Published in final edited form as:

Expert Rev Proteomics. 2012 April ; 9(2): 135–147. doi:10.1586/epr.12.10.

Microscale 2D separation systems for proteomic analysis

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Abstract

Microscale 2D separation systems have been implemented in capillaries and microfabricated channels. They offer advantages of faster analysis, higher separation efficiency and less sample consumption than the conventional methods, such as liquid chromatography (LC) in a column and slab gel electrophoresis. In this article, we review their recent advancement, focusing on three types of platforms, including 2D capillary electrophoresis (CE), CE coupling with capillary LC, and microfluidic devices. A variety of CE and LC modes have been employed to construct 2D separation systems via sophisticatedly designed interfaces. Coupling of different separation modes has also been realized in a number of microfluidic devices. These separation systems have been applied for the proteomic analysis of various biological samples, ranging from a single cell to tumor tissues.

Keywords

capillary electrophoresis; isoelectric focusing; liquid chromatography; microfluidics; 2D separation

Need for 2D separation systems

Proteomic analysis is of importance to unravel the role of proteins in complex biological processes, such as cell differentiation and signal transduction [1–4]. It is also a promising technique for medical diagnosis by identification and quantification of protein biomarkers [5,6]. However, the biological samples are usually very complex; there could be tens of thousands of proteins in a cell or tissue sample, whose abundance can range from six to ten orders of magnitude [7–9]. Analytical methods with high resolving power and sensitive detection are needed for proteomic analysis.

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Financial & competing interests disclosure

The authors thank the financial support from the NIH (grants R21RR024397, R21RR026215 and K25CA149080), the Flight Attendant Medical Research Institute (FAMRI-082502), the Defense Advanced Research Projects Agency (DARPA) via Micro/Nano Fluidics Fundamentals Focus Center at the University of California at Irvine, and the National Science Foundation (OISE-0968313). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Since it was first reported in 1975 [9], 2D gel electrophoresis (2DGE) has become one of the most common methods for proteomic analysis. It utilizes isoelectric focusing (IEF) as the first dimension and SDS-PAGE as the second dimension. Owing to the high orthogonality of the two separation mechanisms, the overall peak capacity is the multiplication product of the peak capacity of each individual dimension [10]. (For those 2D separations where orthogonality is less than 100%, the theoretical peak capacity would be the product of the peak capacities of the two separation dimensions and the orthogonal degree of the two separation mechanisms [10,11]). As a result, thousands of proteins could be resolved in a single run [12]. However, 2DGE has some limitations: its processes are time consuming and labor intensive [13]; and the reproducibility and sensitivity of the method is substandard [14]. Moreover, the gel-based technique cannot be coupled on-line with mass spectrometry (MS) to achieve high-throughput analysis [101], and the overall process is difficult to automate.

Two platforms have the potential to address these limitations. One is capillary electrophoresis (CE), which possesses several advantages, including fast analysis, high separation efficiency and minimal sample consumption. More importantly, high-throughput and automated analysis can be accomplished with CE-based techniques. These advantages result from higher separation voltage in a capillary than in a slab gel, driving protein analysis to CE-enabled platforms. This is analogous to DNA sequencing, which has shifted from slab gel electrophoresis to capillary array electrophoresis [15,16].

The other platform is microfluidics, which enables us to construct miniaturized analytical instruments called 'lab-on-a-chip' devices. The principles of microfabrication and microfluidics, as well as their current and potential applications, have been reviewed in the literature [17,18]. Common analytical assays, including PCR, protein analysis, DNA separation and cell manipulations, have been reduced in size and fabricated in a centimeter-scale device. The size reduction of an analytical instrument has many advantages, including high speed of analysis, minimization of required sample and reagents, and the ability to operate in a high-throughput format and to integrate with a sensitive detector.

In this article, we review the recent advancement of 2D separation systems based on CE and microfluidics, with a focus on three types of platform, including 2D modes of CE, CE coupling with capillary liquid chromatography (LC), and microfluidic devices. The designs of the interfaces to couple different techniques/modes and the performance of these 2D separation systems are discussed.

2D CE

A number of CE modes have been employed for constructing 2D separation systems, including capillary zone electrophoresis (CZE), capillary sieving electrophoresis (CSE), micellar electrokinetic chromatography (MEKC), capillary IEF (CIEF), capillary isotachopheresis (CITP), capillary gel electrophoresis (CGE) and capillary electrochromatography (CEC). CZE separates analytes based on their charge-to-size ratio. CSE separates proteins/peptides based on their differences in size and shape with a capillary filled with sieving medium. Separation of analytes with MEKC is based on their interactions with micelles in the running buffer. CIEF separates and concentrates proteins/peptides based on their differences in isoelectric point. CITP utilizes a discontinuous buffer system, where analytes migrate and form consecutive zones. CGE is similar to CSE, except that it uses a capillary filled with gel. In CEC, a mobile phase is driven through a stationary phase within a capillary by electro-osmotic flow (EOF). Separation of analytes relies on both electrophoretic migration and chromatographic retention. A summary of representative work is provided in Table 1.

Dovich's research group reported the first fully automated 2D CE system for protein analysis [19]. The system utilized submicellar CE separations at different pH in two dimensions. Proteins labeled with a fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde were separated in the first dimension at pH 7.5. The eluted fraction was repeatedly transferred into the second dimension via a flow-gated injection interface and subsequently separated at pH 11.1, as shown in Figure 1a. By using laser-induced fluorescence (LIF) detection in a sheath-flow cuvette, the system enabled the detection of zeptomoles of labeled proteins from HT29 cell lysate. The authors further developed the 2D system by employing CSE based on an SDS-pullulan buffer system as the first dimension and MEKC as the second dimension [20]. After an 11-min prerun in the first dimension, the eluted fractions were repetitively injected into the second capillary and separated by MEKC. The spot capacity of the 2D system was estimated to be approximately 550. This platform allowed the identification of over 150 partially resolved components from *Deinococcus radiodurans* protein homogenate. The same system has been exploited for protein profiling of single mammalian cells [21]. After a 6-min CSE preliminary separation, over 100 fractions from the first dimension were transferred into the second capillary for further MEKC separation over a period of 3.5 h. The system enabled the generation of protein fingerprints from single MC3T3-E1 osteoprogenitor cells and MCF-7 breast cancer cells. Significant differences were found between the single-cell electropherogram of a native MC3T3-E1 cell and that transfected with the human transcription regulator TWIST (Figure 1b), which was in agreement with the overexpression of the transcription regulator and the altered appearance of the cell. The 2D separation system has been further demonstrated for the analysis of mouse tumor cell homogenate, tissue homogenates, single-cell mouse embryo and single macrophage cells [22–25]. The instrumentation has been improved by incorporating an enzyme microreactor at the end of the first capillary, allowing the separated proteins to be digested by pepsin prior to further separation in the second capillary [26]. The peak capacity of this system was estimated to be 590 with MS detection. When the enzymatic microreactor was further advanced using trypsin-modified magnetic beads, two model proteins, β -casein and insulin chain B, were identified based on distinct peptides with sequence coverage of 15 and 100%, respectively [27].

CIEF is a popular mode for coupling with other CE methods to form 2D separation systems [28–34] because of the fact that IEF is the first dimension of the conventional 2DGE. CIEF offers advantages for proteomic separation since it can separate and concentrate analytes simultaneously. Mohan and Lee developed a microdialysis interface for on-line coupling of CIEF with CITP/CZE in a 2D system [28]. Both capillaries were butted inside a polysulfone dialysis tubing within an acetic acid solution, which afforded the anolyte and the background electrolyte for CIEF and CITP/CZE, respectively. Carrier ampholytes used in CIEF were also used as the leading electrolyte in CITP/CZE, facilitating on-line coupling of two dimensions. The system with UV detection was applied for 2D separations of tryptic digests of several standard proteins with a peak capacity of up to 1600. The instrumentation was improved by incorporating ESI-FTICR-MS detection for the analysis of the *Shewanella oneidensis* proteome [29]. The platform enabled the identification of 1174 unique proteins from the soluble cytosolic fraction of bacteria, with 26.5% proteome coverage. Coupling of CITP with CZE has also been reported for the analysis of urine samples [35].

Yang *et al.* developed a CIEF/CGE system by employing a dialysis interface based on a hollow fiber [30]. The interface allowed the reduction of the salt level, replacement of the buffer and preservation of the analytes within the capillary. 2D separation of hemoglobin with diverse variants was achieved in less than 1 h. A similar dialysis interface was utilized to couple CIEF with CZE for separation of ribonuclease [36]. To reduce the dead volume, a new junction for coupling CIEF with CZE was developed by etching a small segment of the capillary to form a porous glass membrane [31]. Such a porous interface was also coupled

with monolithic immobilized pH gradient [32]. The performance of this system was demonstrated by 2D separation of standard proteins and proteins extracted from milk with an analysis time of less than 2 h and a peak capacity of approximately 200. A similar 2D system based on CIEF/CZE with LIF detection was recently reported, with a peak capacity of 125 for separation of standard proteins labeled with fluorogenic reagent Chromeo™ P503 (Sigma-Aldrich) [37].

Zhang and El Rassi developed a platform by coupling CIEF with CEC and the on-line coupling was achieved by using a six-port nanoinjector valve [33]. The effectiveness of the system with UV detection was demonstrated by 2D separation of standard proteins and human serum proteins. Sheng and Pawliszyn demonstrated a 2D separation system that employed CIEF as the second dimension rather than the first dimension [34]. A dialysis interface with a ten-port valve and two conditioning loops was employed for on-line coupling of MEKC (first dimension) to CIEF, and it efficiently removed salt and unwanted components from the fractions collected from the first dimension. Also through the interface, carrier ampholytes were added for the second-dimensional separation. By using whole-column UV detection, 2D electropherograms of tryptic digests of trypsinogen and cytochrome c were obtained. In addition, Sahlin reported a 2D separation system where two capillaries were coupled via tangentially connected channels with circular cross-sections [38]. A small opening at the contact area was used for transferring fractions from the first to the second dimension. By coupling CZE with MEKC via this interface, the system was applied for separation of the tryptic digest of bovine serum albumin (BSA), with an analysis time of 55 min.

CE coupled with capillary LC

A number of 2D separation systems combining a variety of combinations of CE and LC modes have been developed for proteomics analysis. The modes of CE have been discussed in the previous section, while the modes of LC employed include reversed-phase LC (RPLC), size-exclusion chromatography (SEC) and ion-exchange chromatography. The majority of the LC efforts were operated in a capillary or microcolumn to match the flow rate and elution volume of CE. RPLC separates proteins/peptides based on their differences in hydrophobicity. The separation mechanism of SEC is the difference in the molecular size, while that of ion-exchange chromatography is the difference in the net charge. A summary of representative work is provided in Table 2.

2D separation involving CE was reported as early as 1990 by Jorgenson's research group, including the coupling of CE with RPLC or SEC [39,40]. They further developed a 3D system, SEC-RPLC-CZE, for the separation of peptides [41]. On-line combination of CIEF with capillary RPLC (cRPLC) for proteomic analysis was developed much later [42]. In this study, Lee's research group used a microinjector as an interface, which introduced the peptide fractions focused during CIEF into the second dimension, cRPLC separation [42]. The separation profile of the protein digest from the soluble fraction of *Drosophila* salivary glands was obtained using this platform with UV detection. The overall peak capacity was estimated to be approximately 1800, with a run time of less than 8 h. The research group further developed this system by incorporating a new interface and MS detection [43], and the setup is illustrated in Figure 2a. The interface consisted of multiple microselection valves and C18 reversed-phase trap columns. Focused peptide fractions were injected and loaded into individual trap columns consecutively until the entire content of CIEF capillary was sampled. This platform with ESI-MS/MS detection enabled the identification of 1132 unique proteins and 1894 unique peptides from tryptic digest of soluble yeast proteins with a peak capacity of over 2600. The enhanced dynamic range and sensitivity was attributed to the orthogonal 2D separation and high resolving power of CIEF with the concentration

effect (~240-fold). This system has also been employed for proteomic analysis of a microdissected glioblastoma multiforme tumor sample [44]. Figure 2b shows six chromatograms out of 14 that were obtained when 14 trap columns were used to load the focused peptide fractions from the first dimension. Three 2D proteome runs resulted in the identification of 6866 fully tryptic peptides and 1820 distinct proteins. Owing to the use of capillaries in CE and cRPLC, each proteome run consumed only 10 µg microdissected tissues (~20,000 cells), which is beneficial when the amount of tissue samples is limited or the available cell population is small. In addition to tumor tissue, the system has also been used for the characterization of intact yeast proteins and the human salivary proteome [45,46].

Lee's research group developed another 2D separation system by coupling CITP/CZE with cRPLC [47]. The peptide fractions resolved from CITP/CZE were collected into wells of a moving microtiter plate, followed by sequential loading into individual trap columns prior to second-dimensional separation. CITP in the first dimension exhibited advantages over CIEF, including that the resolved peptides/proteins in CITP/CZE are less prone to precipitation, and CITP/CZE could selectively enrich low-abundance proteins and reduce the range of relative protein abundances. By combining this with ESI-MS/MS detection, the system detected a total of 6112 fully tryptic peptides at a 1% false-discovery rate and identified 1479 distinct proteins from a single human saliva sample [47].

Zhou and Johnston reported a similar CIEF-cRPLC system with the use of a microdialysis membrane-based cathodic cell and micro-selection valves/trap column to couple two dimensions [48]. The microdialysis membrane in the cathodic cell allowed the protein fractions to be collected with ampholyte removal prior to cRPLC separations. By using ESI-MS, detection of standard protein mixtures at the low-femtomole level was achieved. Seven CIEF fractions in the isoelectric point range 3–10 could be analyzed in 2 h. The authors later demonstrated the use of this instrumentation for protein profiling of the lysate of green sulfur bacterium. A total of 1162 proteins were identified, which was comparable to both the predicted data from the genome and experimental data from 2DGE [49].

Mao and Zhang developed a 2D separation system by combining CIEF and cRPLC [50]. In contrast to the systems mentioned above, they employed CIEF as the second dimension with cRPLC as the first dimension. Approximately 40–80 fractions from the first dimension were collected and sequentially injected and analyzed by CIEF. By using a LIF detector, this system enabled peptide detection at the femtomole level with a peak capacity of approximately 10,000. Complex protein/peptide samples, including yeast lysate and BSA tryptic digest, were used to demonstrate the effectiveness of this method. The authors further improved the system by coupling cRPLC to an array of CIEF with a whole-column imaging system [51]. This system was used for the analysis of protein extracts from liver cancer tissue with an overall peak capacity of approximately 18,000 and an analysis time of less than 3 h.

Zhang *et al.* demonstrated combining cRPLC and CZE with tandem MALDI-TOF MS detection for proteome analysis [52]. A valve-free hydrodynamic sampling interface was developed to transfer effluents from the first to the second dimension, as shown in Figure 3a. A CE-MALDI deposition interface was made to couple CZE with TOF-MS detection. Using this system, more than 300 proteins from the D20 liver cancer tissue were identified (Figure 3b). Similarly, Bergstrom *et al.* developed a cRPLC-CZE system with the use of ESI-FTICR-MS detection using an interface made from polydimethylsiloxane (PDMS) [53].

Wu *et al.* recently reported a 2D separation system by coupling strong cation-exchange LC in a capillary with reversed-phase pressurized CEC [54]. The fractions eluted from the first

dimension were collected in a collector and a loading loop before injection into the second dimension. This platform was used for the analysis of tryptic digest of BSA and serum samples with a peak capacity of approximately 1200.

2D separation in microfluidic devices

When 2D separation is implemented in a microfluidic device, two schemes may be used. Scheme I is to use one channel for the first dimension and another channel for the second dimension, in a way similar to two capillaries for CE-CE or CE-LC as discussed above. Scheme II is to employ an array of channels for the second dimension, all of which are interfaced with one first-dimension channel. This scheme is more like conventional 2DGE, in which one IEF strip and a number of gel lanes are used. The 2D separation modes used in microfluidic devices include IEF with CZE, IEF with CGE, IEF with RPLC, CGE with MEKC, IEF with DIGE, CEC with CE, and isotachopheresis with CZE. A summary of representative work is provided in Table 3.

Demonstration of 2D separation in microfluidic devices was first reported by Ramsey's research group [55–57]. They microfabricated devices in glass, and each device contained two channels intersecting with each other. Sampling volume and rate were precisely controlled by the gated injection method. Separation modes carried out in the devices included CEC with CE and MEKC with CE. Rapid analyses of protein digests ranging from 10 to 15 min were demonstrated, and an overall peak capacity as high as 4200 was achieved for 2D separation of the BSA tryptic digest. The resolving power demonstrated in the device is superior to slab gel electrophoresis.

Orthogonally coupling CGE with MEKC was introduced by Soper's group [58,59]. The device was made in thermoplastics and the layout is shown in Figure 4a. The effluents from the first dimension (SDS-CGE) were transferred to the second dimension (MEKC) at certain intervals and fixed volumes. When fetal calf serum (labeled with AlexaFluor-633) was analyzed using LIF detection, a peak capacity of approximately 2660 was obtained in a comprehensive 2D map (Figure 4b). The peak capacity was nearly threefold higher and the analysis time was approximately 60-times shorter than the experiment using conventional 2DGE.

Other separation modes have also been described in the literature. Herr *et al.* combined IEF with CE in a thermoplastic device for the analysis of three model proteins [60]. Interestingly, an ampholyte buffer solution was used as the separation media for both IEF and CZE, and the separation mechanism was solely defined by the solution in the reservoirs at the channel ends. Focused protein bands from IEF were mobilized by EOF to the channel intersection, followed by electrokinetic injection to the second dimension. A peak capacity of approximately 1300 was estimated in this device. Cong *et al.* developed a similar IEF-CZE system, but they suppressed EOF through a permanent coating of acrylamide on the channel surface because EOF decreased the separation efficiency and resolution [61]. In addition, two types of separation media were used: carrier ampholytes for IEF and 20 mM H₃PO₄ with methylcellulose for CE. A peak capacity of 540 was obtained when the digest of proteins extracted from *Escherichia coli* was analyzed. The entire process was completed within approximately 50 min.

All efforts discussed above are based on Scheme I, which exploits one channel for the first dimension and another channel for the second dimension. As mentioned above, Scheme II employs an array of channels for the second dimension, all of which are interfaced with one first-dimension channel. Theoretically, Scheme II is advantageous over Scheme I because the former is more like the conventional 2DGE. In Scheme I, effluents from the first dimension must be sequentially transferred to the second dimension and further separated,

during which the electrophoretic separation in the first dimension is suspended. Therefore, separation in the second dimension should be rapid so that the duration between each transfer is short (to minimize the diffusion-based band dispersion). Moreover, sample loss is inevitable owing to this periodic sample injection method, resulting in a detrimental effect in the measurement of the low-abundance proteins from a complex mixture. By contrast, all components in the first dimension could be simultaneously transferred into the second dimension for Scheme II. The simultaneous transfer of the analytes from the first to the second dimension is also the key advantage of microfluidics (Scheme II) over CE-CE and CE-LC, both of which require long operation time due to sequential transfers.

As explained previously [62], the key to realizing 2D separation in a microfluidic device is the interface between the first and the second dimensions. When different separation media are introduced into the intersected channels, cross-contamination at the contiguous interface would take place if no mechanism exists to prevent it. A number of efforts have been reported to address the challenge, as elaborated below.

The first Scheme II device with 2D protein separation was reported by Whitesides and colleagues [63]. The device was made in PDMS and a total of six layers were used. The first dimension (IEF) was performed in a four-layer structure. After IEF, the top and bottom layer were peeled off (owing to reversible bonding) and the middle two layers were assembled with the second set of the top and bottom layers for the second dimension. The focused proteins were transferred from the first dimension through the vertical through-holes to the second dimension. Three model proteins were separated in the device. To simplify the device fabrication, Lee's research group reported a two-layer thermoplastic device [64]. Separation of five model proteins was demonstrated by coupling IEF with SDS-CGE, reaching an overall peak capacity of approximately 1700 in 10 min.

To create an interface, Wang *et al.* utilized pneumatically actuated valves to integrate IEF with CZE (or CGE) [65]. The elastomer-based valves were made from PDMS, created near the junction between two dimensions. The valves in the second dimension were closed when the first dimension (IEF) was in operation. Afterwards, appropriate valves were opened to allow the transfer of the resolved proteins in the first dimension to the second dimension, followed by CZE. Similarly, gel-based pseudovalves have been developed for protein separation in a thermoplastic device using IEF and PAGE [66–69]. Polyacrylamide gels formed *in situ* by photo-polymerization in the microchannels of the second dimension served not only as the separation media, but also functioned as a barrier to reduce diffusion and contamination between two separation media.

In addition, diffusion between two separation media could also be significantly reduced by using a narrow interface channel connecting two dimensions. Mathies' research group reported a glass device consisting of one IEF channel and 20 channels for the second dimension [70]. All channels were 30 μm deep and 200 μm wide, except for the interface between the two dimensions. At the interface, a short segment (400 μm) of channels with much smaller dimension (4 μm deep and 25 μm wide) was created to connect the first and the second dimension. The device was used to compare cell lysate of normal *E. coli* cells (control) with those cells that had been genetically engineered to express maltose-binding proteins (sample). The results show the difference in protein expression between the sample and the control, indicating that the device can be used for proteomic analysis.

Expert commentary

A number of CE-based 2D separation systems have been developed and their capability for proteomic analysis of a variety of samples has been demonstrated. These systems combined two modes of CE or CE with LC, and exhibited comparable or even better performance than

the gel-based system. The analysis can usually be completed within hours, which is faster than 2DGE (which takes days). Some of them were fully automated, requiring much less labor-intensive procedures than the gel-based platform. Among these systems, the CE-CE systems generally utilized a simple interface design, such as flow-gated or microdialysis junctions, because of similar fraction volumes in both dimensions. By contrast, the CE-LC systems usually employed a more complicated interface, such as multiple valves and traps columns. However, higher peak capacity was generally reported for CE-LC systems than CE-CE systems, which is probably due to the higher degree of orthogonality between CE and LC.

Several detection methods have been employed in these 2D separation systems, including UV absorbance, LIF and MS. For the identification of unknown proteins, MS is advantageous since it offers valuable structure information via determination of molecular weights and peptide sequences. For determining the existence and quantity of specific biomarkers, LIF could be a good choice since it is capable of detecting proteins/peptides at the level of zeptomoles to femtomoles.

Microfluidics should be advantageous over CE-based 2D separation systems owing to the higher level of integration and the ability to incorporate an array of channels in a way similar to conventional 2DGE. The device designs enabled the simultaneous transfer of the analytes from the first to the second dimension, which required much less time than sequential transfers in CE-CE and CE-LC. As a result, the operation time in a microfluidic device was often in the region of 10–50 min (Table 3), whereas it was 1–8 h for CE-CE (Table 1) or CE-LC (Table 2). However, the potential of the microfluidics-based 2D separation systems has not yet been realized. The challenges include issues related to device fabrication, requirement of additional electrical or mechanical components for fluid handling, and surface phenomenon and fluid behaviors in microchannels. After these challenges have been successfully addressed and significantly higher peak capacity has been obtained, microfluidics-based 2D separation systems could play a role in proteomic analysis, especially in situations where rapid analysis and easy operation are desirable.

Note that this review is focused on microscale 2D separation systems. There are many other systems that are based on bench-top instruments and that have been used for proteomic analysis. For example, Kobeissy *et al.* developed a cation/anion-exchange chromatography PAGE-RPLC-MS/MS platform for studying biomarkers associated with traumatic brain injury [71]. Reviews are available on multidimensional separation for studying proteomics [72], as well as on MS-based proteomics [73]. The current challenges in obtaining clinically useful results, as well as the complex nature of the proteome, have been discussed [74]. It should be pointed out that a 2D separation system is generally more complicated than an instrument of 1D separation, and the analysis time might be longer. The overall success will depend on the system performance and the adaptation of both academic laboratories and commercial instrument companies.

Five-year view

While most of the current 2D CE-CE systems employed UV or LIF as the detection method, MS could become the choice of the future since it provides more information for protein profiling. MS may also function as the third dimension owing to the fact that molecular weight information can be obtained. As a result, the peak capacity would be greatly enhanced because of orthogonal separation mechanisms. However, challenges in the MS interface must be addressed first. ESI-based interfaces, as well as other methods, have been used to couple CZE to MS [26,29,75,76]. More advances in the development of a novel and

robust CE-MS interface would significantly help the application of on-line MS detection in CE-based 2D systems for proteomic analysis.

Detection of low-abundance proteins is often difficult in 2DGE analysis owing to the broad range of protein abundance in a complex biological sample. The 2D platform employing CITE/CZE has demonstrated its capability for selective enrichment of low-abundance proteins, which would reduce the range of relative protein abundances of the human salivary proteome [47]. Further development of similar techniques and application of them as one dimension for 2D protein separation may help to identify unique biomarkers in low concentrations and enhance protein coverage in complex proteomes.

Microfluidics is promising to achieve comprehensive protein analysis, and most separation techniques have been implemented in a number of microdevices. However, the majority of efforts have been made using highly concentrated protein mixtures or model biological samples; clinical samples were rarely employed. Devices that are truly capable of proteomic analysis of real-world samples have to be realized in the coming years. In addition, elements for pretreatment and postanalysis are expected to be incorporated in the future. The expanding investigation of the fabrication materials (e.g., thermoplastics) that are more amenable to manufacturing processes will facilitate the commercialization of microfluidics. Meanwhile, the development of complementary software and electronics will result in automatic and user-friendly systems.

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Key issues

- Two appropriate separation mechanisms should be selected that offer orthogonality in terms of peak capacity and are compatible with each other in terms of interface and integration.
- A system should demonstrate sufficient peak capacity with comparable or better performance than conventional 2D gel electrophoresis. Note that a short analysis time is expected in a microscale system owing to the dimension advantage and automation.
- The interface between two dimensions is the key to separation efficiency, sample loss (thus detection sensitivity), analysis time, automation and overall performance. Innovation often takes place in the interface design, no matter what platform (capillary electrophoresis [CE]-CE, CE-liquid chromatography or microfluidics) is studied.
- Concentration techniques, such as capillary isoelectric focusing and capillary isotachopheresis, could play a role in proteomic analysis since they enhance the detection sensitivity and dynamic range of a 2D separation system. Selective enrichment of low-abundance proteins would be beneficial for the identification and detection of biomarkers in a complex sample.
- A large number of proteins and peptides must be analyzed in a microfluidic device if the platform is designed for proteomic analysis. The capability must be demonstrated by the analysis of real-world samples, such as mammalian cells and tumor tissues.

Figure 1. 2D separation by capillary electrophoresis

(A) Schematic of the instrument setup. Capillary 1 and capillary 2 were connected via a flow-gated injection interface. The outlet of capillary 2 was placed within a sheath-flow cuvette for post-column fluorescence detection. (B & C) Protein images from single MC3T3-E1 cells. Image (B) was generated from an untransfected cell, whereas (C) was from a single cell that had been transfected with the transcription regulator TWIST. Arb: Arbitrary units; CSE: Capillary sieving electrophoresis; MECC: Micellar electrokinetic capillary chromatography. (A) Adapted with permission from [19]. (B & C) Adapted with permission from [21].

Figure 2. 2D separation by capillary isoelectric focusing with capillary reversed-phase liquid chromatography

(A) Schematic of on-line integration of CIEF with capillary reversed-phase LC (cRPLC) as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of CIEF fractions and the injection of fractions into a cRPLC column, respectively. **(B)** Chromatograms of a representative 2D separation of 10- μg peptide digest from microdissected glioblastoma multiforme tissue sample. Each number represents the sequence of CIEF fractions further analyzed by cRPLC (only six out of 14 are shown). -: Negative voltage; CIEF: Capillary isoelectric focusing; G: Grounded; HAC: Acetic acid; LC: Liquid chromatography; MS: Mass spectrometry.

(A) Adapted with permission from [43].

(B) Adapted with permission from [44].

Figure 3. 2D separation by liquid chromatography with capillary electrophoresis

(A) Schematic diagram of the hydrodynamic interface between the first dimension (LC) and second dimension (CE). The slide bar could be moved up and down by actuation of a step-motor driver and the inlet of the CE capillary was fixed on the slide bar. At the 'injection position' where the inlet end of the CE capillary was in contact with the LC column outlet, the LC effluent droplet accumulated was transported into the CE capillary through hydrodynamic injection due to the elevation. After injection, the CE column was moved to the 'analysis position' – that is, the inlet end of the capillary was dipped into the buffer reservoir, followed by CE separation. (B) Plot of 2D separation for protein tryptic peptides from D20 liver cancer tissue.

CE: Capillary electrophoresis; LC: Liquid chromatography.

Adapted with permission from [52].

Figure 4. 2D separation in a microfluidic device

(A) Layout of a device to carry out 2D separation. The first dimension is CGE while the second dimension is MEKC. The sample was placed into reservoir A and electrokinetically injected into the separation channel C–D. E represents a buffer reservoir, while B and F are waste reservoirs. Each 1-s separation in the first dimension was followed by a 10-s MEKC in the second dimension and the cycle was repeated as needed. (B) 3D landscape map of proteins from fetal calf serum. A total of 159 MEKC cycles were carried out.

CGE: Capillary gel electrophoresis; LIF: Laser-induced fluorescence; MEKC: Micellar electrokinetic chromatography.

Adapted with permission from [59].

Table 1

Summary of 2D separation systems using different modes of capillary electrophoresis.

First dimension	Second dimension	Interface	Detection	Peak capacity	Analysis time (h)	Samples	Ref.
CE at pH 7.5	CE at pH 11.1	Flow-gated interface	LIF		8	Proteins extracted from HT29 cell lysate	[19]
CSE	MEKC	Flow-gated interface	LIF	550	6.7	<i>Deinococcus radiodurans</i> protein homogenate	[20]
CSE	MEKC	Flow-gated interface	LIF	375	3.5	MC3T3-E1 cells and MCF-7 breast cancer cells	[21]
CE at pH 2.7	CE at pH 3.9	Flow-gated interface with microreactor	MS	590		Cytochrome c and heart myoglobin	[26]
CIEF	CITP/CZE	Microdialysis junction	UV	1600		Tryptic digests of standard proteins	[28]
CIEF	CITP/CZE	Microdialysis junction	ESI-FTICR-MS	1600	<3	Cytosolic fraction of <i>Shewanella oneidensis</i>	[29]
CIEF	CGE	Dialysis interface using hollow fiber	UV			Hemoglobin variants	[30]
CIEF	CZE	Etched porous glass interface	UV			Myoglobin and hemoglobin	[31]
CIEF	CZE	Etched porous glass interface	UV	200	<2	Proteins extracted from milk	[32]
CIEF	CEC	Six-port nanoinjector	UV	54,320	168	Human serum proteins	[33]
MEKC	CIEF	Ten-port valve with two conditioning loops	Whole-column UV			Tryptic digest of trypsinogen and cytochrome c	[34]
CZE	MEKC	Tangentially connected channels	UV		<1	Tryptic digest of BSA	[38]

BSA: Bovine serum albumin; CE: Capillary electrophoresis; CEC: Capillary electrochromatography; CGE: Capillary gel electrophoresis; CIEF: Capillary isoelectric focusing; CITP: Capillary isotachopheresis; CSE: Capillary sieving electrophoresis; CZE: Capillary zone electrophoresis; ESI: Electrospray ionization; FTICR: Fourier transform ion cyclotron resonance; LIF: Laser-induced fluorescence; MEKC: Micellar electrokinetic chromatography; MS: Mass spectrometry.

Table 2
Summary of 2D separation systems using capillary electrophoresis-liquid chromatography.

First dimension	Second dimension	Interface	Detection	Peak capacity	Analysis time (h)	Samples	Ref.
CIEF	cRPLC	Microinjector	UV	1800	<8	Soluble fraction of <i>Drosophila</i> salivary glands	[42]
CIEF	cRPLC	Microselection valves and trap columns	ESI-MS/MS	2640		Tryptic digest of soluble yeast proteins	[43]
CIEF	cRPLC	Microselection valves and trap columns	ESI-MS/MS	>6866		Microdissected glioblastoma multiforme tumor	[44]
CITP/CZE	cRPLC	Moving microtiter plate and trap columns	ESI-MS/MS	>6112		Tryptic digest of human saliva	[47]
CIEF	cRPLC	Microdialysis membrane with valves/trap columns	ESI-MS		2	Standard protein mixtures	[48]
SEC	CZE	Transverse flow-gating interface	UV		2	Mixture of protein standards	[40]
cRPLC	CIEF	Fraction collections	LIF	10,000		Yeast cytosol and BSA tryptic digest	[50]
cRPLC	Array CIEF	Fraction collections	Whole-column LIF	18,000	<3	Extracts from liver cancer tissue	[51]
cRPLC	CZE	Hydrodynamic sampling interface	MALDI-TOF-MS			Proteins of D20 liver cancer tissue	[52]
cRPLC	CZE	Cross-structures in PDMS	ESI-FTICR-MS	198	<1	Tryptic digest of BSA and cerebrospinal fluid	[53]
SCXLC	CEC	Collector and loading loop	UV	1200		Tryptic digest of BSA and serum	[54]

BSA: Bovine serum albumin; CEC: Capillary electrochromatography; CIEF: Capillary isoelectric focusing; CITP: Capillary isotachopheresis; cRPLC: Capillary reversed-phase liquid chromatography; CZE: Capillary zone electrophoresis; LIF: Laser-induced fluorescence; MS: Mass spectrometry; PDMS: Polydimethylsiloxane; SCXLC: Strong cation-exchange liquid chromatography; SEC: Size-exclusion chromatography.

Table 3

Summary of 2D separation systems in microfluidic devices.

First dimension	Second dimension	Interface	Detection	Peak capacity	Analysis time (min)	Samples	Ref.
<i>Scheme I</i>							
CEC	CZE	Intersecting channels	LIF	150	13	Tryptic digest of β -casein	[56]
IEF	CZE	No boundary	Fluorescence microscopy	1300	5	Standard proteins	[60]
IEF	CZE	Intersecting channels	LIF	540	50	Digest of BSA and proteins extracted from <i>Escherichia coli</i>	[61]
IEF	CZE/CGE	PDMS valves	Fluorescence microscopy		10	Standard proteins	[65]
MEKC	CZE	Intersecting channels	LIF	4200	<15	Tryptic digest of BSA	[57]
MEKC	CZE	Intersecting channels	Fluorescence microscopy	500–1000	<10	Tryptic peptides from cytochrome c	[55]
CGE	MEKC	Intersecting channels	LIF	1000	12	Ten-protein mixture	[58]
CGE	MEKC	Intersecting channels	LIF	2660	<30	Fetal calf serum proteins	[59]
<i>Scheme II</i>							
IEF	SDS-PAGE	Gel pseudovalves	LIF		<10	Four-protein mixture	[66]
IEF	SDS-PAGE	Gel pseudovalves	Fluorescence microscopy	2880		<i>E. coli</i> cell lysate	[69]
IEF	DIGE	Smaller channels	LIF			<i>E. coli</i> cell lysate	[70]
IEF	CGE	Vertical through- holes	Fluorescence microscopy			Three-protein mixture	[63]
IEF	CGE	Viscous solutions	Fluorescence microscopy	1700	<10	Five-protein mixture	[64]
IEF	RPLC	Smaller channels	MALDI-MS			BSA tryptic digest	[68]

BSA: Bovine serum albumin; CEC: Capillary electrophoresis; CGE: Capillary gel electrophoresis; CZE: Capillary zone electrophoresis; IEF: Isoelectric focusing; LIF: Laser-induced fluorescence; MEKC: Micellar electrokinetic chromatography; MS: Mass spectrometry; PDMS: Polydimethylsiloxane; RPLC: Reversed-phase liquid chromatography.