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Enhanced Neuropeptide Profiling via Capillary Electrophoresis Off-line Coupled with MALDI FTMS

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Abstract

An off-line interface incorporating sheathless flow and counter-flow balance is developed to couple capillary electrophoresis (CE) to matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI FTMS) for neuropeptide analysis of complex tissue samples. The new interface provides excellent performance due to the integration of three aspects: (1) A porous polymer joint constructed near the capillary outlet for the electrical circuit completion has simplified the CE interface by eliminating a coaxial sheath liquid and enables independent optimization of separation and deposition. (2) The electroosmotic flow at reversed polarity (negative) mode CE is balanced and reversed by a pressure-initiated capillary siphoning (PICS) phenomenon, which offers improved CE resolution and simultaneously generates a low flow (<100 nL/min) for fraction collection. (3) The pre-deposited nanoliter volume DHB spots on Parafilm-coated MALDI sample plate offers improved substrate for effective effluent enrichment. Compared with direct MALDI MS analysis, CE separation followed by MALDI MS detection consumes nearly 10-fold less sample (50 nL) while exhibiting 5 to 10-fold enhancement in S/N ratio that yields the limit of detection down to 1.5 nM, or 75 attomoles. This improvement in sensitivity allows 230 peaks detected in crude extracts from only a few pooled neuronal tissues and increases the number of identified peptides from 19 to 43 (*C. borealis* pericardial organs (n=4)) in a single analysis. In addition, via the characteristic migration behaviors in CE, some specific structural and chemical information of the neuropeptides such as posttranslational modifications and family variations has been visualized, making the off-line CE-MALDI MS a promising strategy for enhanced neuropeptidomic profiling.

Keywords

Capillary electrophoresis; Siphoning; Off-line coupling; MALDI FTMS; Neuropeptides; *Cancer borealis*

Introduction

Neuropeptides (NPs) are an important class of signaling molecules that regulate many essential physiological functions of nervous system.¹ The identification and characterization of NPs represent significant challenges because they are often active at very low and wide dynamic ranges (pM-nM level *in vivo*). Furthermore, the analysis is often complicated by the interferences such as high salt content from the circulating fluids or abundant lipids and proteins present in complex tissue samples. Recently, the advances in mass spectrometry (MS) have provided a new and powerful analytical platform for studying NPs, which leads to the characterization of more than 1,000 peptides.² Our lab has developed several combined MS-

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based peptidomics strategies^{3, 4} for neuropeptide profiling, with an overall goal to obtain simultaneous visualization and identification of the entire complement of peptides as well as the posttranslational modifications (PTMs) in a given tissue. However, numerous endogenous peptides present in extremely low abundance are below the detection limits of our current techniques. Therefore, there is a great need to develop new analytical strategies with high resolution and specificity for the global analysis of NPs.

Capillary electrophoresis (CE) is a powerful tool for complex biological mixture separations.^{5, 6} Since its introduction,⁷ the hyphenation of the highly efficient CE separation with MS has gained increasing acceptance for proteomics and glycomics research.^{8–11} The on-line CE/electrospray ionization (ESI) MS hyphenating method is more suitable for automation;⁹ however, it demands a MS instrument of fast data acquisition rate¹² to gather sufficient data points across the CE peak as well as a robust interface to introduce CE flow into MS analyzer. Various interfaces have been constructed to improve the CE-MS coupling, with the low sheath flow^{13, 14} and sheathless nano-flow^{15, 16} interfaces showing the greatest promise for sensitivity improvement.

Off-line coupling of CE to MALDI MS represents an alternative to the on-line CE/ESI MS,^{17, 18} which offers increased flexibility for independent optimization of CE and MS experiments, making the majority of the CE fractions available for further biochemical characterization such as the on-target fast derivatization¹⁹ or digestion.^{20, 21} Furthermore, the interface design for off-line coupling of CE and MALDI is relatively straightforward and faces less difficulty than the on-line CE/ESI MS by avoiding the need for a co-electrical field between the CE outlet and the ESI-emitter.

Several previous off-line CE-MALDI MS coupling methods involved fraction collection in vials^{22–24} or elution onto the sample target by a larger coaxial sheath flow with or without MALDI matrix.^{21, 25–29} While effective a problem of sensitivity loss was noted due to significant dilution caused by a much larger volume of supplemental fluid to the CE effluent. To address this problem, Johnson *et al.*³⁰ reported the use of a prestructured sample support (with Teflon coating) for fraction collection. In this design, dilution by the matrix/buffer solution was recuperated by the concentrating effect of the hydrophobic Teflon surface. However, the use of such prestructured sample support is not readily available to most labs and increases the analytical cost greatly.

To address some of these issues, we used a porous joint^{28, 31} made of cellulose acetate membrane³² near the capillary outlet for the off-line CE-MALDI experiment. This sheathless interface enables decoupling of separation and deposition as compared to the conventional coaxial sheath liquid assisted deposition. The elimination of sheath flow also minimizes dilution problem and maintains high separation efficiency of CE as no dead volume is involved. Furthermore, the CE effluents were directly deposited onto a Parafilm coated nanoliter DHB spots³³ for more homogenous crystallization and better sample enrichment due to concentration effect on hydrophobic surface.

In addition to sensitivity improvement, another important consideration for successful CE-MS coupling experiments is enhancing resolution. One approach to resolution improvement is to reverse the electroosmotic flow (EOF) direction (by reversing polarity or employing inner wall coating) while directing the flow towards outlet to keep the analytes longer in capillary for better separation. This was first realized by Jorgenson and coworkers via pressure application on the capillary column during separation,³⁴ termed as pressure-induced flow counterbalanced capillary electrophoresis (FCCE) technique. The idea has since been used to improve the separation of capillary electrochromatography³⁵ or to selectively elute the species of interest while stabilizing others in the capillary.³⁶

In this paper, we modified the FCCE technique by combining “pressure initiation” and subsequent “siphoning-driven” stream in the capillary to counterbalance the flow. We termed this method “pressure initiated capillary siphoning (PICS)” and employed this phenomenon to produce a bulk flow toward the outlet of capillary while reversing the polarity of CE to direct the EOF toward the inlet under a suitable electric field. This new simple method has effectively improved the resolution of CE separation by increasing the duration of analytes retained in the capillary at a given CE system, thus extending both the time the analytes migrate in the electric field and the effective length, which greatly facilitates separation of complex NP mixtures of tissue extracts. This improvement in resolution in combination with the sheathless flow interface and nanoliter matrix-precoating on parafilm substrate for CE fraction collection enables the construction of an improved CE-MALDI MS platform for NP analysis.

The electrophoretic migration theory and models of peptide separation in CE have been utilized to study the migration behaviors of peptides.^{37–39} CE has also gained increasing attention for the analysis of a variety of signaling molecules—from small amines and amino acids to NPs and larger proteins.⁴⁰ However, the applicability of CE based on the electrophoretic migration behaviors for NP mapping has not been fully demonstrated. Here, we employed characteristic electrophoretic migration pattern for global NP analysis. Because NPs exist in super-large families NPs sharing similar structural motifs either at the C-termini or N-termini, this unique feature provides an excellent means to verify the NP identity⁴¹ or to discover novel member (s) of a known NP family based on similar migration patterns. We developed a migration matching factor based on the observed migration time versus calculated value for enhanced NP identification and peptide family assignment. By doing this, some specific structural and chemical information such as pyroglutamylation modification and family variations have been visualized via the characteristic migration behaviors of the NPs in CE, which facilitates the subsequent classification and identification of novel NPs.

Experimental Section

Chemicals and Materials

Methanol, acetonitrile (ACN), ammonium hydroxide, trifluoroacetic acid (TFA) and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Cellulose acetate, 39.7 wt%, average MW 50,000, Acetyl chloride, formaldehyde-H₂ (FH₂, 37% in H₂O), and formaldehyde-D₂ (FD₂, Isotec, ~20% in D₂O) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). 2, 5-dihydroxybenzoic acid (DHB) was obtained from ICN Biomedicals Inc (Costa Mesa, CA). Parafilm “M”® was obtained from Pechiney Plastic Packaging (Menasha, WI). C18 Ziptip column was manufactured by Millipore and all water used in this study was doubly distilled on a Millipore filtration system (Bedford, MA). The physiological saline consisted of (in mM): NaCl, 440; KCl, 11; MgCl₂, 26; CaCl₂, 13; Trizma base, 11; maleic acid, 5; pH 7.45.

Neuropeptide Standards

A collection of 25 neuropeptide (NP) standards was used to generate a mixture for the method evaluation. Table 1 gives the details including the accurate molecular mass, sequence, net charges from neutral to acidic conditions, and the concentrations used for experiments. Among these NPs, **P** 1, 3, 4, 7, 9, 10, 14, 16–19, 25 were purchased from the American Peptide Company (Sunnyvale, CA). **P** 2, 5, 6, 8, 11–13, 15, 20, 21 were synthesized at the Biotechnology Center of the University of Wisconsin at Madison. **P** 22–24 were synthesized at the Biotechnology Center of the University of Illinois at Urbana-Champaign (kind gift of Professor Jonathan Sweedler).

Animal Dissection and Tissue Extraction

Jonah crabs *Cancer borealis* were purchased from the Fresh Lobster Company (Gloucester, MA) and maintained without food in an artificial seawater tank at 10–12 °C. Details of the animal treatment and dissection were described previously.^{42, 43} Briefly, animals were cold-anesthetized by packing in ice for 15–30 min prior to dissection, the brain, pericardial organs (PO), commissural ganglia (CoG) were dissected in chilled physiological saline. Neuropeptides were extracted as described previously,⁴⁴ briefly, the organs were combined and homogenized and peptides were extracted using ice-cold acidified methanol (methanol: glacial acetic acid: water/90:9:1). The extract was dried down and resuspended with 5–10 μ L of water containing 0.1% formic acid.

In Solution Formaldehyde Labeling of Pericardial Organ Extract

In solution formaldehyde labeling was performed as described previously.⁴⁴ Briefly, the extract contains one *C. borealis* PO was added with formaldehyde- H_2 (FH_2 , 4% in H_2O , 1 mL) and then vortexed prior to the addition of sodium cyanoborohydride ($NaCNBH_3$, 26 mM, 1 mL, prepared fresh daily). The mixture was vortexed again and then reaction was allowed to proceed at room temperature for 5 minutes prior to analysis. Deuterium labeling was performed with another PO tissue, which was treated identically except formaldehyde- D_2 (FD_2) was added in place of FH_2 in the procedure. Two equal aliquots (15 μ L each) of the above labeled products were mixed together prior to analysis.

Sample Preparation Procedure

The above samples were ready for CE analysis without further treatment. For direct MS analysis, 10 μ L of the resulting solution was desalted by ZipTipC18 and eluted in 5 μ L of 50% acetonitrile/50% water containing 0.1% TFA.

Cellulose Acetate Membrane-Coated Porous Joint

The procedure for construction of an on-column fracture was modified from Whang and Chen.³² The fused-silica capillary of 82-cm-long (50 μ m i.d./360 μ m o.d. Polymicro Technologies, Phoenix, AZ) was placed over a 1-cm $\times$ 0.3-cm glass slide, on which two small drops of QuickGrip® glue (Beacon Adhesives Co., Mt Vernon, NY) were preloaded on each end to form a V-shaped notch in the center. The capillary was affixed near one end (about 7 cm) by the glue onto the glass slide by leaving a distance of *ca.* 1–2 mm from glass surface. After glue dried in hood, a small scratch was made on top of the exposed column using a capillary cutter (Chromatography Research Supplies, Inc. Louisville, KY). The capillary was then pushed up gently from the bottom, directly under the scratch, with a small pointed stylet thereby forming the fracture. A small drop of 12% (w/v in acetone) cellulose acetate (CA) solution was dripped onto the fracture covering the glass surface and the fracture. Under a gentle stream of air and with slight rotation of the whole glass slide, a thin film of CA membrane was uniformly coated over the fracture region.

The fracture assembly was left to dry for more than 1 hour and then placed in a 0.6-mL plastic vial (Fisher Scientific, Pittsburgh, PA) with the short section (6 cm) stretched out of a hole (1 mm) sealed at the bottom. The vial was then filled with buffer solution and a platinum-wire electrode was dipped in the solution. An enlarged scheme of the polymer-coated capillary joint is illustrated in Figure 1A.

“Pressure Initiated Capillary Siphoning (PICS)” in CE

The home-built CE apparatus consisted of a high voltage power supply (HV30KVD, dual-polarity, 0 to $\pm$ 30 kV, Unimicro Technologies Inc., Pleasanton, CA), the capillary assembly described above and some small accessories. As shown on the right side of Figure 1B, the

running buffer (background electrolyte) containing sodium phosphate (or ammonium formate, or their cocktails with varying concentration and pH) was introduced into a syringe, with a Pt electrode and separation capillary mounted via ferrules (Upchurch Scientific, Inc. Oak Harbor, WA). The procedures of generating PICS include the following steps: 1) place the outlet of capillary at a Δh (up to 8 cm) below the inlet; 2) insert the capillary into the running buffer reservoir (through the adapter); 3) apply a micro-pressure of ~ 0.2 psi for 10s to initiate the siphoning; 4) retract the pressure; 5) take the capillary out of the buffer and place in air for a brief moment up to several seconds to disconnect the capillary inlet with the buffer to release the pressure; 6) place the capillary inside the buffer again. The siphoning flow was thereby generated and flowed toward the outlet automatically (see the “PICS” flow in Figure 1B).

Capillary Treatment and Sampling

Prior to use, new capillary was rinsed/flushed with (1) 75:25/ NaOH (1.0 M):MeOH, (2) water, (3) 0.1 M NaOH, (4) air, (5) water again, and (6) running buffer under ~ 0.5 psi in sequence for 5 min in each step, followed by electrophoretic equilibration with the separation buffer for 10 min prior to injection of sample. Except for the first two steps, the remaining steps were repeated between CE runs to remove any residual peptides adsorbed onto the capillary wall.

Prior to moving the capillary into running buffer to start the PICS, the injection of sample was performed by pressure at 0.5 psi for 3s (~ 50 nL). The pressure injection was chosen for two reasons. The first one was that it only needs 2–5 μ L starting solution to immerse the capillary tip instead of both the capillary and the electrode used in electrokinetic injection. The second reason for selecting pressure injection is that a large volume injection can be performed and the electric-discrepancy for some ions can be avoided.⁴⁵ Electrophoresis was initiated by applying a voltage between the inlet and the on-column porous joint. In most cases, we used a reversed polarity (negative) mode CE (from -14 to -20 kV) by reversing the polarity to yield an EOF toward the inlet end (see Figure 1B).

Flow Rate Measurement and Fraction Collection by on-Parafilm Spots

The flow rates of PICS alone and PICS with EOF were determined by using a pipette tip (GPS-L10, RAININ Inc. Oakland, CA). The tip has accurate scale labels at 2- μ L. The flow rate was determined by measuring the time used for collecting 2- μ L liquid into the tip through the orifice. The fractions were deposited, in every 30 s (or 60 s), onto the tiny matrix spots pre-deposited onto the hydrophobic Parafilm. The custom-built Parafilm-supported nanoliter volume DHB spots were described elsewhere.³³ Briefly, a narrow piece of Parafilm M was cut to the size of 2.5 cm (l) $\times$ 0.4 cm (w), stretched to 8.0 cm and directly placed onto the MALDI sample target. About 50 nL DHB matrix (150 mg/mL in 50:50/ methanol: water) was deposited by capillary onto the film. Upon drying small spots of about 400 μ m were formed due to the solvent repellent property of the film. The fraction collections were conducted by moving the sample target up and down to make a gentle contact between the DHB-predeposited spot and the newly formed droplet at the capillary tip.

MALDI FTMS

Mass spectra of the CE fractions were recorded on a Varian IonSpec Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0 T actively shielded superconducting magnet. The FTMS instrument consisted of an external high-pressure MALDI source. A 355 nm Nd: YAG laser (Laser Science, Inc., Franklin, MA) was used to create ions that can be accumulated in the external hexapole storage trap before being transferred through a quadrupole ion guide to the ICR cell. All mass spectra were collected in positive ion mode. The ions were excited prior to detection with an rf sweep beginning at 7050 ms with a width of 4 ms and amplitude of 150 V base to peak. The filament and quadrupole trapping plates

were initialized to 15 V, and both were ramped to 1 V from 6500 to 7000 ms to reduce baseline distortion of peaks. Detection was performed in broadband mode from m/z 108.00 to 2500.00.

MALDI FTMS SORI-CID Analysis

Fragmentation of peptides was accomplished by sustained off resonance irradiation and collision-induced dissociation (SORI-CID). An arbitrary waveform with a ± 10 Th isolation window was introduced to isolate the ion of interest during the period of 2000–2131 ms. Ions were excited with a SORI burst excitation (2.648 V, 2500–3000 ms). A pulse of N_2 was introduced through a pulse valve from 2500 to 2750 ms to elevate the pressure to 10^{-6} Torr that induced collisional activation leading to subsequent fragmentation.

Peptide Net Charge Calculation

The net charge q of a peptide at a certain pH can be estimated by using the formula ⁴⁶

$$q = \sum_i N_i \frac{10^{pK_{a_i}}}{10^{pH} + 10^{pK_{a_i}}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pK_{a_j}}} \quad (\text{where } N_i \text{ is the number and } pK_{a_i} \text{ is the } pK_a \text{ values of the N-terminus and the side chains of R, K, and H. The } j\text{-index pertains to the C-terminus and the side chains of D, E, C, Y amino acids. The residues are assumed to be independent of each other. N- and C-termini have fixed } pK_a \text{ values, except modified termini which are ignored. Modified residues, e.g. phosphorylated serines (pS) are ignored. Cysteines are assumed not to take part in disulfide bridges, i.e. they may contribute to the charge). The details including the } pK_a \text{ values of twenty amino acid residues can be found in reports.}^{38, 46, 47}$$

Results and Discussion

Evaluation of the Cellulose Acetate Membrane-Coated Porous Joint Used in CE

The main reason for choosing the cellulose acetate (CA) membrane porous joint is its reliability for use. Compared to the etched porous joint (by HF) which is often very fragile, the CA membrane joint is much more robust when installing into a buffer reservoir. Furthermore, the porous joint can maintain stable performance for a long time (> one month), which is important when repetitive experiments need to be performed. Other features of the CA membrane joint include the zero dead volume, good electric conductivity, and easily reproducible fabrication without the need for extensive training or special apparatus.

A well-fabricated porous cellulose acetate (CA) joint should possess the following characteristics: 1) Care should be taken to prevent the clogging of the porous joint by CA. This was tested by flushing the capillary with water or CE buffer at 0.5 psi. A fluent flow of *ca.* 200 nL/min seen from the column outlet verifies the good connection of the joint. 2) No leakage. This was tested by flushing the capillary with air while keeping the joint in water and no bubbles should appear, verifying that the CA coating was uniform and no leakage was observed. 3) Good electrical conductivity. This was inspected by performing electrophoresis between the joint and the capillary inlet with buffers. Nice linear relationship between current (0–70 μ A) and voltage (0–30 kV) was obtained with 0.5% ammonium acetate buffer, pH 4.5 as running buffer (Figure S-1, Supporting Information, SI). Capillaries that meet the above criteria are ready for use, and can usually be stored for more than 6 months without losing performance. The CA joint is stable in the presence of 25% (V/V) organic additives in the running buffer such as methanol and ACN but could be damaged by less than 10% acetone solution.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

PICS and the Flow Rate under Different Polarity Modes

In contrast to the conventional integrated CE systems where capillary siphoning is considered or controlled⁴⁸ to a negligible level, on this custom-built CE device the siphoning flow was observed as a steady large flow lasting for 2 to 4 hours once initiated by the micro-pressure. Briefly, we applied a micro-pressure to start the siphoning inside capillary and the pressure was retracted prior to the CE run, thus no external pressure was applied during the separation, indicating that the bulk flow was maintained by siphoning only. Therefore, “pressure-initiated capillary siphoning” (PICS) is fundamentally different from the terms such as “pressure-driven” or “pressure-assisted” because this specific counter-flow CE technique via siphoning is initiated by a pressure but relies on siphoning in the capillary to maintain its flow. Unlike pressure-driven flow that would produce a parabolic flow leading to reduced resolution, the flow profile produced by PICS is not well understood. A complete understanding of the fluidic pattern of siphoning-dominated bulk flow in the presence of EOF would require further investigation by some special techniques such as whole column imaging⁴⁹ detection⁵⁰ or computer simulation⁵¹

The PICS flow rate (v_s) was determined to be ~ 75 nL/min ($n=3$) by using ammonium formate buffer (50 mM, 10% ACN, pH 3.5, 25 °C) with the height difference ($\Delta h = h_{\text{inlet}} - h_{\text{outlet}}$) of 7.5 cm. When electrophoresis was started under voltage of ± 18 kV, the flow rates of CE stream changed to ~ 96 nL/min ($n=3$) and ~ 61 nL/min ($n=3$), corresponding to the flow in positive mode CE ($v_s + v_{\text{eof}}$) and negative (reversed polarity) mode CE ($v_s - v_{\text{eof}}$), respectively. Note that the evaporation of the liquid at the capillary outlet and inside the pipette tip was not taken into account for the flow rate measurement, therefore the determined flow rate can be viewed as an “apparent” flow rate. Further studies will be performed to determine the extent of evaporation and its effect on the CE experiment. Another important issue of concern is the gas formation at the electrodes, as described previously in a comprehensive review on CE/MS.⁹ Several factors including the CE current, flow rate and the buffer pH change would affect the amount of generated bubbles in CE. In our CE assembly (Figure 1) the electrode is positioned about 2-mL (~ 3.5 cm in distance) away from the capillary inlet (see Figure S-2 for the detailed schematic drawing of the CE inlet). This is a fairly large distance as compared to conventional CE in which the electrode and the capillary inlet are usually put together in a small vial. Furthermore, the current was controlled to a low level (~ 20 μ A) by applying low electric field and low concentration buffer, which minimized the production of hydrogen gas at the inlet end. Therefore, the bubble formation was not taken into account in the PICS technique reported here.

The siphoning flow was several times greater than the EOF and resulted in the migration of all of the analytes towards the capillary outlet. The significant suppression of the flow rate of EOF was mainly due to two factors. Firstly, the EOF mobility (μ_{eof}) was very low when using a low pH (pH 3.5) buffer solution, which was about $5.0 \times 10^{-5} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$, eight to nine times lower than that at high pH (e.g., pH 9.0).⁵² Secondly, the electric field strength ($E = 18 \text{ kV}/75 \text{ cm}$) was also very low in comparison with a routine CE experiment. The theoretical EOF velocity ($v_{\text{eof}} = \mu_{\text{eof}} \times E$) under this condition was ~ 14.4 nL/min, which matched well with the experimental result. On the other hand, the unique construction of CE setup produced siphoning free from backpressure because the capillary outlet was completely open in contrast to the format being surrounded with buffer in conventional CE.

The flow balance was successfully achieved inside the capillary with the PICS technique, by adjusting the voltage and the Δh to control the flow rate toward two ends. It was shown that the highest positively charged peptide was kept in the capillary for much longer time (over 35 minutes) than the negatively charged ones, resulting in greatly improved resolution of the CE separation. The siphoning-assisted flow counterbalance and sheathless deposition offers

several attractive features such as simplicity for construction and steady but reduced flow rate (and thus increased mass sensitivity) for fraction collection and deposition.

Evaluation of the Method with a Complex Mixture of Neuropeptides

A collection of 25 neuropeptides (NPs) with concentrations of 10^{-6} to 10^{-7} M was selected for the evaluation of this method (Table 1, for the $q/M^{1/2}$ and M_f values, see the discussion below). About 50 nL of samples were injected into the capillary for electrophoresis under both positive (+16 kV) and negative (−15 kV) modes of CE in the presence of the PICS flow. Running buffer was a cocktail of ammonium formate (100 mM): H₂O: ACN with a ratio of 5:4:1, at pH 3.9. The fractions were collected every 30s for a total of 40 min followed by MALDI FTMS analyses of these off-line deposited fractions. As expected, the separation under positive mode CE with PICS was much faster than that under negative mode, whereas the latter separation provided much better resolution for the mixture components.

The improvement of resolution also has an effect on sensitivity enhancement. With the same sampling volume, separation buffer, and MS detection settings, the MS signal intensities of the peptides under reverse polarity mode CE/PICS were found to be about 5-fold higher than that under positive mode. As a result, all 25 NPs were detected in reverse polarity mode CE but 4 of the 25 NPs were not detected under positive CE mode because of inadequate ion abundance. One possible reason was due to longer and more effective focusing of the peptides in capillary and better separation in reverse polarity CE mode which reduced possible analyte suppression from co-eluting peaks. Sensitivity improvement was evident with S/N >200 for peptides at 1×10^{-7} M. Limit of detection of 1.5 nM was obtained for 75 attomole peptide from 50 nL volume of solution at S/N = 3.

Linear Fitting of $q/M^{1/2}$ Values vs. Migration Time (t)

The classical semi-empirical formula in electrophoresis reveals a linear correlation between the electrophoretic mobility (m_e) and $q/M^{1/2}$ value (where q is a peptide's net charge, M is the molecular mass).^{38, 53} Based on this, it is possible to evaluate electrophoretic migration behavior,³⁷ study structural modifications and conformational changes of peptides and proteins with CE.³⁹

The studied NPs were plotted as q against migration time (t) in Figure 2, left panel. The closed circles represent the peptides being separated using a positive mode CE with PICS. The open circles represent those separated with negative mode CE. The migration order was observed linearly related to the net charges, as extracted and shown in the inset below. It was observed that the larger m_e the peptide has, the more quickly it elutes in CE when operating in positive mode. Therefore, the migration time (t) of a peptide is inversely proportional to its m_e in the positive CE. In contrast, the t value is proportional to its m_e , and accordingly proportional to its $q/M^{1/2}$ value in the negative CE mode. These observations are plotted in Figure 2 (a–d) showing the relationships of the q and $q/M^{1/2}$ values and CE migration time under negative CE. It is noted that the pyroglutamylated peptide corazonin, pQTFQYSRGWNa (MH⁺ = 1369.7) migrates very early in the negative CE, and elutes very late in the positive CE together with other acidic peptides such as the orcokinin family peptides. The calculated net charge at pH 3.9 is +2 if the N-terminal pGlu-modification is neglected, whereas the observed migration behavior indicates a −0.2 net charge for the pyroglutamylated peptide, slightly higher than the orcokinin family peptides (−0.33). Other known pyroglutamylated peptides were also observed to elute earlier during the negative CE separation, as is shown later in the results section.

Figure 2b depicts the linear fitting of $q/M^{1/2}$ values for all the 25 peptides against migration time ($q/M^{1/2}$ - t), a fairly linear correlation is obtained ($R^2=0.903$). With the removal of the badly deviated spot (MH⁺ = 1369.7), the correlation improves to $R^2=0.979$ (Figure 2c), which is

consistent with the classical semi-empirical relationship. It is observed that the fitting with only q against t (Figure 2a) also shows a linear correlation with even better R^2 (0.98) than the $q/M^{1/2}$ - t curve, indicating a direct linearity between q and t , which is independent of the molecular mass, M . The peptides with the earliest ($n=2$) and latest ($n=2$) migration time were used for another $q/M^{1/2}$ - t linear fitting, as shown in Figure 2d, yielding a line that is very close to that with the remaining 24 peptides included in 2c. This result suggests that the acquired fitting curve with greatly reduced number of peptides deviates only slightly from that with all peptides included. Such simplified method should be useful for plotting the fitting curves in real sample analysis where the $q/M^{1/2}$ values for most of the peptides are unknown. Since only limited data is needed to generate the fitting curve, it is often possible to identify a few known peptides from both ends of the electrophoretic migration order. Therefore, it is applicable for complex extract analysis, provided that the regressive validation of the fitting curve with all of the assigned peptides can give a satisfactory matching. This approach offers an increased confidence for peptide identification and was employed for the following real sample analysis.

Peptide Migration Behavior Matching Evaluation

A matching factor (Mf.) is defined here as $Mf. = q/M^{1/2}_{cal}/(k \cdot t_{obs} + b)$ according to the fitting curve for the peptides' migration behavior matching evaluation (where $q/M^{1/2}_{cal}$ is the calculated q over $M^{1/2}$ of the peptide, t_{obs} is the observed migration time, k and b is the slope and intercept for the fitting line, respectively). The Mf. values of the model peptides from Figures 2c and 2d are given in the Mf.(c) and Mf.(d) columns in Table 1, respectively. As seen, in both cases, most Mf. values of the peptides are around 1.0, the theoretical value, indicating that the fitting line matches perfectly with the peptides' experimental distribution. Statistical analysis was performed with SAS software version 9.1 according to these data, average values of 1.032 ± 0.138 and 1.039 ± 0.135 were obtained for the Mf.(c) and Mf.(d) columns, respectively. Note that the corazonin ($MH^+ = 1369.7$) was identified as an outlier from statistical analysis due to its exceptionally large Mf. values (Mf.(c) = 7.3, Mf.(d) = 9.27). Another peptide, allatostatin IV, DRLYSFGLamide ($MH^+ = 969.5$, Mf.(c) = 1.4, Mf.(d) = 1.5) was also observed to deviate substantially from the confidence interval (CI, $p < 0.05$) of 0.76~1.30. These elevated matching values (or matching errors) indicate the peptides' earlier elution in CE as compared to their predicted times ($t_{obs} < t_{pre}$). This deviation often stems from post-translational modifications (PTMs) occurring on a given peptide (e.g., pQTFQYSRGWTNa) or the presence of specific amino acid residue (see discussion below), presenting a useful tool for elucidation of unique features of peptides.

Enhanced Profiling of Neuropeptides from Tissue Extracts of *C. borealis*

We have previously employed numerous MS-based strategies including direct tissue MALDI MS and capillary LC coupled to tandem MS for neuropeptide discovery and characterization. 42, 43, 54-56 To date, more than 250 neuropeptides from various decapod crustacean model organisms have been identified, with some being commonly present in several tissues, while others uniquely present in a specific tissue. However, numerous putative neuropeptides remain unidentified and their relevant physiological actions are unknown due to the low concentrations, extreme chemical diversity and PTMs of these trace-level signaling molecules. 57

Compared to capillary LC, CE consumes much less sample. For a typical LC-MS/MS experiment, often 50 to 100 animals' tissues are pooled and homogenized for peptide extraction that are used for subsequent LC fractionation. In contrast, the minimal sample consumption of CE enables the use of tissue from a single animal or a few animals. In addition, the sample preparation procedure is simplified when coupling CE to MALDI MS detection, due to its tolerance to salts and denaturants. The reconstituted tissue extracts containing about 5 μ L supernatant were used for CE injection. In each run, ~50 nL of the sample from pooled *C.*

borealis POs (n=1 or 4), brains (n=5), or CoGs (n=6), were injected respectively for analysis by off-line CE and MALDI FTMS.

CE fractions of PO extract were collected with an interval of 30 s for 50 min. A total of 62 fractions were found to contain peptide signals. The obtained CE-MALDI-MS data set is displayed as a two-dimensional (2D) plot in Figure 3A. A total of 160 putative peptide peaks were detected in the MALDI spectra, 43 peaks could be assigned to known NPs by searching the home-built database, and 8 peaks were assigned to the known NPs fragments or adduct. Reconstructed CE-MALDI electropherograms of these identified NPs are shown in Figure S-3. Nine SORI-CID fragmentation spectra were obtained on the fractions with high ion abundance, with one representative fragmentation spectrum shown for the peptide with precursor mass of 1030.5 in Figure 3B.

For comparison, the crude PO extract was desalted and concentrated by ZipTipC18, and a 0.5- μ L aliquot of the processed extract was loaded and analyzed directly by MALDI FTMS. The direct analysis allowed identification of 19 peptides. Comparisons of mass spectra obtained using these two methods are shown in Figure 4. Three spectra of individual CE fractions are shown in Figures 4B–D. Although 10-fold less samples were used for CE-MALDI-MS than for the direct MALDI MS mixture analysis, the former experiments with CE separation allowed detection of significantly more peptides with up to 10-fold improved sensitivity. This improvement in peptide coverage was mainly due to alleviation of signal suppression from the complex mixture by incorporating CE separation step. In some cases, overlapping peptides close in mass can be better separated in CE process. Furthermore, as is shown in Figure S-3, the intensity varies greatly (over 300-fold) from peptides to peptides, indicating a wide dynamic range of the complex sample. However, this quantitative information was masked in the direct MALDI MS analysis.

Duplicate analyses of a single PO extract sample were carried out to evaluate the reproducibility of the method. Repetitions of the same extract that conducted in a time span of two weeks showed very similar results in both spectral pattern and peptide intensity (see Figure S-4). Similar analytical results were obtained with the brain and CoG extracts (data not shown). These results were combined together into a table (see Table S-1) for subsequent analysis and comparison. For more confident identity assignment, the mass error (*Er.*) and peptide electrophoretic behavior matching factor (*Mf.*) were introduced for peptide assignment from more than 220 detected peaks.

Formaldehyde Labeling of PO Extracts

In addition to comparing peptide migration matching factor, chemical derivatization represents an alternative method to give complementary information for neuropeptide identification. The N-terminal isotopic dimethylation reaction⁵⁸ with formaldehyde was used to label the peptides prior to CE separation. The labeling with formaldehyde- H_2 (FH₂, light) and formaldehyde- D_2 (FD₂, heavy) can produce peaks with +28 and +32 Da mass shifts, respectively. In combining with CE, this protocol can provide increased confidence for peptide identification. For example, an observed single peak (without expected mass shift) after labeling reaction indicates that the peptide is N-terminally blocked, which is consistent with the observation of a unique earlier migration behavior in CE. On the other hand, a pair of peaks with 4.02 Da (+28/+32 mass shifts after derivatization) difference suggests that the original peptide is N-terminal free or N-terminally blocked but contains one lysine (K).

Peptide Identity Assignment

As seen in Table 2, sixteen representative peptides were selected from approximately 230 peaks to illustrate the procedure of peptide assignment used in CE off-line MALDI MS experiment.

Typically, four cases were considered for the identity assignment. *Case 1*: Highly confident assignment of the peptides (No. 1–3 in Table 2) with high mass measurement accuracy ($Er.$ = 5.0 ppm) and good CE migration matching factor ($Mf.$ $\approx$ 1.0) (for the $Mf.$ value shown as 2.4/D, see the discussion below for the modified $Mf.$ values for these peptides). The assignment of peptide 4 is incorrect due to the greatly deviated $Mf.$ value (2.4). *Case 2*: Assignment of the pyroglutamylated peptides 5 and 6 according to the evidence of “earlier migration” in CE and non-reactivity to formaldehyde labeling as a result of N-terminal blockage. This information together with the SORI-CID MS/MS spectrum (Figure 3B), verified the pyroglutamylation modification on the N-terminus (E) of peptide 5, pEGFYSQRYa (MH^+ = 1030.5). Peptides 7 and 8 are two suspected examples of similar structures with amino acid sequences yet to be determined. *Case 3*: Assignment of the correct identity from two sequences with very close masses, either by $Er.$ and $Mf.$ values (peptide 9) or via labeling reaction (peptide 10). For example, the assigned peptide QRAYSFGLa (MH^+ = 940.5) has a better match with the observed mass and $Mf.$ value. In the case of peptide assignment for GAHKNYLRFa, both +28/+32 and +56/+64 Da mass shifted pairs are observed in the spectra, indicating a lysine (K) containing N-terminal free sequence GAHKNYLRFa (MH^+ = 1104.6). Peptide 11 is an unknown peptide with the labeled pairs also detected by CE-MS. *Case 4*: Peptides belonging to a specific family that share significant sequence homology (peptides 12–15) are observed to co-migrate or elute closely in CE. As shown in Figure 5, four orcokinin-like peptides coeluted in the CE separation and were found to be with similar abundance distribution in both brain and CoG samples. Another putative novel member of the orcokinin peptide family with even lower abundance also eluted together with the four peptides, with the identity yet to be *de novo* sequenced in future study. Similarly, by comparing eluting profiles of reconstructed electropherograms of the 43 identified peptides (Figure S-3), it was observed that members from the same peptide family often co-migrate and peptide families were eluted in the following order with reversed polarity CE mode: pGlu-structure > Orcokinin-like peptide > AST-A > AST-B > RYamide > RFamide. Some peptides with more specific acidic (D, E) or basic (K, R) amino acid residues will exhibit more pronounced deviations in the migration order, as discussed above. Overall, the incorporation of CE fractionation has added another dimension of important information to increase the confidence of peptide assignment and also exhibited great potential for novel peptide discovery.

Earlier Migration Peptides and PTM Characterization

In Table 2, highly increased $Mf.$ values, as denoted with 6/pE and 8/pQ, were observed for several pyroglutamylated peptides. According to the definition of the $Mf.$, the increased value indicates a lower observed net charge (q) as compared with the calculated one using the equation, and also suggests an earlier migration in CE than the predicted time. As we know that in the pGlu-structure, the free amine group ($-NH_2$) and the carboxyl ($-COOH$) side chain of the E or Q forms a cyclic amide ($-CONH-$) at N-terminus, which has blocked the protonation of both groups and thus leads to the lower net charge in an acidic environment.

Other identified peptides in Table 2 **P3**: DRNFLRFa (MH^+ = 966.5) and in Table S-1, DV RTPALRLRFa (MH^+ = 1342.8) with the $Mf.$ values of 2.4/D and 1.82/D, also exhibited great deviations and indicated slightly earlier migration than predicted time with calculation. As observed above for allatostatin IV, DRLYSFGLa, the increased $Mf.$ value was congruously found to occur in peptides with similar structures that are C-terminally amidated and contain an acidic residue (D) at the N-terminus. A modified net charge calculation method is therefore proposed for those and other similar peptides being subsequently identified in all samples (including others in Table S-1). The resulting q value is in close match to the experimental value by subtracting a basic residue such as K (or H, R) from the sequence (if there has one) when carrying out the calculation with the equation. These findings are believed to be useful

for the construction of predictive models of peptide migration behavior based on the same calculation method.^{37, 59}

Conclusions

In summary, we have described an improved design of CE off-line coupled to MALDI FTMS and its successful application for neuropeptide analysis. Improved neuropeptidome coverage was achieved with less sample consumption and enhanced ability for prediction and characterization of novel neuropeptides and their corresponding peptide families. Over 220 putative peptide peaks were detected with wide dynamic range from the crude extract of several pooled *C. borealis* neuronal tissues. Among these, 70 neuropeptides that belong to ten families were identified with increased confidence by incorporating CE separation. The CE fractionation shows great potential for the global analysis of neuropeptides from complex samples by providing effective preconcentration, desalting, and separation to decrease the ionization suppression of peptides prior to MALDI MS analysis.

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Figure 1.

Schematic diagram of the off-line CE-MALDI MS setup. Panel A) An enlarged scheme of the polymer-coated capillary joint. Panel B) From right, the capillary inlet and Pt cathode are mounted into a pump device containing the running buffer. A cellulose acetate membrane-coated porous polymer joint is made on the capillary near the outlet end to complete the electric circuit. The "PICS" denotes the pressure-initiated capillary siphoning flow that directs toward the capillary outlet. The outlet is placed at a Δh (cm) below the inlet to maintain the siphoning flow after retraction of pressure. CE is run under negative mode to yield reversed EOF to counter the PICS flow. MALDI sample plate pre-deposited with nanoliter matrix spots on Parafilm is used for CE effluents collection.

Figure 2.

Distribution of the experimental data of the peptides and linear fittings of the q (a) and $q/M^{1/2}$ (b-d) against t under negative CE (where q is net charge of peptide, M is molecular mass, t is migration time). (a) and (c) are fittings with the removal of modified peptide ($MH^+ = 1369.7$) as compared to (b). (d) fitting with two peptides in the earliest and latest eluted fractions, respectively.

Figure 3.

A) Bubble mapped 2D plot of the off-line CE-MALDI MS data. The bubble size is related to ion abundance of a peptide. Larger bubbles indicate more intense peptide signals. SORI-CID spectra are recorded from spots a) to h) indicated by arrows, with the precursor masses listed in the margin. B) Fragmentation ion spectrum of pEGFYSQRYamide ($MH^+=1030.5$) with sequence-specific fragment ions labeled.

Figure 4.

Comparison of the MS spectra of direct MALDI analysis and three of the CE fractions (total 43 peptides were identified by the CE/MS method). A) 19 peptides (a-s) are identified with direct MALDI MS analysis. B) spectrum of fraction 18, with two identical (c and i) and three additional (u, v, w) peptides identified as compared to A. C) spectrum of fraction 53, with six identical (a, b, l-n, p) and four additional (x, y, z, aa) peptides identified. D) MS spectrum of fraction 57, with six identical (e, g, h, l, m, p) and two additional (aa, ab) peptides identified. The absolute intensity is shown on the right of each spectrum. The asterisks show unidentified peptides.

Figure 5.

Coelution of four orcokinin-like family peptides in CE, with similar abundance distribution in both *C. borealis* brain and CoG extracts. A novel low abundance peptide MH^+ 1563.7 is observed to coelute with other members of the orcokinin peptide family, the isotopic peaks are shown in the inset.

Table 1
The neuropeptide standards used for the off-line CE-MALDI MS experiments

P	m/z (MH ⁺)	Name	Sequence	Conc. (pM)	q/M ^{1/2}	Mf.(c)	Mf.(d)
01	689.36	UP	DLWQK	4.15	0.019	0.75	0.81
02	784.41		FVNSRY ^a	2.50	0.071	1.02	1.05
03	956.37	CCAP	PFCNAFTGC ^a	1.05	0.032	1.04	1.10
04	969.52	AST-IV	DRLYSFGL ^a	0.86	0.048	1.42	1.50
05	976.46		SGFYANRY ^a	4.10	0.064	1.03	1.06
06	1037.55	Cab Pk-II	SGGFAFSRPL ^a	0.38	0.062	1.12	1.16
07	1046.54	Ag-II	DRVYIHFP	1.19	0.046	1.05	1.10
08	1051.57	Cab Pk-I	TNFAFSPL ^a	0.51	0.062	1.11	1.15
09	1067.56	AST-I	GDRLYAFL ^a	0.42	0.045	1.09	1.14
10	1084.45	[Arg ⁸] Vasopressin	CYFQNCPRGa	0.55	0.061	1.05	1.08
11	1104.64		KHKNYLRF ^a	1.15	0.134	0.99	1.01
12	1105.59		GAHKNYLRF	0.20	0.091	1.00	1.03
13	1107.52		QWSSMRGAW ^a	0.90	0.060	1.04	1.07
14	1172.67	Kinetensin	IARRHPYFL	5.08	0.088	0.97	0.99
15	1260.66		SGKWSNLRGAW ^a	1.18	0.084	0.98	1.00
16	1282.67	[Val ⁵] Ag-I	DRVYVHPHL	0.52	0.070	0.99	1.02
17	1335.72	AST-I	APSGAQLYGFGL ^a	0.21	0.055	1.02	1.05
18	1347.74	Substance P	RPKQQFFGLM ^a	0.47	0.082	0.99	1.02
19	1369.65	Corazonin	pQTFQYSRGWTNa	0.41	0.054	7.3 ^d	9.27 ^d
20	1470.70	CbAST-B1	VPNDWAHFRGSW ^a	0.91	0.064	0.97	1.00
21	1473.68		NFDEIDRSFGFGA ^a	1.36	0.025	0.75	0.79
22	1474.66	[Ala ¹³]-Ok	NFDEIDRSFGFGA	2.02	-0.010	1.19	0.98
3	1502.69	[Val ¹³]-Ok	NFDEIDRSFGFGV	2.05	-0.010	1.17	0.97
24	1517.67	[Asn ¹³]-Ok	NFDEIDRSFGFGN	0.17	-0.010	1.17	0.97
25	1637.72	Somatostatin	AGCKNFFWKFTSC	1.37	0.049	0.85	0.88
CI value (p<0.05): low to upper				0.76~1.30			0.77~1.30
Mean ±Std Dev				1.032±0.138			1.039±0.135

CCAP, Crustacean Cardioactive Peptide; AST, Allatostatin; CabAST-B1, *C. borealis* B-type allatostatin 1; UP, Uremic Pentapeptide; Pk, Pyrokinin; Ag, Angiotensin; Ok, Orcokinin. q is net charge of peptide, M is molecular mass. Mf., the custom defined matching factor of the peptide for observed and predicted migration time in CE. The Mf. (c) and Mf. (d) values resulting from the linear fitting in Figure 2 (c) and (d). CI, confidence interval.

^a Outlier in statistical analysis due to the extra large Mf. value.

Table 2
Representative examples of the peptide assignment via off-line CE-MALDI MS

P	Obsd MH ⁺	PO		Br	CoG	Er.	Mf.	Theor.	Sequence	Family
		N	(p)							
1	<u>965.548</u>	+	+	+	+	5.4	1.01	965.5428	NRNFLRFa	RFamide
2	<u>1260.661</u>	+		+	+	0.4	1.11	1260.6596	SGKWSNLRGAWa	AST-B
3	<u>966.528</u>	+	+		+	1.2	2.4/D	966.5268	DRNFLRFa	RFamide
4	<u>1486.724</u>			+	+	-4	2.4	1486.7294	GKKNVEMMTARGFa	
5	<u>1030.465</u>	+		+	+	-9	6/pE	1030.4741	PEGFYSQLYFa	RYamide
6	<u>1369.635</u>				+	-13	8/pQ	1369.6522	pQTFQYSRGWWTNa	Corazonin
7	<u>969.486</u>	+		+				Unk		
8	<u>1608.719</u>	+		+				Unk		
9	<u>940.503</u>	+	+		+	-8.7	1.57	940.51116	RFVGGSRYa	
		+	+		+	3.3	1.05	940.49993	ORAYSFGLa	AST-A
10	<u>1104.608</u>	+	+	+	+	-1.3	0.83	1104.6095	AQPSMRLRFa	
		+	+	+	+	1.7	1.05	1104.6061	GAHKNYLRFa	RFamide
11	<u>1602.851</u>	+	+					Unk		
12	<u>1474.659</u>			+	+	-1	0.99	1474.65973	NFDEIDRSFGFAG	Orco-LP
13	<u>1502.701</u>			+	+	6.6	1.01	1502.69103	NFDEIDRSFGFV	Orco-LP
14	<u>1532.718</u>			+	+	11	1.02	1532.70161	NFDEIDRSFGFV	Orco-LP
15	<u>1547.674</u>			+	+	-1	1.03	1547.67611	NFDEIDRSFGFN	Orco-LP
16	<u>1563.703</u>			+	+			Unk		

Mass underlined, the unidentified peptides. Samples are from tissue(s) of pericardial organs (PO), Brain (Br), commissural ganglia (CoG), respectively. The columns under PO: the N column, normal sample without isotopic formaldehyde labeling; the (p) column, peptides detected as pairs with +28/+32 Da mass shift upon labeling; the (s) column, peptides detected as unlabeled single peaks. "+," shows that the peptides are detected in the samples. Er., the mass error in ppm. Mf., the custom defined matching factor of the peptide for observed and predicted migration time in CE. AST -A, -B: Allatostatins A and B types. Orco-LP: Orcokinin-like peptide. See the text for the details of these data analysis.