright 2005 American Chemical Society.

material, and coupled it on-line with the separation capillary (Fig. 4). This method was utilized in the purity profiling of synthetic peptides. An interesting system developed by Liu and Pawliszyn [73], integrated solid-phase microextraction (SPME) and CIEF with LIF–WCID (Fig. 5). The catholyte in the CIEF stage was also the desorbing agent in SPME. By this system, a CLOD in the low picomole level of *R*-phycoerythrin in water was obtained. Janini et al. [102] assembled a miniaturized, membrane-based solid-phase extractor (mSPE) using a C_{18}-impregnated extraction disk. In combination with an in-house developed sheathless interface, a mid-attomolar mass limit of detection and a low nanomole concentration limit for some proteomic samples were achieved.

As a means to overcome problems on detection limits and interferents in microdevices, the on-chip incorporation of SPE has been studied as well. Oleschuk et al. [104] fabricated a chromatographic bed on a glass substrate. A double weir design was used to construct a cavity in which beads coated with octadecylsilane (ODS) could be trapped. Such a system yielded concentration enhancement of up to 500 times. On the other hand, Yu et al. [105] prepared monolithic porous polymers by photoinitiated polymerization within the microchannels. Using this monolithic concentration device, the concentrations of a hydrophobic tetrapeptide and a green fluorescent protein was enriched by a factor as high as 1000.

4. Conclusions

A wide range of techniques for sample enrichment is currently available for CE practitioners. The technique of choice depends on the nature of the sample to be analyzed and the extent of the desired sensitivity enhancement.

In the area of protein and peptide analyses, much emphasis has been given to improving current systems or developing new ones, and exploring the feasibility of combining methods to achieve optimum performance. Despite the impressive gains in sensitivity demonstrated in many cases, their (except, perhaps, CIEF) use in biological samples were still limited. This could be due to the fact that the proteins and peptides of interest are often present in low concentrations together with interfering proteins in high concentrations. Given the limited dynamic concentration range of most systems, the first group remains beyond the detection of CE even after focusing. Thus, there is a need to develop more selective sample enrichment procedures. These, alongside advances in other aspects of CE, will help propel it towards full utilization as a bioanalytical tool.

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