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Optimization of Field-Amplified Sample Injection for Analysis of Peptides by Capillary Electrophoresis−Mass Spectrometry

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A versatile experimental approach is described to achieve very high sensitivity analysis of peptides by capillary electrophoresis−mass spectrometry with sheath flow configuration based on optimization of field-amplified sample injection. Compared to traditional hydrodynamic injection methods, signal enhancement in terms of detection sensitivity of the bioanalytes by more than 3000-fold can be achieved. The effects of injection conditions, composition of the acid and organic solvent in the sample solution, length of the water plug, sample injection time, and voltage on the efficiency of the sample stacking have been systematically investigated, with peptides in the low-nanomolar (10^{-9} M) range readily detected under the optimized conditions. Linearity of the established stacking method was found to be excellent over 2 orders of magnitude of concentration. The method was further evaluated for the analysis of low concentration bioactive peptide mixtures and tryptic digests of proteins. A distinguishing feature of the described approach is that it can be employed directly for the analysis of low-abundance protein fragments generated by enzymatic digestion and a reversed-phase-based sample-desalting procedure. Thus, rapid identification of protein fragments as low-abundance analytes can be achieved with this new approach by comparison of the actual tandem mass spectra of selected peptides with the predicted fragmentation patterns using online database searching algorithms.

High-performance capillary electrophoresis (CE) has many desirable features for the analysis of biological compounds and pharmaceuticals due to its unparalleled separation efficiency and resolving power.[1−3] Compared to HPLC, CE offers complementary selectivity, higher separation efficiency, shorter analysis time, and lower sample consumption. Coupling of capillary electrophoresis with electrospray ionization-mass spectrometric detection systems (ESI-MS), especially with mass spectrometers that have tandem mass spectrometric capability, allows the molecular weight or structural information of the analytes to be obtained.[4,5] Therefore, the direct interfacing of CE with ESI-MS provides a powerful combination of separation and detection.[3,6] However, compared to other techniques such as electrochemical detection or laser-induced fluorescence detection where low-zeptomole-level detection limits have been realized,[7−9] ESI-MS has not yet achieved a similar status as a very sensitive technique, with the concentration limits of detection of current CE-MS instruments in the 1−10 μM range,[10,11] which is too high for many real-world samples, especially those arising in many types of proteomic applications.[12,13] The very small volume of sample and the low abundance of the analytes that can be loaded into the narrow-bore capillaries for such applications is the main reason for this poor sensitivity. As a thumb of rule, only ∼1−2% of the whole capillary volume can be filled with the sample in order to not impair the separation efficiency by overloading.[11] Moreover, the most common and robust CE−MS interface, the coaxial liquid sheath flow, causes dilution and band broadening of the sample zone,[14] resulting in the reduced sensitivity in the mass spectrometer. On the other hand, ESI-MS functions as a concentration-sensitive detector under many conditions.[15] The intensity of analyte ions from electrospray ionization is dependent on ESI efficiency, ion sampling efficiency into the vacuum, and ion transmission efficiency through ion optics

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and mass analyzer.[16,17] Although the ESI efficiency is relatively high, the ion transmission efficiency is very low. According to the literature, only ~1 in 10^4 ions generated from the ESI source reach the detector.[17]

Many chromatographic and electrokinetic on-column and off-column sample stacking approaches, such as solid-phase extraction,[18] pH-mediated sample stacking,[19] on-column isotachophoresis,[20] and field-amplified sample injection (FASI)[10,21−24] have been applied to increase the amount of polar analytes such as drug substances, which can be loaded into the capillary in order to improve the sensitivity of the detection. Of these, field-amplified sample injection is a robust approach that has been used with organic analytes to provide ~1000-fold sensitivity enhancement, while maintaining the other advantages of CE, such as rapid separation and high efficiency.[10,24,25] In this modified CE technique, charged solutes contained in a relatively large volume of sample can be effectively stacked at the head of a CE capillary prior to their separation.[21,26] After filling the capillary with buffer electrolyte, a short low-conductivity zone (usually a water plug) is introduced hydrodynamically at the inlet end prior to electrokinetic sample injection of a sample solution, also of low conductivity. The preinjection of the water plug (typically of several millimeters in length for a 50-μm-i.d. capillary) creates a nonuniform electric field strength across the capillary during the electroinjection, with the electric field strength within the water plug much higher than that within the more highly conductive buffer.[21,23,26] The high electric field at the injection point allows the rapid migration of charged analytes into the capillary. Upon reaching the boundary between water and the separation buffer electrolyte, the velocity of these charged analytes drops abruptly because they encounter a much lower electric field. Such a process literally stacks charged analytes at the interface of the water/separation buffer electrolyte. As a result, charged analytes can be effectively concentrated prior to their electrophoretic separation. Moreover, little sample solvent is coinjected because the net electroosmotic flow at low pH is much smaller than local electrophoretic transport.[10,22,23] In field-amplified sample injection (also termed electroextraction injection[27]), theoretically all of the charged analytes present in sample volumes of up to several hundred microliters can be injected into a capillary,[22] giving rise to a very high sample stacking factor. Moreover, the injection of large sample volumes that may deteriorate the CE separation is avoided as a consequence of the FASI process. This unique feature of FASI makes it a very promising approach among the sample stacking techniques. The inevitable enrichment of contaminants present in the sample solution is a shortcoming of FASI; however, the major advantage of this technique is that it can be applied to a wide range of charged analytes under CE conditions.

Field-amplified sample injection has been applied for the analysis of inorganic and small organic ions,[28−30] drugs in biological fluids,[10,23] and other molecules.[25,31,32] However, very few studies[22,24] have been carried out to optimize field-amplified sample injection procedures with biomolecules, such as peptides and proteins, to achieve maximal sample enrichment. In this study, we have used several synthetic peptides to investigate the effects of injection conditions, the properties of sample solution, the length of water plug injected, the electrokinetic injection voltage applied, and time on the efficiency of FASI in a CE−ESI-MS system. Very effective sample stacking was obtained under the developed conditions as indicated by the sample depletion injection experiments. As a result, over 3000-fold enhancement of sensitivity was achieved with synthetic peptides detected in the low-nanomolar concentration range by utilizing the optimized FASI process. To explore the potential of this analytical technique in proteomic studies, this new method was further evaluated using low-abundance, complex mixtures of peptides and peptidic fragments generated via enzymatic digestion of proteins.

EXPERIMENTAL SECTION

Chemicals and Materials. Acetonitrile, methanol, and 2-propanol (HPLC grade) were obtained from Biolab Scientific Pty Ltd. (Sydney, Australia). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). Ammonium formate and 100% (v/v) acetic acid were purchased from BDH Chemicals Australia Pty. Ltd. (Kilsyth, Australia). NaOH and formic acid 99% (v/v) were obtained from AJAX Chemicals (Sydney, Australia), while trifluoroacetic acid (TFA, peptide synthesis grade) was purchased from Auspep (Melbourne, Australia). The peptide standards (bradykinin, bradykinin fragment 1−5, substance P, [Arg^8]-vasopressin, luteinizing hormone releasing hormone, bombesin, leucine enkephalin, methionine enkephalin, and oxytocin) and proteins (cytochrome *c* and myoglobin from horse heart) were obtained from Sigma Aldrich (St. Louis, MO). Sequencing grade trypsin was purchased from Promega (Madison, WI). The surfactant RapiGest SF was obtained from Waters Corp. (Milford, MA). The ZipTip$_{C18}$ pipet tips were received from Millipore. The untreated fused-silica capillaries were purchased from Agilent Technologies (Waldbronn, Germany).

The stock solution of 100 mM ammonium formate buffer, pH 3.0, was made by titrating 100 mM ammonium formate with formic acid. All the CE running electrolytes were prepared by mixing appropriate proportions of the stock solution, MilliQ water, and the organic solvent and were degassed by ultrasonication for 10 min before use.

The peptide **P1** (Ac-AAEAAHKAY-NH_2) and analogues **P2** (Ac-AHEAAHKAY-NH_2) and **P3** (Ac-AAEHAHKAY-NH_2), used in this study to optimize field-amplified sample injection, were

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synthesized and purified using procedures reported previously.[33] The peptides were first dissolved in water at a concentration of 1 mg/mL and then serially diluted to the designated concentration (10^{-5}–10^{-9} M) with different solvent combinations (see legends to Figures 1–6 for further details).

Protein Digestion and Sample Cleanup. For protein tryptic digestion, cytochrome *c* and myoglobin from horse were dissolved in 50 mM NH_4HCO_3 with 0.1% (w/v) RapiGest SF. After incubating the protein solution in a 37 °C water bath for 5 min, 1 μL of sequence grade trypsin at a concentration of 1 mg/mL was added into 99 μL of protein solution to start the proteolytic reaction. The digestion was stopped 4 h later by adding pure formic acid to a final volume fraction of 1%.

A reversed-phase extraction protocol using the ZipTip C18 system (Millipore, Milford, MA) was employed to desalt the protein digests before the field-amplified sample injection. The ZipTip pipet tip was first conditioned three times with 10 μL of 0.1% TFA in 50% (v/v) aqueous acetonitrile and three times with 10 μL of 0.1% TFA in H_2O. A 20-μL aliquot of protein digest was then loaded on the tip by pipetting the solution 10 times in and out of the tip. After that the tip was rinsed three times with fresh 0.1% TFA in H_2O and the peptides were eluted with 2 volumes of 10 μL of 0.1% TFA in 50% (v/v) aqueous acetonitrile.

CE–ESI-MS Instrumentation. CE–MS experiments were carried out on an Agilent CE capillary electrophoresis system coupled to an Agilent 1100 series LC/MSD-SL ion trap mass spectrometer through an Agilent G1607A orthogonal electrospray interface (Agilent Technologies, Waldbronn, Germany). Electrical contact at the electrospray needle tip was established via a liquid sheath flow delivered by an Agilent 1100 series isocratic LC pump. All system control and data acquisitions were conducted with Agilent ChemStation and MSD Trap Control software.

CE–ESI-MS Conditions. For CE separations, untreated fused-silica capillaries with an internal diameter of 50 μm and a total length of 82 cm were used and thermostated to 25 °C during all experiments. The capillary was conditioned by flushing with water for 3 min, 0.1 N NaOH for 3 min, water again for 3 min, and separation buffer electrolyte for 10 min. Between runs, the capillary was flushed with fresh separation buffer electrolyte for 2 min. During all these flushing steps, the mass spectrometer was set to the "standby" mode. For all the field-amplified sample injection procedures, a fixed volume of 20 μL of low-concentration samples were used and loaded into the capillary by electrokinetic injection after hydrodynamic injection of a water plug at 50 mbar. The nebulizing gas pressure at mass spectrometer side was set to 2 psi during the injections to avoid the coinjection of sample solvent by the aspirating effect of high nebulizing gas pressure.[34] All the experiments were conducted under positive polarity mode at 25 kV (with the anode at the CE inlet and cathode at the MS side). The sheath liquid consisted of 0.1% formic acid in 50% MeOH (v/v) and was delivered at a flow rate of 3 μL/min.

ESI-MS analysis was carried out in the positive ion mode. The electrospray voltage was 3.5 kV, and ion trap mass spectrometer was operated in full scan mode (partially varied in the range of 50–1200 m/z). The nebulizing gas (N_2) pressure, the drying gas (N_2) flow rate, and the drying gas temperature were set at 10 psi, 5 L/min, and 150 °C, respectively. The ion accumulation time was automatically adjusted using the ion charge control (ICC) feature of the instrument. The maximal accumulation time was set at 300 ms, and the ICC target was set at 30 000. Smart parameter setting of the Agilent MSD Trap Control software was utilized to simplify the ion trap operation. The target mass was set at 600 m/z, the compound stability was set at 50%, and the trap drive level was set at 100%. For the tandem mass spectrometry experiments, the instrument was automatically switched from MS to MS/MS mode when the intensity of a particular ion exceeded the preset threshold. Automatic data analysis was achieved by postprocessing with Agilent DataAnalysis software (Version 4.2).

RESULTS AND DISCUSSION

Optimization of Field-Amplified Sample Injection. The choice of separation buffer electrolyte, including its pH and ionic strength, is an important factor that influences the efficiency of field-amplified sample injection. In CE with naked fused-silica capillaries, separation buffer electrolytes of low pH are preferred since positively charged analytes tend to adsorb onto the negative charged capillary walls at intermediate pH range. However, our initial experiments, in which 200 mM formic acid and 1 M acetic acid were employed as the background electrolyte, showed no significant stacking effect for synthetic peptides used in this optimization study, although these volatile background electrolytes are often favored choices for the separation of peptides and other biomolecules in the CE–ESI-MS system.[3,35] Nevertheless, this outcome was not unexpected. According to the basic principles of field-amplified sample injection, better sample enrichment should be obtained with buffer electrolytes of higher ionic strength.[21,26] This conclusion was confirmed by our initial results obtained when a 40 mM ammonium formate buffer, pH 3.0, was used. Moreover, to avoid the generation of excessive Joule heating by using this high ionic strength buffer electrolyte, a small amount of acetonitrile was added to the buffer electrolyte to stabilize the current.[36] The composition of the buffer electrolyte used for the subsequent optimization studies was 20/40/40 acetonitrile/100 mM $HCOONH_4$, pH 3.0/water (v/v/v). One major advantage of this binary buffer electrolyte system is that it is suitable for the stacking and separation of hydrophilic as well as hydrophobic peptides.

The properties of the sample solution, including its acidity and conductivity, have significant impact on the sensitivity of a field-amplified sample injection as demonstrated by Zhang and Thormann[10] with aromatic amines. Sample solutions of low pH and conductivity tend to give the highest sensitivity. Moreover, the presence of a small amount of acid in the sample solution can facilitate the protonation of peptidic analytes and enhance their stacking efficiency.[10,30] Formic acid, acetic acid, and phosphoric acid have previously been used for the field-amplified sample injection of drugs and heterocyclic aromatic amines.[10,22,24,30] However, the proton donating capability of these acids is different, which results in differential effects on the sample stacking. Figure 1 shows the effect of formic acid ($pK_a = 3.74$), acetic acid ($pK_a =$

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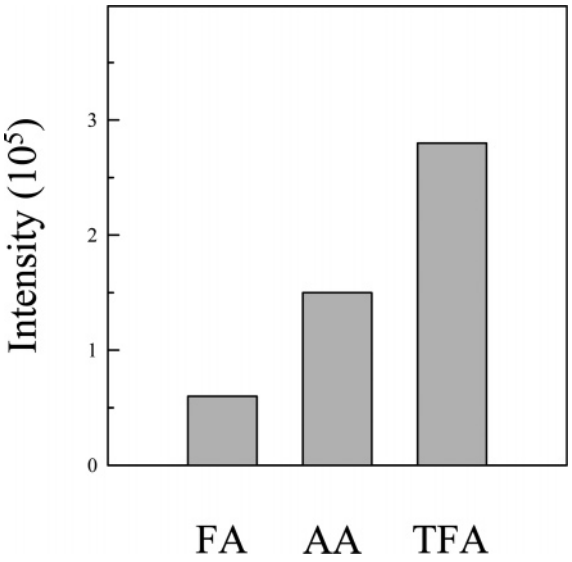

Figure 1. Effects of different acids, formic acid (FA), acetic acid (AA), and trifluoroacetic acid (TFA), in sample solution on detection sensitivity. Separation buffer electrolyte, 20/40/40 acetonitrile/100 mM $HCOONH_4$, pH 3.0/water (v/v/v); separation voltage, 25.0 kV; field-amplified sample injection procedure, hydrodynamic injection of water for 10 s at 50 mbar followed by electrokinetic injection of sample for 120 s at 10 kV; sample, the synthetic peptide **P1** at a concentration of 1×10^{-5} mg/mL dissolved in 50% (v/v) aqueous acetonitrile with 0.1% acid (v/v).

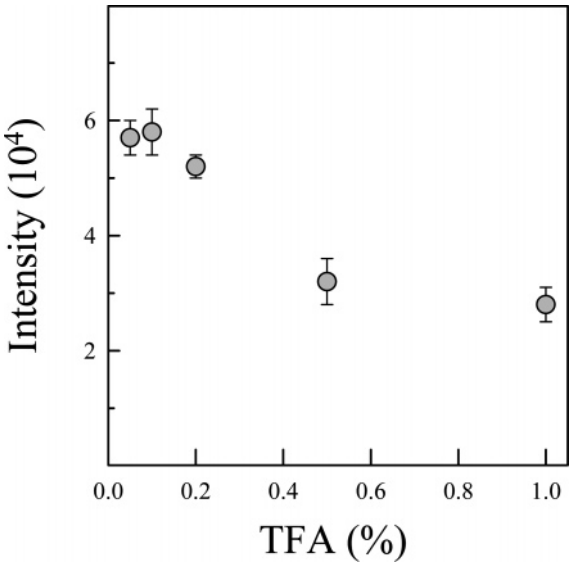

Figure 2. Effects of TFA content in sample solution on detection sensitivity. Sample: the synthetic peptide **P1** at a concentration of 5×10^{-6} mg/mL dissolved in 50% (v/v) aqueous acetonitrile with different contents of TFA (v/v). Other experimental conditions are given in the footnote to Figure 1.

4.79), and trifluoroacetic acid ($pK_a = 0.53$) on the detection response with the synthetic peptide **P1**, Ac-AAEAAHKAY-NH_2. The peak intensity of the synthetic peptide in these experiments was expressed as the peak height in the extracted ion chromatogram (EIC) of the 486.75 m/z ion species (the double charged ion of the peptide). As illustrated in Figure 1, TFA was more effective than formic acid in enhancing the detection sensitivity with this peptide. This is the consequence of TFA being the strongest acid among the three acids examined. At a given concentration, the addition of TFA to the sample solution produced the lowest pH. Nevertheless, the increase in the conductivity of sample solution from the addition of TFA was limited compared to the other two acids due to the larger molecule size and therefore the smaller electrophoretic mobility of the trifluoroacetate ions.

The effects of the TFA volume percentage in the sample solution on the sensitivity of FASI were further investigated, documenting that the optimal content was 0.1% (v/v) (Figure 2) Although increasing the volume percentage of TFA results in a lower pH, the conductivity of the sample solution also increased concomitantly, giving rise to an adverse effect on sample stacking. On the other hand, if the volume percentage of TFA in the sample solution was too low, the analytes were not as effectively protonated, resulting in insufficient sample stacking. Best sample enrichment and detection sensitivity was thus achieved at an optimal TFA content of 0.1% (v/v) in the sample solution.

Addition of organic solvents to a sample solution is a straightforward approach to reduce its conductivity and to enhance sample stacking with low molecular weight polar compounds.[10,30] The effects of three common organic solvents, acetonitrile, methanol, and 2-propanol, on the detection sensitivity were thus investigated. 2-Propanol has the lowest dielectric constant and, therefore, was anticipated on the basis of this criterion to produce the lowest sample solution conductivity at a specified volume percentage. Surprisingly, addition of 2-propanol, however, did not give rise to the highest detection sensitivity. Compared to the acetonitrile−water and the methanol−water binary system at the same volume percentages, the 2-propanol−water system has the highest viscosity.[37] Since the mobility of a solute in a binary buffer electrolyte solvent system is proportional to $\epsilon^{1/2}/\eta$, where ϵ is the dielectric constant and η is the viscosity of the solvent,[10] a charged solute will have the lowest mobility in the 2-propanol−water system, and this will significantly influence its stacking in an electric field. The highest detection sensitivity was obtained when the peptide was dissolved in acetonitrile−water. This is consistent with the properties of the binary system, i.e., a high dielectric constant and a low viscosity, which in turn results in a high mobility for the charged solute. Figure 3 illustrates the effects of acetonitrile content in the sample solution on the detection sensitivity. As demonstrated in Figure 3, the sensitivity was nearly linearly increased with the volume fraction of acetonitrile up to a maximum near 60% (v/v), after which a decline in sensitivity was observed. Compared to the pure aqueous system, a more than 8-fold sensitivity enhancement was achieved when the peptide was injected as a 60% (v/v) acetonitrile solution.

Preinjection of a short water plug before the sample injection has been proposed in FASI[21] to increase the ruggedness of the technique and achieve higher sample stacking efficiency.[21,26] Preinjection of other low-conductivity media, such as a methanol plug, has also been reported.[30] However, some controversy still remains about the necessity of a water plug preinjection.[10,22,23,38] For instance, Sun et al.[38] utilized FASI for the analysis of *Coptidis* alkaloids but found no peak height enhancement with the preinjection of a short water plug before the electrokinetic sample injection. According to theory,[21,26] the total amount of analyte

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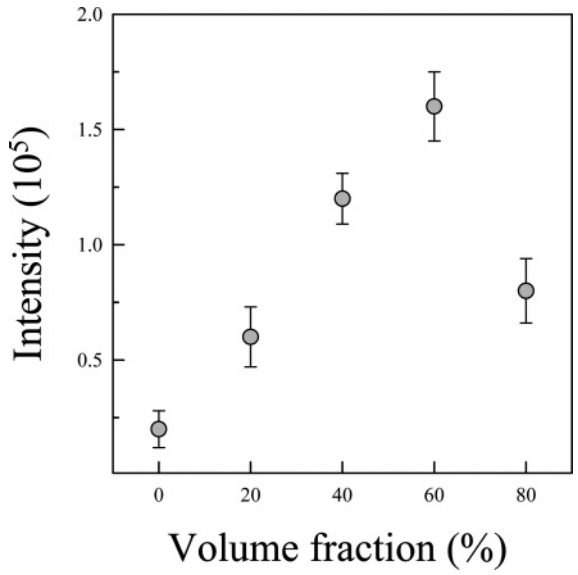

Figure 3. Effects of acetonitrile content in sample solution on sensitivity. Sample: synthetic peptide **P1** at a concentration of 1×10^{-5} mg/mL dissolved in different contents of acetonitrile with 0.1% TFA (v/v). Other experimental conditions are given in the footnote to Figure 1.

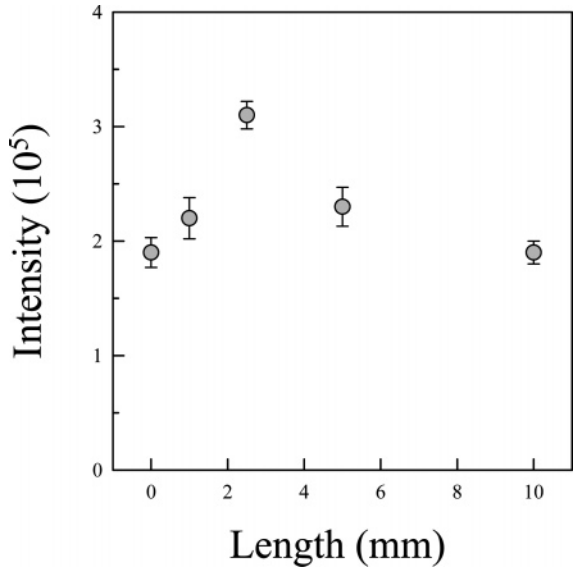

Figure 4. Effects of water plug length on sensitivity. Injection conditions: hydrodynamic injection of water at different time intervals followed by an electrokinetic sample injection for 120 s at 10 kV. Sample: synthetic peptide **P1** at a concentration of 1×10^{-5} mg/mL dissolved in 60% (v/v) aqueous acetonitrile with 0.1% TFA (v/v). Other experimental conditions are given in the footnote to Figure 1.

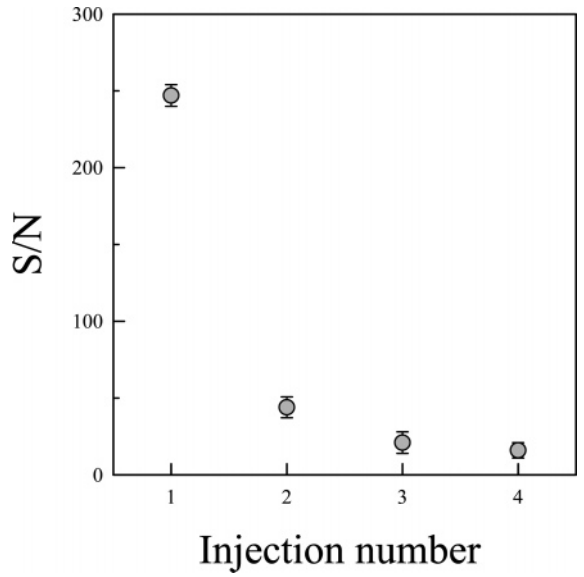

Figure 5. Depletion of the peptide sample by repetitive injections. Conditions: hydrodynamic injection of water for 5 s at 50 mbar followed by an electrokinetic sample injection for 180 s at 10 kV. Sample: synthetic peptide **P1** at a concentration of 1×10^{-5} mg/mL dissolved in 60% (v/v) aqueous acetonitrile with 0.1% TFA (v/v). Other experimental conditions are given in the footnote to Figure 1.

introduced into a capillary by FASI decreases with increasing water plug length. Therefore, the length of the water plug should be kept as short as possible in order to achieve the maximal analyte enrichment and best sensitivity. However, if the water plug is too short, its conductivity will be increased by the introduced charged solutes so quickly and significantly that stacking processes may become impaired and stop prematurely. From several preliminary experiments, as demonstrated in Figure 4, an optimal water plug length that enables the maximal sample enrichment can be readily established. With the system employed, the optimal water plug length was found to be ~2.5 mm, corresponding to a 5-s hydrodynamic injection at 50 mbar. Compared to a control experiment, e.g., no water preinjection, more than 50% sample enrichment could be obtained using this condition.

The influence of electrokinetic sample injection voltage and time on sensitivity was also investigated as the last step of the optimization process. When high injection voltages, e.g., ~20 kV, were applied, the electric current frequently dropped to zero. This was probably due to excessive heat generation and bubble formation under these high electric field strength conditions. On the other hand, if the injection voltage was too low, a much longer injection time is required, resulting in band dispersion of the already stacked sharp sample zone and deterioration in detection sensitivity. In these investigations, as a compromise, a 10-kV injection voltage was selected, which gave rise to an efficient sample stacking and stable injection current even if long injection times were employed. The injection time was further optimized at an injection voltage of 10 kV, and it was found that the best injection time was 180 s. No significant sensitivity improvement was observed when the injection time was longer than 180 s.

Efficiency and Linearity. To test sample enrichment efficiency under the optimized conditions, a series of experiments involving sample depletion injections were carried out. Unlike the previous optimization studies, where the peptide sample was employed only once due to the changes in the composition of the sample solution during electroinjection (although the volume of the sample stayed unchanged), in the sample depletion injection experiments, on the other hand, the same sample was used for several consecutive injections. Figure 5 shows the S/N ratio obtained from the repetitive FASI injections of a 20-μL peptide **P1** sample. The S/N ratio was calculated according to the EIC of the peptide and was obviously a function of the injection number. It is clear that more than 80% of the analyte was injected in the first injection, and more than 90% of the analyte was consumed after two injections. This clearly shows the high

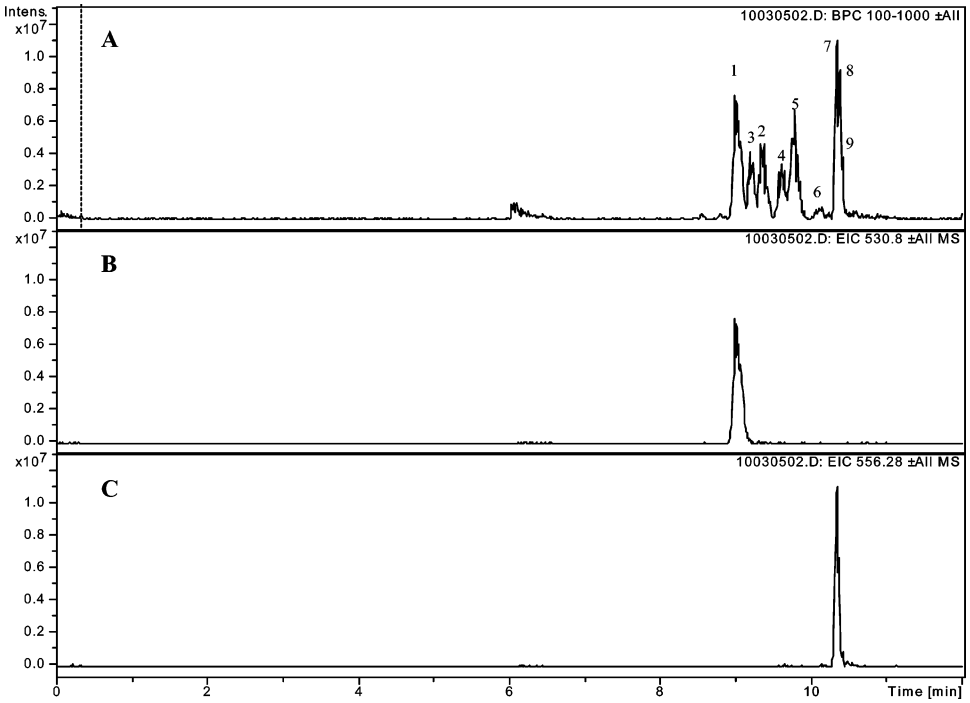

Figure 6. CE−ESI-MS analysis of a standard peptide mixture. (A) Basic peak chromatogram of peptides: (1) bradykinin; (2) bradykinin fragment 1−5; (3) substance P; (4) [Arg^8]-vasopressin; (5) luteinizing hormone releasing hormone; (6) bombesin; (7) leucine enkephalin; (8) methionine enkephalin; (9) oxytocin, (B) The extracted ion chromatograms of bradykinin; (C) the extracted ion chromatograms of leucine enkephalin. Sample concentration: 0.2 μM, diluted from a stock solution with 0.1% TFA in 60% (v/v) aqueous acetonitrile; hydrodynamic injection of water for 5 s at 50 mbar followed by an electrokinetic sample injection for 180 s at 10 kV; other experimental conditions are given in the footnote to Figure 1.

efficiency of the optimized method in sample enrichment. Moreover, by comparing the peak intensity of the peptide obtained from the hydrodynamic injection of a concentrated sample and the FASI injection of a diluted sample, the sensitivity enhancement factor can be calculated. When the peptide sample at a concentration of 5×10^{-6} M was injected hydrodynamically for 5 s at 50 mbar, the peak intensity obtained was 6.3×10^4. After the peptide was diluted 1000-fold to a concentration of 5×10^{-9} M and injected into the capillary with the optimized FASI method, a peak intensity of 21.0×10^4 was obtained. This corresponded to an enhancement factor of more than 3000 in terms of sensitivity. Moreover, to validate the optimized FASI method, parallel experiments with two analogues of peptide **P1**, namely, **P2** (Ac-AHEAAHKAY-NH_2) and **P3** (Ac-AAEHAHKAY-NH_2), were further carried out, whereby similar sensitivity enhancement factors were obtained.

In addition, the dependence of peak area on the injected peptide concentration in the 5−1000 ng/mL range was examined with the developed FASI stacking method. For these experiments, the synthetic peptide **P1** was serially diluted with 0.1% TFA in 60% acetonitrile (v/v) and loaded into the capillary with the optimized FASI method. A good linear relationship, with a correlation coefficient $r^2 = 0.9977$, was found over this concentration range, indicating the suitability of the method for quantitative analysis.

Applications to Bioactive Peptides and Protein Tryptic Digests. The successful application of capillary electrophoresis−mass spectrometry in proteomics lies to a great extent in its capability to analyze complex mixtures of biomolecules present in low abundance. To evaluate the viability of the established FASI method for the analysis of such samples, a peptide mixture and several protein tryptic digests were employed to replicate such proteomic applications. The peptide mixture contains nine bioactive peptide hormones that have diverse physicochemical properties and structures. The stock solution of these peptides was consecutively diluted to a concentration of 0.2 μM with 0.1% TFA in 60% (v/v) aqueous acetonitrile. The diluted sample was then injected and concentrated by FASI and separated by capillary electrophoresis. As shown in Figure 6, most peptides have excellent signal-noise ratios, and despite the fact that some peptides were only partially resolved, they could be easily identified by mass spectrometry.

Analogous signal-to-noise enhancement results were also obtained when the method was applied to the analysis of low-abundance tryptic fragments derived from proteins. Two proteins, cytochrome *c* and myoglobin, at a concentration of 0.5 μM were digested with a modified procedure. An acid-labile surfactant, RapiGest SF, was added to 50 mM NH_4HCO_3 to enhance the enzymatic in-solution digestion of proteins. As a result, rapid and efficient protein digestion was achieved, although the protein digests could not be directly applied for field-amplified sample injection due to the presence of high concentration ammonium bicarbonate buffer and surfactant. In this case, a ZipTip desalting procedure was employed. One advantage of the method is that the optimized composition of the sample solvent, 0.1% TFA in 60% acetonitrile, is very close to the standard ZipTip elution solvent, i.e., 0.1% TFA in 50% acetonitrile. Therefore, protein digests can be applied directly in the subsequent field-amplified sample injection and CE separation after a ZipTip desalting process. Due to the high sample stacking efficiency of the method, the mass signal of the individual tryptic peptides was significant. When the

Table 1. Tryptic Peptides Observed from the Digestion of Horse Cytochrome *c* and Matched with the Correct SwissProt Database Accession Number P00004

obsd *m/z* value[a]	calcd mass MH^+ (Da)	residue	sequence
634.4 (+1)	634.4	9−13	IFVQK
584.9 (+2)	1168.6	28−38	TGPNLHGLFGR
812.3 (+2)	1623.8	61−73	EETLMEYLENPKK
403.7 (+2)	806.5	73−79	KYIPGTK
779.4 (+1)	779.4	80−86	MIFAGIK
454.2 (+2)	907.5	80−87	MIFAGIKK

[a] Charge state shown in parentheses.

Table 2. Tryptic Peptides Observed from the Digestion of Horse Myoglobin and Matched with the Correct SwissProt Database Accession Number P68082

obsd *m/z* value[a]	calcd mass MH^+ (Da)	residue	sequence
536.4 (+3)	1606.9	17−31	VEADIAGHGQEVLIR
636.4 (+2)	1271.7	32−42	LFTGHPETLEK
395.7 (+2)	790.4	57−63	ASEDLKK
618.9 (+3)	1853.9	80−96	GHHEAELKPLAQSHATK
374.8 (+2)	748.4	134−139	ALELFR
471.3 (+2)	941.5	146−153	YKELGFQG

[a] Charge state shown in parentheses.

mass intensity of a peptide exceeded a preset threshold, an automatic MS/MS experiment was triggered. After data analysis on the obtained tandem mass spectra, the results were submitted to the MASCOT database searching program. By comparison of actual tandem mass spectra of selected peptides with the predicted fragmentation patterns, the two investigated proteins, cytochrome *c* and myoglobin, were successfully identified as top candidates, with sequence coverage higher than 40% for both proteins. The tryptic peptides observed and identified by MASCOT database searching algorithms are listed in Table 1 and Table 2. Due to the limitation of current database searching algorithms,[39] some of the tryptic peptides could not be directly identified by these bioinformatics techniques. Nevertheless, manual interpretation of the obtained mass spectra and tandem mass spectra gave rise to higher levels of sequence coverage.

(39) Chamrad, D. C.; Koerting, G.; Stuehler, K.; Meyer, H. E.; Klose, J.; Blueggel, M. *Proteomics* **2004**, *4*, 619−628.

CONCLUSIONS

Field-amplified sample injection is an electrokinetic sample enrichment technique that has been used for a wide range of pharmaceuticals, due to its simplicity and high efficiency. However, much less attention has previously been paid to optimize the technique for peptide analysis so that maximal sample enrichment and better detection sensitivity can be achieved for the analysis of low-abundance samples, such as those derived from protein digestion in proteomic studies. As shown in this investigation, the injection conditions, such as the properties of sample solution with regard to its acidity and conductivity, the length of water plug, sample injection time, and voltage have significant influence on the efficiency of FASI for peptide stacking. After optimization of these conditions, very effective sample stacking was obtained as indicated by the sample depletion injection experiments. As a result, greater than 3000-fold sensitivity enhancement was achieved and detection of peptides in the subnanomolar concentration range could be readily achieved with the optimized method. The sensitive analysis of peptide mixtures of low concentration and identification of low-abundance proteins with high confidence clearly demonstrate the great potential of the technique for proteomic applications.

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