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Applications of capillary electrophoresis to high-sensitivity analyses of biomolecules*

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Abstract: To increase the detection sensitivity of capillary electrophoresis (CE) and thereby widen its application to biomolecular analyses, we have developed a number of on-line preconcentration strategies, i.e., techniques to increase sample loading without compromising resolution and efficiency. Two such techniques, namely, dynamic pH junction and field-enhanced sample injection (FESI) are covered in this work. Dynamic pH junction is predicated on a sharp reduction in an analyte's migration velocity following a reversal of its electrophoretic direction from the acidic sample zone to the basic background solution (BGS) zone. FESI, on the other hand, depends on the retardation of analyte velocity as it transits from the low-conductivity sample zone to the high-conductivity milieu of the BGS zone. Their applications to high-sensitivity analyses of peptides and proteins are discussed.

Keywords: capillary electrophoresis; biomolecules; analytical chemistry; sample enrichment; concentration sensitivity.

INTRODUCTION

Capillary electrophoresis (CE) has emerged as one of the most important tools in separation science over the last two decades. Several separation modes—capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), capillary gel electrophoresis (CGE), and capillary electrochromatography (CEC)—are available, enabling rapid and highly efficient analyses of both charged and neutral species. Because the capillaries employed are typically less than 100 μm in internal diameter, only a small volume (a few nanoliters at most) of the sample is required for analysis. Excellent limits of detection in terms of absolute amount (attomole level) have been reported. However, its concentration sensitivity (micromolar level), which is dependent on the volume of sample loaded, is rather poor. This problem becomes aggravated since most commercial CE instruments are equipped with absorbance-related detectors. With the usual capillaries, the available path length for detection is short. For these reasons, CE has lagged behind chromatography-based systems in terms of application to routine analyses, particularly of biomolecules.

To improve concentration sensitivity, CE practitioners have adopted a number of approaches, including the use of capillaries with extended path length (z-cell, bubble cell) in the case of absorbance detectors [1], more sensitive alternative detector formats (laser-induced fluorescence, chemilumines-

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cence, amperometric, conductimetric) [2], and sample enrichment techniques, both off-line [3] and on-line [4–16]. Of these, the on-line sample enrichment (preconcentration, focusing) schemes have generated the most interest, because they require only judicious manipulation of the composition and concentration of background solutions (BGSs) and sample matrices without modification in instrument configuration. The analytical conditions are designed such that the BGS and the sample matrix differ in terms of some physicochemical property (e.g., conductivity [4–6,15], salt content [7], pH [8,9,11–14], presence of an additive [10]), which brings about an alteration in the migration velocity of an analyte as it transits the boundary between these two regions. By exploiting an intrinsic property of the analyte (e.g., pK_a) or changing its environment (e.g, local field strength), it can be made to speed up or slow down in discrete sections of the capillary, enabling compression of long sample bands into narrow, concentrated zones. As such, large volume injections of low-concentration samples may be introduced into the capillary without causing a breakdown in resolution and efficiency.

Our group has worked extensively in developing on-line focusing schemes. Two of such schemes, namely, dynamic pH junction and field-enhanced sample injection (FESI), are discussed in this work, and their usefulness for effecting high-sensitivity analyses of peptides and proteins is presented.

DYNAMIC pH JUNCTION

Several pH-mediated focusing strategies have been developed, mostly for high salt-containing matrices. Similarly, changes in analyte velocity as a direct consequence of pH differences in multi-section capillaries have also been exploited [8,9,11]. In such cases, the change is prompted by an alteration in the analyte's ionization state as it passes between two zones where a distinct pH difference exists. The pH gradient inside the capillary dissipates, or at least becomes shallow; hence, the technique is called "dynamic pH junction". For example, analytes with weakly acidic functionalities are dissolved in a low pH solution and injected as a long plug into a capillary filled with a high pH BGS. When voltage is applied, a moving pH boundary sweeps through the sample zone, and the analytes, initially neutral and migrating at the same speed as the electroosmotic flow (EOF), are converted into their anions. Their net migration velocities subsequently decrease since they are now directed electrophoretically against the EOF, and their bands are compressed into sharp zones. We have previously shown its application to metabolome analysis [12,13].

Peptides are rarely found in their uncharged states, and changes in local pH conditions can bring on large changes in their ionization; hence, a dynamic pH junction technique to focus them within the capillary is an attractive option. In a pH-sectioned capillary, the analytical conditions can be tuned such that the peptide experiences a reversal of charge as it goes from the sample plug region to the BGS region. A model for focusing acidic peptides is depicted in Fig. 1. Briefly, a plug of acidified sample is introduced into an uncoated capillary filled with basic BGS (A). When positive voltage is applied, the local pH at the front end of the sample plug rises sharply because of the titration of the H^+ ions from the sample plug by the OH^- ions from the BGS. Fast-moving cationic peptides, upon reaching this juncture, are converted to anions, and they experience a drastic reduction in their migration velocities, since they must move now against a high, cathode-directed EOF. A moving pH boundary sweeps across the sample zone until the whole region is compressed (B), and the focused peaks separate and migrate to the detector (C).

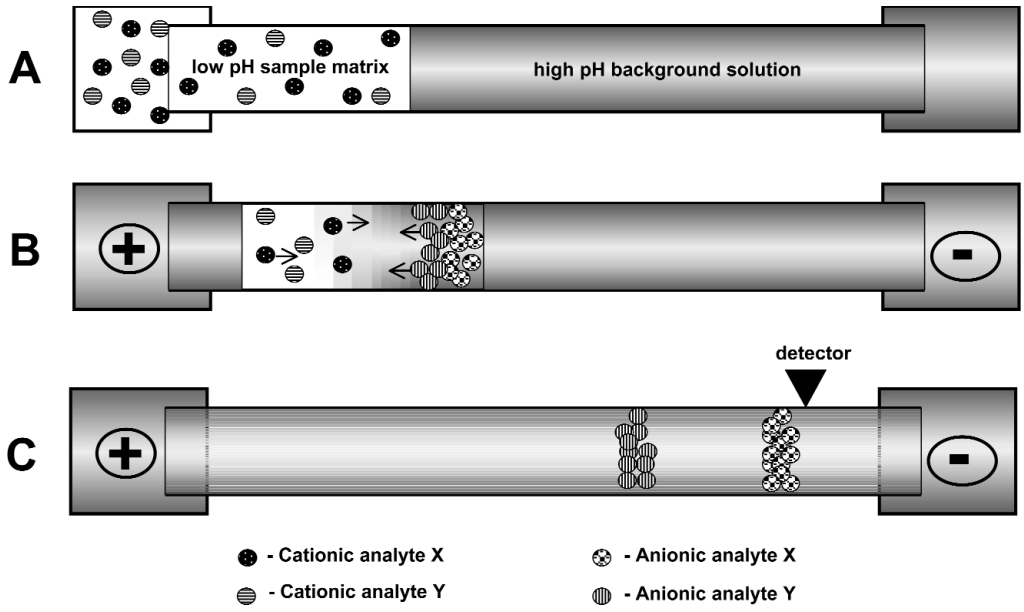

Fig. 1 A dynamic pH junction model for peptides.

For the model peptides listed in Table 1, the effect of changing the pH of the sample matrix while keeping that of the BGS constant is shown in Fig. 2 [14]. The strongly basic peptide bradykinin (peak 1, p*I* 12.00) concentrated poorly because it was cationic in both the sample and the BGS, while the other three weakly acidic peptides (p*I*s between 6.74 and 8.75) were reasonably well focused within the range studied. These results can be explained in terms of the changes in the peptides' ionization states. Under optimum conditions found in this work (i.e., a pH 4 sample matrix for a pH 11 BGS), the change in net charge (ΔQ, Table 1) of bradykinin was approximately one unit only, and it remained positive within the range under consideration. In contrast, [Sar^1, Ile^8]-angiotensin II, angiotensin I, and angiotensin II were changed by approximately 3, 5, and 4 units, respectively, and all three underwent transition from cation to anion. A more pronounced change in an analyte's effective charge should translate to a greater change in its electrophoretic mobility, resulting in a more abrupt reduction in its migration velocity, and possibly, to enhanced focusing.

A convenient way to evaluate a focusing technique is by comparing the detector response between it and a conventional (short) injection, and expressing the effectiveness as an *x*-fold improvement in signal. One typical measure is known as sensitivity enhancement factor (SEF, Table 1). In this case, the peak heights were compared, and as much as two orders of magnitude enhancement in response were obtained. The concentration limit of detection (CLOD) at the signal-to-noise ratio (S/N) equal to 3 was estimated to be 3×10^{-8} M, with % relative standard deviation (RSD) in peak height of not more than 11 % [14]. It should be noted that these results were evidently independent of conductivity-based stacking, the best results having been obtained when the conductivity of the sample matrix was slightly higher than that of the BGS. This should be advantageous for the analyses of real samples, without the necessity of rigorous clean-up steps.

Table 1 Model peptides used in this study.

(Peak number) peptide	Amino acid sequence	p*I*[a]	ΔQ[b]	SEF_{height}[c,d]
(1) Bradykinin	RPPGFSPFR	12.00	1	–
(2) [Sar[1], Ile[8]]- Angiotensin II	Sar-RVYIHPI	8.75	3	65
(3) Angiotensin I	DRVYIHPFHL	6.92	5	113
(4) Angiotensin II	DRVYIHPF	6.74	4	124

[a]Isoelectric point calculated using <http://kr.expasy.org/tools/pi_tool.html>. Glycine was substituted for the nonstandard amino acid sarcosine for computational purpose.

[b]Approximate change in charge using pH 4 in the sample matrix and pH 11 in the BGS, calculated using <http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html>.

[c]$SEF_{height} = \frac{\text{Height with dynamic pH junction}}{\text{Height with conventional injection}} \times \text{dilution}$

[d]Under optimum conditions: 1000 mM acetic acid, pH 4.0 as sample matrix, 60 s injection. Other conditions are the same as given in Fig. 2.

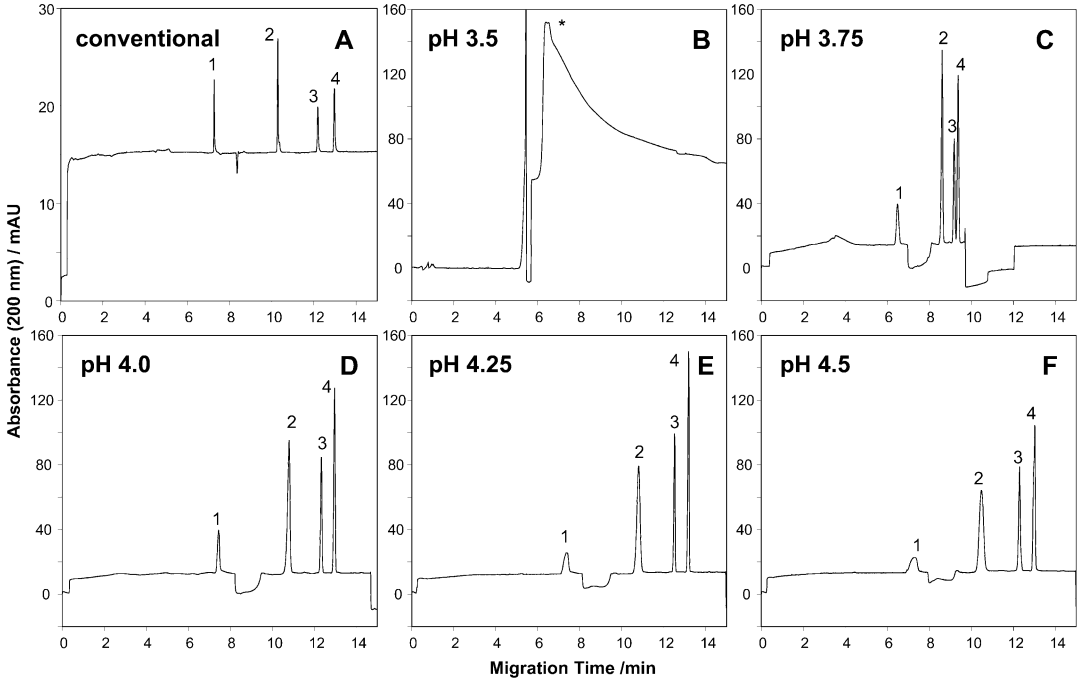

Fig. 2 Effect of sample matrix pH on focusing. Conditions: capillary, uncoated, 50 µm. i.d. × 360 µm o.d., 50 cm effective length; BGS, 200 mM borate, pH 11.0; sample matrix, 350 mM acetic acid with pH varied as shown; injection, 1 s of a mixture containing 100 ppm of each peptide (A), 100 s of a mixture containing 10 ppm of each peptide (B–F); applied voltage, +15 kV; detection, 200 nm. Peak identification, bradykinin (1), [Sar[1], Ile[8]]-angiotensin II (2), angiotensin I (3), angiotensin II (4).

FIELD-ENHANCED SAMPLE INJECTION

FESI is one of the most popular on-line preconcentration techniques in CE. It is known to provide as much as three orders of magnitude improvement in detector response. In FESI, the sample is prepared in water or in a low-conductivity solution and then injected electrokinetically into a capillary filled with high-conductivity BGS. The high resistivity of the sample solution results in high electric field strength at the injection point and enables introduction of large amounts of ions, which subsequently slow down and focus upon reaching the BGS region. A FESI model for cations, using an electrospray/mass spectrometry (ESI/MS) detector coupled to CE via a sheath liquid interface, is shown in Fig. 3. Initially, the capillary is filled with the BGS, followed by a hydrodynamic injection of a short plug of water, which guarantees the presence of a sufficiently long zone of low conductivity (A) [5]. Thereafter, the analytes, prepared in an acidic (to promote protonation), low-conductivity matrix, are injected electrokinetically by application of positive voltage at the inlet for a period of time longer than usual injections. The analytes move rapidly into the capillary across the water plug and stack at its boundary with the high-conductivity BGS (B). After substituting the sample vial with a BGS vial at the inlet, the CE and ESI voltages are turned on, and the focused analytes separate according to their charge and size (C). Upon reaching the tip, they mix with the sheath liquid and the nebulizer gas, are ionized, and electrostatically propelled into the mass analyzer (D).

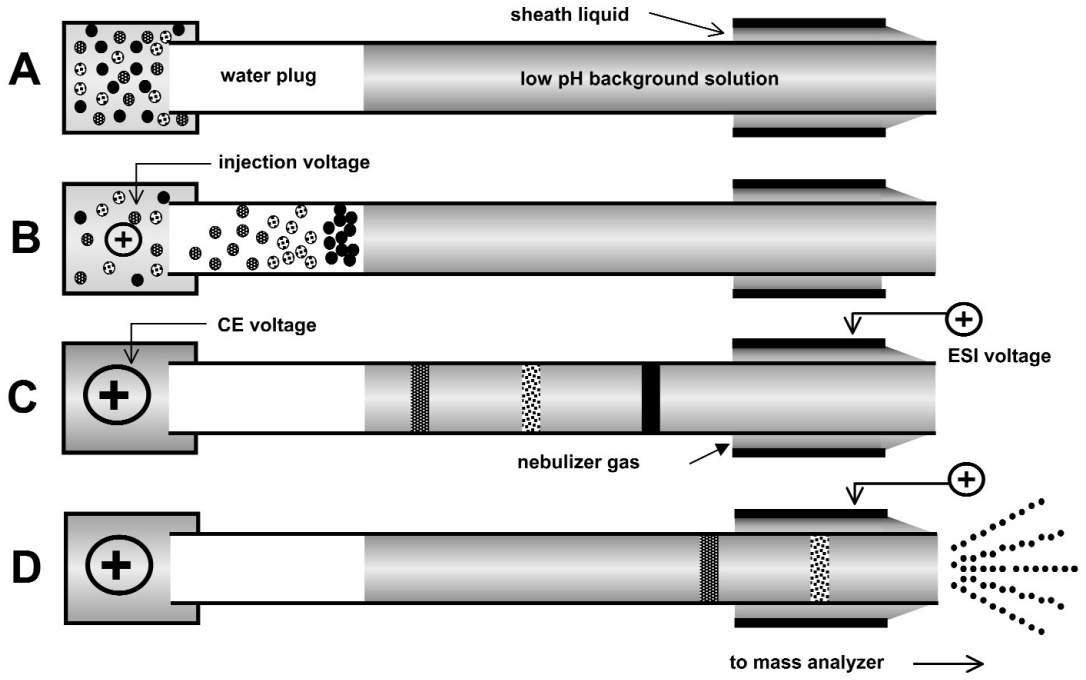

Fig. 3 Evolution of analyte zones in FESI/CE/ESI/MS.

A typical 1-s injection (panel A) and FESI (panel B) of a synthetic mixture of some peptides are shown in Fig. 4 [15]. By comparing corresponding components, it can be inferred that as much as 3600-fold increase in signal intensity has been obtained. Despite the use of a sheath liquid interface, which tends to reduce sensitivity because of dilution at the exit end of the capillary, at S/N = 5, the CLOD is estimated to be in the low nanomolar range (3×10^{-9} M) (10 % RSD in highest ion intensity), comparable with published results using more sensitive nanoelectropray emitters.

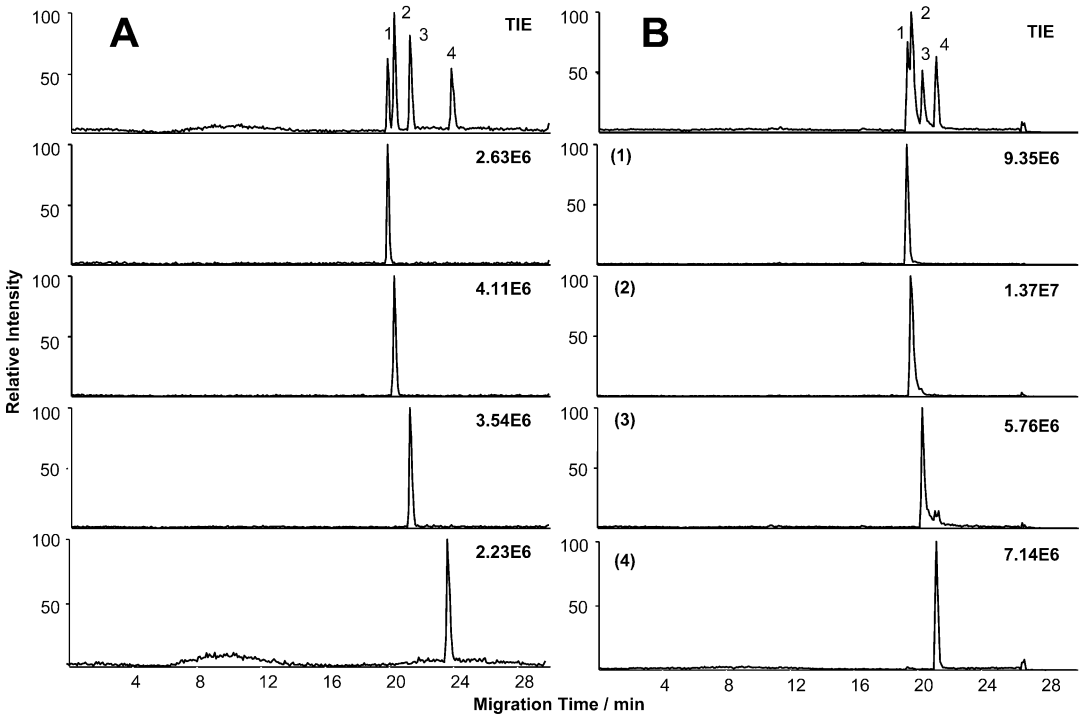

Fig. 4 CE/MS of some standard peptides. Conditions: BGS, 100 mM ammonium formate, pH 3.0; capillary, uncoated fused silica, 60 cm × 50 μm I.D.; CE voltage: +14 kV; ESI voltage, +4.5 kV; sheath liquid (50 % methanol, 49 % water and 1 % acetic acid), 5 μl/min; injection, 1 s at 50 mbar of samples ca. 200 ppm each (A), 180 s at +7 kV of samples ca. 200 ppb each (B); peak identification, [Sar^1, Ile^8]- angiotensin II (1), bradykinin (2), angiotensin I (3), angiotensin II (4). Selected ion monitoring mode. Samples were diluted with 0.1 mM formic acid.

FESI FOR PROTEIN IDENTIFICATION

With FESI, very little of the sample matrix is coinjected because the net electrosmotic velocity is much lower than electrophoretic velocity [5]. In addition, with prolonged injection times, it is possible to significantly deplete the sample matrix of the analytes [6], i.e., maximize the amount that can be injected. These clearly underscore the usefulness of the technique for low-abundance samples.

We thought that one practical application will be in the area of protein identification from proteolytic digests. Proteolytic agents cleave the protein at very specific points, and such cleavages produce a mixture of peptides that is characteristic of the protein. While a protein may be identified by means of only 4–6 of its peptides, it is better to be able to account for as many peptides as possible in order to resolve any ambiguity between closely related species. Hence, for the analysis of low-concentration digests by CE/MS, an on-line enrichment procedure for the peptides will be very helpful in amplifying their signals.

One method to identify the protein is to use the molecular masses of the peptide fragments in a database search in order to determine the best possible match. For example, ca. 40 nM of tryptic digest of bovine serum albumin (BSA) was analyzed by FESI/CE/MS, with the resulting electropherogram shown in Fig. 5. The molecular masses of 29 fragments were used in a search (MS Fit, <http://prospector.ucsf.edu>) which did not impose any restriction on the species of origin. Not surprisingly, the top candidate protein encoded was serum albumin from Bos taurus (cow), accession CAA41735. It was possible to distinguish it from the next-ranked protein, serum albumin precursor (Allergen Bos d 6), ac-

cession P02769. These two proteins differ in their 607-amino acid sequence only at 214, where threonine in CAA41735 is changed to alanine in P02769.

For even more unequivocal protein identification, the MS/MS spectra of the peptides can be used in addition to their molecular masses in a database search. In this case, the actual MS/MS data are compared with putative fragmentation patterns, and any ambiguity resulting from the presence of isobaric peptides is removed. Again, we were able to obtain positive identification of the original proteins after on-line enrichment by FESI of their component peptides in low-concentration digests.

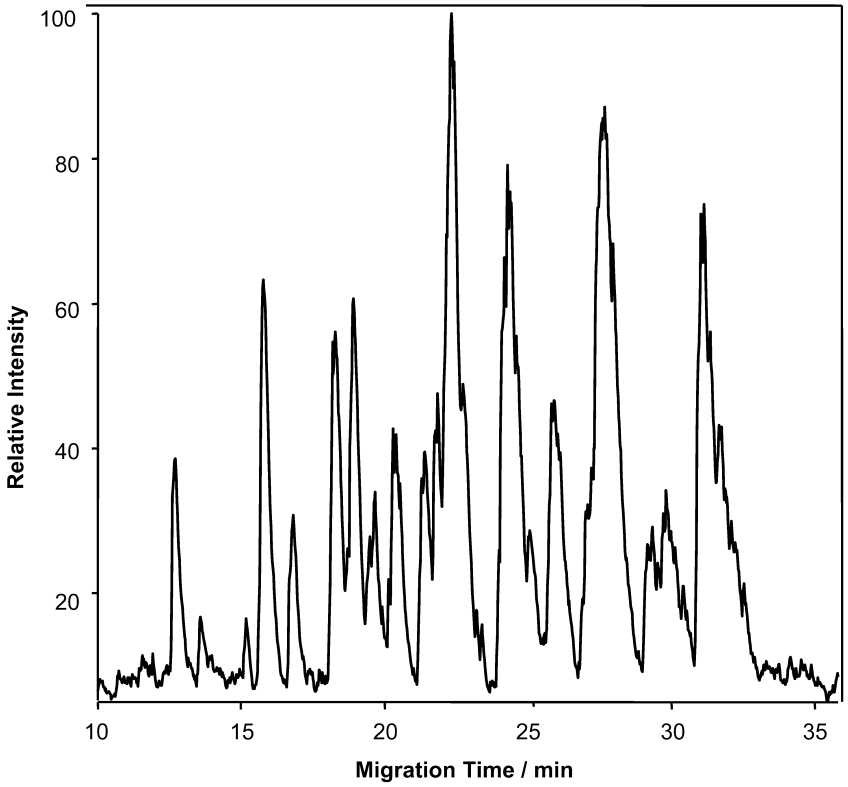

Fig. 5 FESI/CE/MS of tryptic digest of BSA. Conditions: BGS, 75 mM morpholine, pH 3.0; capillary, uncoated fused silica, 70 cm × 50 μm i.d.; CE voltage, +30 kV; ESI voltage, +4.5 kV; sheath liquid (50 % methanol, 49 % water and 1 % acetic acid), 3 μl/min; injection, ca. 40 nM at +10 kV for 450 s. Full scan acquisition (570–1700 m/z).

FESI FOR DISCRIMINATE PRECONCENTRATION OF MODIFIED PEPTIDES

Analyses of low-abundance components in complex mixtures are often compromised by the limited dynamic concentration range of most analytical systems. Modified proteins and peptides, for instance, are generally present in small quantities relative to nonmodified species. Given that many of these have important cellular regulatory functions, there is widespread interest in developing strategies for their selective determination.

In FESI, the amount of analyte injected depends on its concentration as well as its charge. The implication is that for mixtures, there exists a bias against less mobile species and low-abundance components. If the dynamic concentration range is wide, one way to work around the concentration bias is to set up the analytical conditions such that there is discriminate injection of the minor components by virtue of their charges, i.e., to use charge bias to offset concentration bias. We thought that this feature

could be exploited in devising a sensitive, CE/MS-based technique for analyzing modified peptides, specifically phosphopeptides and sulfated peptides. These are characterized by higher acidity compared to their nonmodified analogs; hence, they can be converted into their anions more easily, and be more efficiently introduced into the capillary when negative voltage is applied for injection. A diagram depicting our on-line preconcentration scheme using MS detection is shown in Fig. 6. A long plug of water (to concentrate the analytes deeper into the capillary) is injected hydrodynamically into a successive multiple ionic polymer layer, SMIL(–)-coated capilllary, after filling it with a moderately acidic BGS (A). The SMIL(–) coating causes strong, cathode-directed EOF even under acidic conditions. The more strongly acidic components in the sample, prepared in a low-conductivity matrix, are then preferentially injected into the capillary by applying negative voltage at the inlet for an extended period of time (B). A marked difference in field strengths causes the injected ions to slow down and focus into narrow bands upon reaching the high conductivity milieu of the BGS region. After substituting the sample vial with a BGS vial, a positive voltage is applied at the inlet for separation, and the focused analyte zones separate and migrate to the detector (C). At the tip, the capillary effluent mixes with the sheath liquid, is ionized, and the ions electrostatically propelled into the mass analyzer (D).

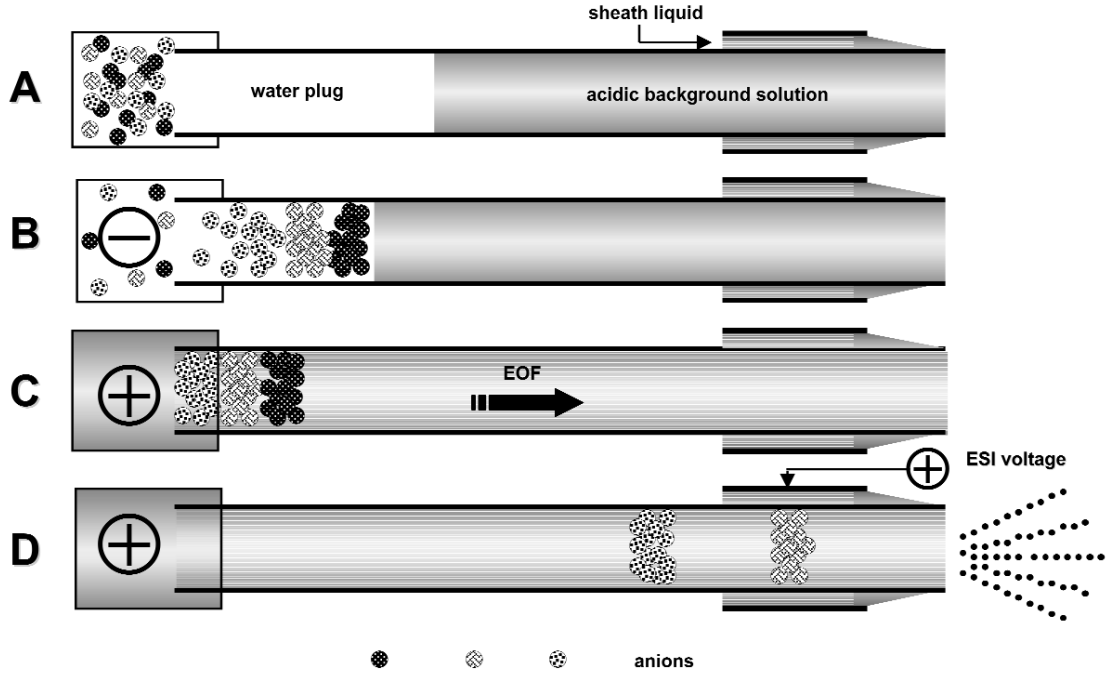

Fig. 6 Schematic illustration of FESI (with polarity switching)/CE/ESI/MS.

A comparison of a typical injection (A) of a synthetic mixture of nonphosphorylated (peaks 1 and 2) and phosphorylated (peaks 3 and 4) peptides, and FESI (B) with the pH of the sample matrix adjusted to 4 are shown in Fig. 7 [16]. Obviously, the phosphopeptides were focused well, whereas the non-phosphopeptides were not, the former being more easily injected from an acidic sample matrix upon application of voltage. As much as 4000-fold enhancement in signal was obtained. Using the same technique, the phosphopeptides in tryptic digests of naturally occurring phosphoproteins (e.g., bovine β-casein, chicken ovalbumin) could be clearly detected even at low nM concentrations of digests, and the specific sites of phosphorylation could be identified via MS/MS. A phosphopeptide spiked in the digest of HCT116-C9 cell lysate at 1:2400 was clearly detected as well.

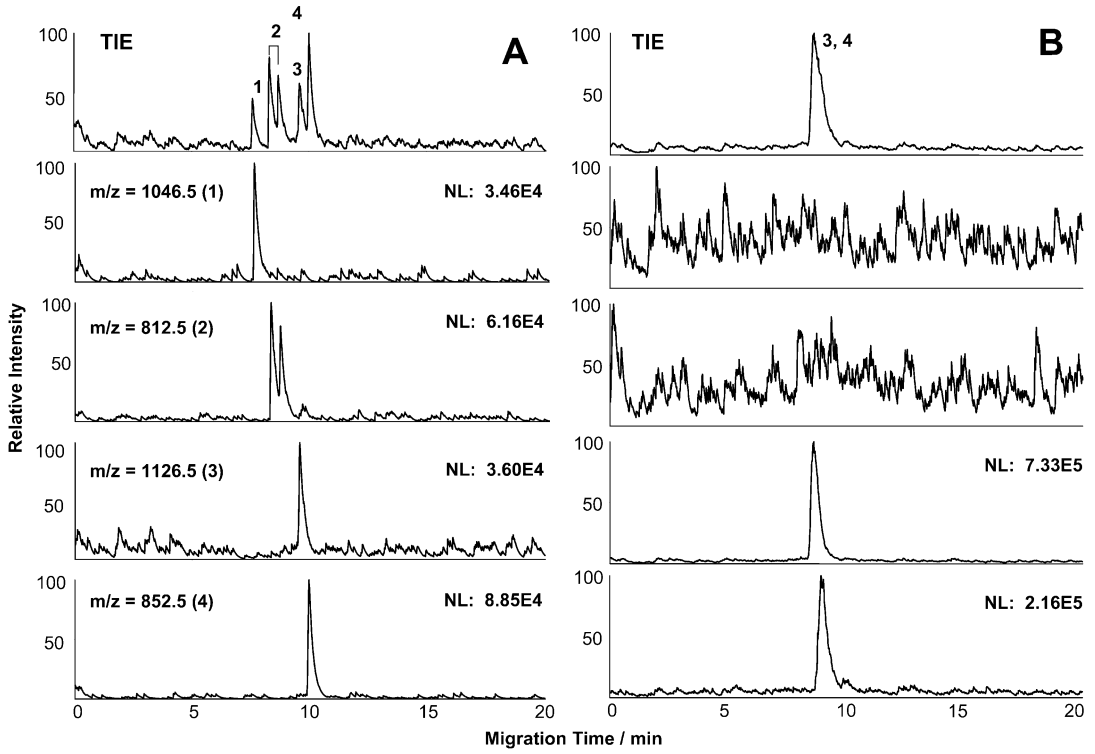

Fig. 7 CE/MS of some nonphosphorylated and phosphorylated peptides. Conditions: BGS, 75 mM ammonium acetate, pH 5.5; capillary, SMIL(–)-coated, 50 μm i.d. × 360 μm o.d., 60 cm effective length; injection, 3 s at 50 mbar of samples 100 ppm each prepared in water (A), 120s of water at 50 mbar followed by 180 s at –20 kV of samples 500 ppb each prepared in 1 mM acetic acid pH 4.0 (B); CE voltage: +21 kV; ESI voltage, +4.5 kV; sheath liquid (50 % methanol, 49 % water and 1 % acetic acid), 3 μl/min; peak identification: (1) angiotensin II (pI, 6.74), (2) insulin receptor (pI, 5.79) (2), angiotensin II substrate (3), kinase domain of insulin receptor-2 (4).

Preferential injection of sulfated peptides over their non-sulfated analogues was also observed (in this case, however, the capillary used was SMIL(+), in which the EOF was directed toward the anode for faster analysis time; MS detection was in the negative ion mode). As much as 3700-fold increase in signal was obtained. The sulfated and nonsulfated forms of leucine-enkephalin were spiked in the digest of HCT116-C9 cell lysate at 1:2000, and only the sulfated form could be detected, as shown in Fig. 8 [16].

FESI, in this case, confers a two-fold effect. First, it enables efficient introduction into the capillary of fast-moving ions that are otherwise compromised by their low abundance. Since the efficiency of injection depends on an analyte's p*I*, the detection cannot be tuned exclusively for one species alone since all other components which are similarly acidic can be concentrated as well; hence, the technique cannot be strictly described as selective. However, it significantly cuts down on the number of species that are injected into the capillary and limits potential interference in the detection and separation of the target analyte. Second, considerable enhancement in signal is achieved simply by narrowing the analyte bands within the capillary, and this does not necessitate any off-line pretreatment of sample nor changes in existing instrument configuration.

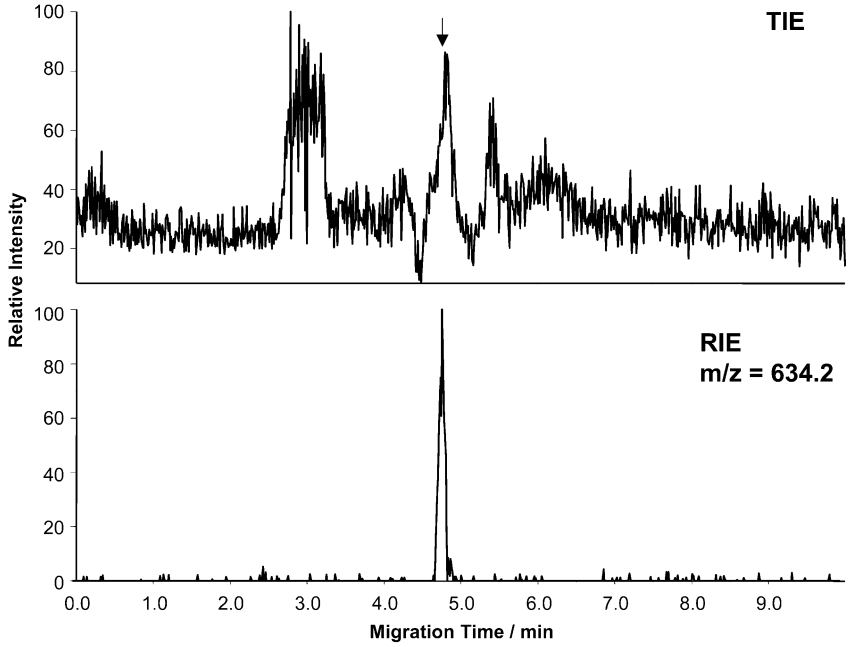

Fig. 8 Detection of sulfated peptide spiked in the digest of HCT116-C9 cell lysate. Conditions: BGS, 200 mM formic acid –50 mM ammonia, pH 3.0; capillary, SMIL(+)-coated, 50 µm i.d. × 360 µm o.d., 60 cm effective length; injection, 90 s at –25 kV of ca. 1.5×10^{-5} mg/mL of sulfated leucine spiked in 3.0×10^{-2} mg/mL of digested cell lysate. CE voltage: –25 kV; ESI voltage, –5 kV; sheath liquid (60 % methanol, 40 % water), 4.5 µl/min.

CONCLUSION

Despite many advances in CE instrumentation and methodologies over the last 20 years, it has yet to gain widespread acceptance in standard biomolecular analyses where the analytes are often present in submicromolar levels. Hence, strategies to improve the CLODs are indispensable. To this end, we explored the use of on-line preconcentration techniques. Using the techniques we described in the preceding sections, the concentration sensitivities were improved to low nanomolar levels, making CE compatible with the biomolecular analyses.

The main attraction of on-line sample enrichment strategies lies in their relative simplicity. By using discontinuous electrolyte systems, the migration behavior of analytes can be altered as means of controlling normal band broadening. In this way, sample loading in CE can be increased without degrading resolution and efficiency. Better understanding of how these techniques work, as well as knowledge of analyte properties, should help in the rational design of CE systems to bring about optimum performance in terms of sensitivity and selectivity, if so warranted. By building on these and earlier efforts, it is expected that the popularity of CE as an analytical tool will grow, and its advantages will be better appreciated and exploited.

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