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Review

Recent progress in microchip electrophoresis–mass spectrometry

Fumihiko Kitagawa*, Koji Otsuka

Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

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ABSTRACT

This review highlights the methodological and instrumental developments in microchip electrophoresis (MCE)–mass spectrometry (MS) from 1997. In MCE–MS, the development of ionization interface is one of the most important issues to realize highly sensitive detection and high separation efficiency. Among several interfaces, electrospray ionization (ESI) has been mainly employed to MCE–MS since a simple structure of the ESI interface is suitable for coupling with the microchips. Although the number of publications is still limited, laser desorption ionization (LDI) interface has also been developed for MCE–MS. The characteristics of the ESI and LDI interfaces applied to the electrophoresis microchips are presented in this review. The scope of applications in MCE–MS covers mainly biogenic compounds such as bioactive amines, peptides, tryptic digests and proteins. This review provides a comprehensive table listing the applications in MCE–MS.

Contents

1. Introduction

In the analysis of biogenic compounds such as peptides, proteins, bioactive amines, sugar chains and metabolites, combination of mass spectrometry (MS) with an appropriate separation technique has been desired. So far, liquid chromatography (LC) and capillary electrophoresis (CE) have been recognized as the best selection in the coupling with MS, and thus the development of LC–MS [1] and CE–MS [2,3] have progressed. In LC–MS, reversed-phase (RP) mode is commonly employed for bioanalyses [4,5]. However, target biogenic compounds are sometimes too hydrophilic to be retained by the RP column. To overcome this problem, the ion-pair RP or ion-exchange mode are selected in LC–MS but an appropriate mobile phase is not available in some cases due to involatility of mobile-phase components, which reduces the sensitivity in MS. In the LC–MS analysis of biocompounds, the application of the hydrophilic interaction chromatography (HILIC) mode has been exhaustively investigated for the past few years [6]. CE also exhibits excellent analytical performances for biogenic compounds [7,8]. In CE, high separation efficiencies and selectivities can be obtained due to the plug-like electroosmotic flow (EOF) and various separation modes, respectively. Consequently, CE–MS

* Corresponding author. Tel.: +81 75 383 2448; fax: +81 75 383 2450.
E-mail address: fkitagawa@anchem.mc.kyoto-u.ac.jp (F. Kitagawa).

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has been regarded as a powerful analytical tool since MS detection shows high identification ability based on structural information of detected analytes [9]. Among various bioanalyses, 'omic' analyses including proteomics, peptidomics, metabolomics and glycomics require particularly massive measurements of biogenic compounds. Therefore, high-speed and high-throughput analysis systems employing MS detection scheme should be introduced for elucidating biological functions.

As an alternative separation scheme coupling with MS detection, microchip electrophoresis (MCE) has attracted much attention. In addition to several superior characteristics obtained in CE, MCE can realize rapid analyses in seconds, high efficiencies with low sample consumption because of an extremely reduced injection length of a sample and higher electric field strength compared to CE [10–12]. Furthermore, high-throughput analyses can be performed by employing microchips with highly integrated separation channels. The integration ability of other chemical processes such as extraction, desalting and preconcentration of analytes prior to the MCE separation and MS detection on single microchip is useful to realize the high-throughput bioanalyses without any connector. Therefore, coupling of MCE with MS has been investigated for the past dozen years [13,14].

In MCE–MS, the development of ionization interface is one of the most important issues to obtain good analytical performances. Among several types of the interface, electrospray ionization (ESI) and laser desorption ionization (LDI) have been applied to MCE–MS. In this article, successful ionization interfaces for MS detection combined with the electrophoretic "separation" microchips are mainly reviewed. The following sections provide an overview of microchip designs, ionization interfaces, coupling of a separation channel with packed beds and applications in MCE–MS.

2. Microchip design

In MCE–MS, it is important to combine a separation channel with an ionization interface on a single microchip. Classical microchip for the electrophoretic analyses consists of four reservoirs, a cross-type injector, and a separation channel. In the conventional MCE analysis, a sample solution can be injected as a short plug with a length of tens of micrometers. Since the Joule heating dissipation ability in the microchannel is superior to that in CE, higher electric field can be applied to the separation channel. These features of MCE provide high separation efficiencies. In this section, chip materials and channel geometries applied to MCE–MS are briefly overviewed.

2.1. Chip material

In the early stage of the development of MCE–MS, quartz and glass substrates have been mainly employed as a chip material [10–12]. Since these glass materials give a faster EOF, a separation time can be reduced. In addition, several microchannel coating techniques based on silane chemistry, which have been developed in fused silica capillary based electrophoresis, are available in the glass microchips. A wide variety of the coating techniques is advantageous for suppressing the adsorption of analytes such as peptides and proteins onto the microchannel. However, the production of the glass chips is expensive, time-consuming, and labor-intensive because the microdevices are fabricated by using photolithography and wet-etching techniques, which generally needs a clean room. Thus, cheap polymeric materials have been employed as the chip substrates.

Poly(dimethyl siloxane) (PDMS) is the most popular polymer in microfabrication technology. Since PDMS is an elastomeric material which can be cured by a heat treatment, microchannels are fabricated by replica-molding: soft lithography [15]. O_2 plasma treatment, which is often used for bonding channel and cover substrates, gives a faster EOF in the PDMS channel. Furthermore, the PDMS substrate seals readily with not only PDMS but other materials such as quartz, glass, and poly(methyl methacrylate) (PMMA) without any bonding process. These characteristics have allowed prototyping of PDMS chips for MCE–MS.

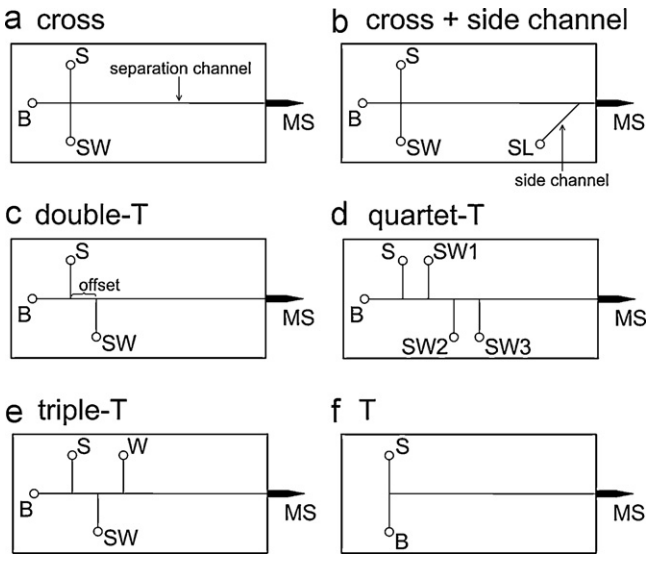

Fig. 1. Microchip channel geometries for MCE–MS. S, sample; B, BGS; SW, sample waste; SL, sheath liquid; W, washing liquid reservoirs.

PMMA is a popular polymer material and suitable for the use as the substrate for microfluidic devices because of its high thermal conductivity, low cost, high dielectric constant, and ease of fabrication. Several techniques for fabricating microchannels on the PMMA substrate, e.g., injection-molding [16], hot embossing [17], laser ablation [18] and wire-imprinting [17], have been introduced. Cyclo-olefin polymer (COP) is also a commercially available polymeric material. COP exhibits several superior characteristics such as low water absorption, low contamination, high transparency, good moldability, high thermal resistance, biocompatibility, ease of metallization, and high resistance to dissolution by common chemicals, e.g., methanol, acetonitrile, acids and bases [19]. Since polymer components and impurities which elute into a background solution (BGS) due to the dissolution by organic modifiers may interfere with the detection of target analytes, low contamination and high chemical durability of the polymeric materials are suitable properties in fabricating the microdevices for MCE–MS. Recently, a negative photoresist, SU-8, has been utilized as the chip substrate for MCE [20]. SU-8 microstructures of desired shape and size can be fabricated by a standard UV lithography. SU-8 is also a chemically stable material with glass-like surface properties with respect of the surface charge and EOF. Application of these polymeric materials to the fabrication of MCE–MS devices allows the disposable use. Since a clogging occurs frequently in the microchannel, the disposability is one of the important factors for selecting the chip material in MCE–MS.

2.2. Channel geometry

As one of the most useful features of microfabricated devices, multiple channels can be combined without any connector. To realize high performance analyses, several types of injectors and separation channel configurations have been developed in MCE–MS (Fig. 1). As mentioned above, simple cross-type channel geometry has often been employed to a microchip for the

electrophoretic separation prior to MS detection (Fig. 1a). In the cross-channel microchip, a sample solution is introduced into a separation channel by the pinched [12] or gated [21] injection technique. Since an extremely short sample plug can be introduced by the pinched injection, higher efficiencies can be obtained. On the other hand, the gated injection technique allows the sample introduction with a desired plug length by adjusting an application time of an injection voltage. As shown in Fig. 1b, a side channel connecting to the end of the separation channel has been fabricated for MCE–MS [22]. From the inlet of the side channel, a sheath liquid, which is employed to stabilize the ionization, can be introduced to an ESI interface. In this type of microchip, a sample dilution by the sheath liquid should cause the decrease of the MS sensitivity. To compensate for the dilution, the gated injection technique on the cross-channel or T-formed channel chip has often been employed in combination with a sheath liquid-assisted ionization interface.

Among several microchannel chips containing T-injectors, the double-T channel geometry is widely used, which allows the injection of a fixed volume of a sample solution into a separation channel (Fig. 1c) [23]. During the loading step, the sample solution are loaded into the offset between two T injectors, and then the sample loaded in the offset channel is flushed into the separation channel by a BGS. Since a larger volume of the sample can be injected compared to that in the pinched injection on the cross-channel chip, the MS sensitivity reduction caused by introducing the sheath liquid is compensated on the double-T channel chip in spite of the risk of the decrease in the plate numbers. To overcome this drawback on the double-T channel chip, the application of on-line sample preconcentration by transient-isotachophoresis has been investigated [23]. The preconcentration of the sample injected as a long plug into the separation channel on the double-T channel chip will enhance both the plate numbers and sensitivities. Zhang et al. have developed a quartet-T channel chip which contains three sample waste reservoirs and hence short, medium and long sample injection lengths are available in the single microchip (Fig. 1d) [24]. They have also developed a triple-T channel chip (Fig. 1e) [25]. Sample injection is performed via the double-T channel and other T-injector is used for introducing a washing solution after the measurements. Mao et al. have fabricated a single-T channel chip as shown in Fig. 1f [26]. A sample solution can be introduced through the T injector by applying a high voltage to an ionization interface with the sample reservoir grounded and the BGS reservoir floating. In these T channel-based microchips, the number and configuration of T-injector can determine specific functions which are useful to improve the plate numbers, MS sensitivities and/or analytical performances.

3. Ionization interface

In MCE–MS, an ESI has been mainly employed as the interface since it is the most suitable for ionizing analytes dissolved in a liquid phase. Additionally, the ESI interface should not require a complicated structure on microchips. Various ESI interfaces have been developed for MCE–MS, which can be roughly classified in three types: (i) microsprayer with a similar structure of CE–ESI-MS interface, (ii) tapered capillary spray (nanospray tip) inserting in an access hole of a separation channel, and (iii) monolithically fabricated spray tip structure. On the other hand, an LDI interface has also been applied to MCE–MS. In MCE–LDI-MS, a laser for ionization has been irradiated to a separation medium directly or after depositing a matrix which is used in conventional matrix-assisted LDI (MALDI). In this section, the ESI and LDI interfaces coupled with the MCE separation and MS detection are mainly reviewed.

3.1. ESI interface using tapered capillary tip

In the first microchip–ESI-MS report, an electrospray has been simply emerged from a channel opening fabricated at an edge of planar substrate [22]. The channel opening from where an electrospray is generated has been fabricated by nicking and breaking the end of a separation channel. The fabricated glass microchip contains a conventional cross-type channel and a linear-polyacrylamide (LPA) coated side-channel connecting to the separation channel. By applying the voltage between sample-inlet and side-channel reservoirs, the EOF is generated toward the opening of the separation channel, forming the Taylor cone and electrospray at the channel opening. On the fabricated microchip, tetrabutylammonium iodide can be detected with an ion-trap mass spectrometer under an infusion mode. However, it is supposed that the separation performance may be insufficient to analyze a complexed mixture because the Taylor cone generated at the channel opening is very large (~20 nL volume). Figeys et al. have reported that conventional fused-silica capillary of 12 cm in length is attached to the terminus of the separation channel on the planar edge of the microchip via a Teflon sleeve and a stainless steel tubing [27]. A 3 cm length of the capillary is inserted at the other end of the tubing to form a liquid junction for applying the ESI voltage. In the fabricated microdevice, the sequential infusion of a small amount of a sample solution can be realized and tryptic digests of proteins are successfully analyzed by ion-trap MS. Unfortunately, the MCE separation on the microchip has not been reported but the microdevice is apparently pioneering in the tapered capillary-based ESI interfaces.

In 1999, a first microchip device for coupling the MCE "separation" with ESI-MS detection has been developed [28]. In the fabricated device, an opening is drilled at the end of a separation channel, perpendicular to a planar surface of a glass microchip with a single cross channel or cross/side channel configuration. A commercially available tapered capillary (360 μm o.d., 50 μm i.d., 5 μm i.d. at the tip) is inserted into the channel opening, which works as the ESI spray (nanospray) tip. The ESI voltage of 1.3–1.6 kV is applied to the microchip through a platinum electrode inserted in an inlet reservoir. The microchip is positioned in front of the orifice of a time-of-flight (TOF) mass spectrometer. It should be emphasized that TOF-MS, which is a non-scanning detector, is suitable for MCE–MS since monitoring of narrow peak profiles with their time scale of a few seconds or less is generally required. In an infusion analysis, 400 zmol of gramicidin S can be detected. When a five peptide mixture is analyzed by MCE–MS, three peptides are detected as a single peak due to serious peak broadening. This band broadening effect may be caused by several factors, e.g., dead volume, flow flux mismatch between the separation channel and the nanospray tip, laminar flow induced by the negative pressure at the ESI tip, sample adsorption onto the inner wall of the channel, and so on.

To clarify the band broadening at the ESI spray inserted into the access hole to the separation channel, several investigations have been carried out. Bings et al. have reported that the shape of the access hole strongly affects the efficiencies [29]. When the access hole is created by a standard pointed drill bits, conically shaped bottom is formed. This leads to a dead volume of 0.7 nL at the junction. Consequently, such dead volume causes a significant band broadening. By removing the conical area with a flat-tipped drill bit, the dead volume is almost eliminated. As a result, the obtained plate number is 98% of the predicted value. On the other hand, the effect of pressure flow generated by the difference of the EOF rate between the separation channel and narrower ESI capillary tip has been investigated [30]. In case where there is EOF in channel (100 μm width) and capillary tip (11 mm length, 20 μm i.d.), theoretical calculation indicates that the original EOF will be reduced to 23%, which agrees well with the experimental values. Since the pressure flow induced by such EOF restriction reduces the sepa-

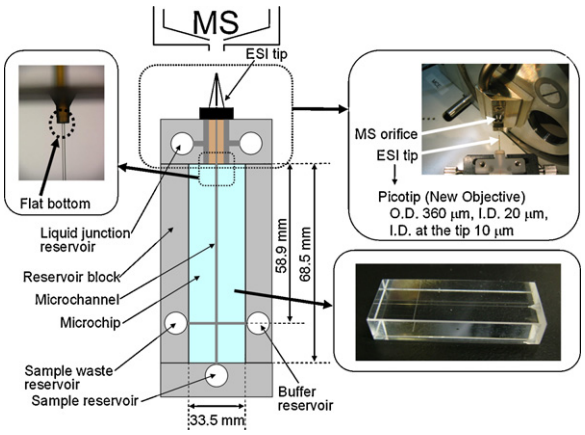

Fig. 2. Schematics of the microdevice and photographs of tapered capillary-based sheathless ESI interface.

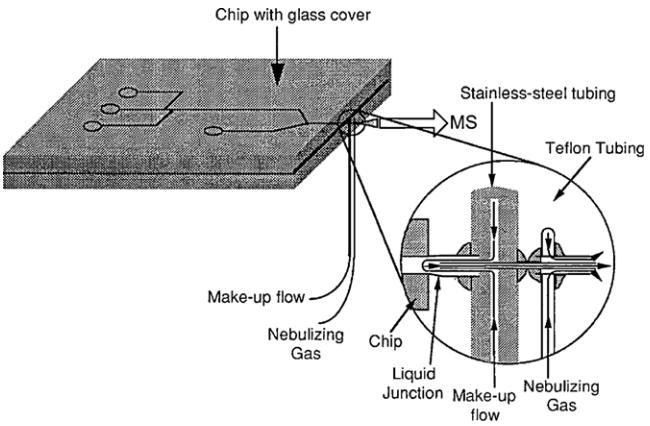

Fig. 3. Schematic drawing of the glass chip-based CE/MS apparatus and the expanded view of the coupled microsprayer.
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ration efficiency, the length of the inserted capillary tip should be kept to a minimum. Tachibana et al. have developed a microchip to which the spray tip can be attached using a PEEK screw without adhesive (Fig. 2) [31,32]. They studied the effect of the ESI tip size and the length of the separation channel on the separation and detection performances. In the analysis of basic drugs, the separation is improved significantly by using 20 μm i.d. spray tip compared to 50 μm i.d. This is probably due to a negative pressure generated at the tip of the ESI capillary. The adverse effect on the ESI tip can be minimized by using a microchip with a long separation channel, resulting in the improved efficiency. The microchip modified along these findings can realize the MCE–MS analysis of drug components, peptides, tryptic digestions [31,32], and proteins [33].

As a more sophisticated tapered spray interface, a glass microchip having a monolithically integrated tapered ESI emitter has been fabricated [34–36]. In the fabrication, a little cone with a diameter of 0.3 mm is milled, and then the cone encloses the open end of a separation channel centrically to give a dead-volume-free coupling of the MCE microchip with the ESI spray. The protruding cone is drawn to a sharp tip by a heating puller. The fabricated ESI spray has a very fine tip structure with tapered microchannel, whose dimension is 15 μm o.d. and 5 μm i.d. at the tip. When the voltage of 4 kV is applied to an inlet reservoir of the developed device and a MS orifice is grounded, a BGS is efficiently sprayed without external pressure. The analytical performance of the microchip is also excellent to separate four pharmaceutical components within 100 s with their plate numbers of 10,000–20,000 [34]. By coating the channel surface with hydroxypropylmethyl cellulose (HPMC), the efficiency can be improved to be 89,000 [35]. Furthermore, the combination of the developed chip with the high electric field of 5800 V cm^{-1} and fast TOF-MS provides extremely rapid separation within only 2 s [36]. Although the sheathless and dead-volume-free MCE–MS device is very attractive especially for the proteomic and metabolomic analysis, a clogging in the fine ESI tip and/or narrow separation channel will be troublesome during replicate measurements. Thus, the introduction of disposable chip substrates such as PDMS, PMMA and COP is desired in such highly integrated microchips.

3.2. Sheath-liquid and gas assisted microsprayer ESI interface

In CE–ESI-MS, a sheath liquid and a sheath gas are generally introduced in a microsprayer-type interface to stabilize the ESI. These sheath flows not only compensate for the low flow flux in CE but suppress the corona discharge at the spray tip. In MCE–MS, the sheath-liquid and gas assisted microsprayer ESI interface has been developed. As a prototype of this ESI interface for MCE–MS, Li et al. have reported that a traditional microsprayer interface developed in CE–ESI-MS is coupled to a separation microchip via a low dead-volume connector and capillary tubing [37]. In the ESI interface, the electrospray voltage is independently applied to a stainless steel needle, which is also used to deliver the sheath solution via a tee located near the exit of the capillary tubing. The ESI interface provides relatively high separation efficiencies ranging from 26,000 to 58,000 for peptide mixtures. This device can be also employed to the analysis of protein digests whereby peptide sequence segments identified in the MS/MS are used to search database using only 150 fmol of the original protein.

To miniaturize the ESI sprayer for coupling the MCE chip with MS detector, Teflon/stainless-steel tubes and liquid junction based microsprayer has been developed [38]. This microsprayer employs a free-standing liquid junction formed via continuous delivery of a sheath flow which carries the MCE effluent through a pneumatically assisted electrospray needle positioned in front of an orifice of atmospheric pressure ionization (API) triple–quadrupole mass spectrometer (Fig. 3). The liquid junction acts as a dynamic and flowing liquid bridge between the exit of separation channel of the microchip and the inlet of the microsprayer. For the MCE separation, a glass microchip with double T injector and 4.2 cm separation channel is connected to the microsprayer [39]. In the MCE–ESI-MS analysis, carnitine and three acetylcarnitines in human urine are separated in less than 48 s with the plate numbers of 2860. The determination of the same analytes by MCE–MS can be performed on a disposable COP microchip combined with the microsprayer interface [40]. The ESI interface can be applied to the MCE–MS analysis of fortified human plasma samples containing imipramine and desipramine [41]. By using internal standards, good calibration curves ranging from 5 to 500 μg mL^{-1} are obtained with intra-assay accuracy of 94.0–104%. Thus, quantitative determinations of real samples containing drug components and biogenic amines can be carried out with the microsprayer-type MCE–ESI-MS devices.

Mao et al. have been reported that an ESI interface is made with a deliver capillary connecting to a separation glass microchip coated with 3-aminopropyltriethoxysilane (APTS), a commercial tapered capillary for spraying, and a stainless steel tube which are connected together through a tee unit [26]. High ESI voltage and a sheath liquid with the flow rate of 100–200 nL min^{-1} can be applied via the stainless tube. In the fabricated MCE–ESI-MS device, glycoproteins and glycopeptides are successfully analyzed with a limit of detection (LOD) at femtomole level [26]. An ESI interface has been developed to allow the introduction both of a sheath liquid and gas

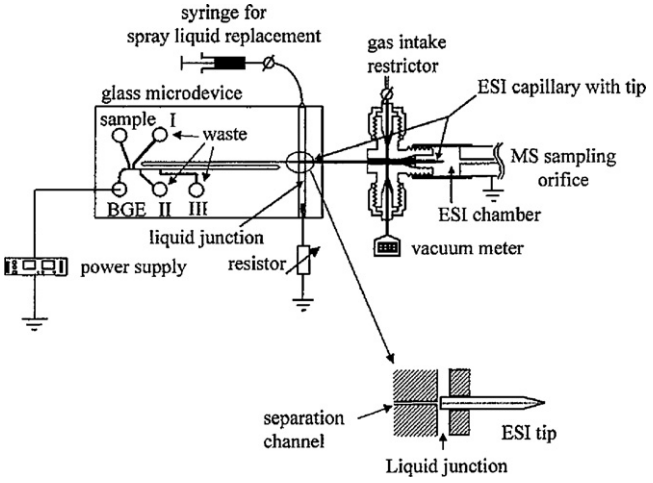

Fig. 4. Diagram of the microdevice with a sub-atmospheric electrospray interface. The expanded view shows the coupling of the ESI tip with the separation channel in the liquid junction.
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by using two tee units [42,43]. The developed ESI can be coupled to a glass microchip with cross-type microchannel. The MCE–ESI-MS device provides successful determinations of glycopeptides [42] and peptides [43]. Although there are significant problems concerning the sample dilution by employing these sheath flow-assisted microsprayer interfaces, they are effective for stabilizing the electrospray in MCE–MS.

3.3. Chamber-type ESI interface

The nanospray and the microsprayer ESI interfaces are based on the downsized CE–ESI-MS interfaces. In this section, an ESI interface originally developed for MCE–MS is overviewed. Karger and co-workers have developed a sub-atmospheric chamber-type ESI interface to combine an electrophoresis glass chip and mass spectrometer [23,24]. The developed MCE–MS device integrates a sample inlet, a separation channel, a liquid junction, and guiding channel for inserting an ESI spray capillary, which is enclosed in the sub-atmospheric chamber of an ion-trap mass spectrometer (Fig. 4). In this device, the ESI capillary can be aligned with the exit of the separation channel by the guiding channel. The sub-atmospheric chamber equips with a gas intake restrictor and vacuum meter to maintain a constant pressure of 78 kPa in the chamber, resulting in a stable flow volume and ESI. By using the developed ESI interface, eight-angiotensin peptide mixture, four-protein mixture, and bovine serum albumin (BSA) tryptic digests are successfully separated and detected within 8 min. The plate numbers as high as 31,000 are achieved with the LOD in the attomole range [24]. It should be noted that the developed MCE–ESI-MS system is independent of the EOF in the microchannel since the flow rate can be controlled by the pressure in the ESI chamber. This is highly advantageous for utilizing all separation modes including the MCE analysis in a neutral surface coatings modified channel or in a polymer-filled channel.

Furthermore, the MCE–MS device with the sub-atmospheric ESI chamber has been coupled with automated sampling from a 96-well sample microwell plate for high-throughput analysis [25]. Buffer reservoirs external to the microdevice and a computer-activated electropneumatic distributor are used for automated sample loading and device washing after each run. The analysis begins with loading of the sample from the microwell plate by applying a vacuum at a sample loading port. A short vacuum pulse is applied to an injection port to fill a sample loop, and then the vacuum is turned off and the voltage is applied for the MCE separation and ESI-MS detection. After the analysis, all of the channels are washed by applying pressure on the liquid reservoirs, which effectively reduces the sample carry over during replicate runs. In the MCE–MS analysis of a tryptic digest of BSA, a partial separation of 29 peaks is obtained within 10 min with the efficiencies over 10 000 plates. A total of 28 of the 29 peaks can be directly identified from a database. The reproducibilities of the migration times and the peak height are ~2% and ~7%, respectively. The combination of such fast, reproducible, automated MCE–MS system with disposable polymer chips and/or microdevices with multiplexed separation channels should considerably enhance the throughput of bioanalysis.

3.4. ESI interface using metal coated capillary

In the MCE–ESI-MS systems mentioned in the previous sections, the ESI voltage is generally applied via a liquid junction or an inlet reservoir. In this section, an ESI interface with a metalized nanospray, which allows the direct application of the ESI voltage, is described. Li et al. have reported that a gold coated nanospray is inserted into an access hole fabricated at the end of a separation channel of a microchip to form an ESI interface [44]. The inner surface of the separation channel is coated with [(acryloylamino)propyl]trimethyl ammonium chloride (BCQ) to suppress the irreversible adsorption of sample peptides. Since the coating generates a reversed EOF, the tip of the gold nanospray applying the ESI voltage acts as the anode. Although proteolytic digests are analyzed in less than 90 s with peak widths of 2 s for individual peptides and sample proteins can be indentified with the peptide mass fingerprint database searching, the resolutions are still poor probably due to a short separation length. To improve the detection sensitivity, furthermore, on-line sample preconcentration by field-amplified stacking and solid phase extraction (SPE) with a C_{18} membrane filter has been carried out in the developed MCE–MS device. In the analysis of standard peptides, the LODs of 0.3 nM in the stacking and 2.5 nM in the SPE can be obtained. It should be emphasized that the metal coated nanosprays are damaged by Colona discharge during applying the ESI voltage, resulting in a short lifetime of the nanospray. The insertion of a resistor of 10–100 MΩ into the ESI voltage circuit is effective to suppress the discharge.

To obtain longer lifetime and robustness of the coated spray, a sheathless ESI interface with a conductive rubber coated nanospray has been developed [45]. In the conventional nanospray ESI interface, a tapered capillary is simply inserted into an access hole and hence a dead volume produced at the connection point is often problematic, i.e., reduced separation efficiency and MS sensitivity. To overcome this problem, a wire assisted method has been applied to allow an easy alignment between a PMMA electrophoresis channel and a nanospray. Before the connecting point is filled with the epoxy resin, a wire is inserted into a separation channel and nanospray capillary to minimize the dead volume. However, the obtained separation efficiencies for standard peptides are apparently poor. The adsorption of the peptides onto the PMMA surface by the hydrophobic interaction has been proposed as the major reason for the poor efficiencies.

A hybrid device has been fabricated by combining capillary and PDMS microchip with integrated ESI nanospray [46]. In the fabrication of the PDMS microchip, stainless steel wires are used to define a microchannel and the shape of the nanospray is drilled into the mould. PDMS prepolymer is cast to cover the metal wires and the mould, which overcomes the bonding and alignment problems. The PDMS nanospray structure fabricated at the end of the separation channel is coated with graphite powder to provide electric conduc-

tivity. The graphite coated emitter tip sprays continuously for more than 180 h at the flow rates ranging from 0.1 to 1.0 μL min^{-1}. The stability of the ESI is also confirmed by recording the MS ion current in which the RSD value of the signal is 3.9% for a 30 min run. In the straight channel fabricated on the chip, five standard peptides can be separated and detected by MS within 2 min with similar efficiencies as for conventional CE. Thus, such hybrid nanospray ESI interface is effective for suppressing band broadening effects originating from the connection between the separation channel and the nanospray.

3.5. ESI interface monolithically integrating sharp-pointed structure

In the development of the ESI interface in MCE–MS, the tapered capillary has been mainly employed to the spray emitter. Recently, spray emitters with more simple structures have been developed. A microchip integrating a sample injection, a separation channel, and an ESI emitter has been fabricated [47]. The emitter is designed as triangular-shaped structure with 8-mm long, forming 42° pointing angle. A thin layer of uncured PDMS followed by a methanolic dispersion of graphite powder is applied on the emitter structure to keep electric contact at the tip. The ESI performance is sufficient to detect a 10 μg mL^{-1} to some extent separated four-peptide sample. However, the separation efficiencies on the fabricated device are apparently low due to irreversible adsorption of the analytes onto the bare PDMS surface and the applicable BGS is limited to higher pH. To improve the separation performance, a dynamic coating with a cationic polymer, PolyE-323, has been introduced in the PDMS channel on the same device [48]. As a result, three peptides can be separated with acceptable resolutions. Furthermore, a reversal EOF produced by PolyE-323 can expand the applicable pH to allow the use of acidic and neural BGS. Shinohara et al. have fabricated a COP microchip with an ESI tip directly machined on the end of a separation channel [49]. The tapered tips with the angle of 180°, 90°, 60° and 30° are structured by a circular saw. To obtain an electric contact, the ESI tip structure is coated with gold by electron beam evaporation. By applying the ESI voltage of 1.8 kV to the spray tip via the gold coating, the most stable ESI is observed at the tip angle of 30°. On the fabricated COP device, an amino acid and a drug component are separated and detected by MCE–ESI-MS. However, the separation efficiencies and detection sensitivities are insufficient. This may be due to the formation of a large Taylor cone at the ESI emitter tip.

A unique approach for integrating a triangular-shaped ESI spray has been developed. A microchip is fabricated fully of a negative photoresist SU-8 by a standard photolithographic process to pattern three-layered design incorporating a fluidic inlet, an enclosed microchannel, and a sharp-pointed ESI tip [20]. In the developed device, 100 μm × 50 μm sized spraying channel is formed at the apex of the emitter structure. A side arm channel is also integrated at the end of the separation channel to introduce a sheath flow. When the ESI voltage of 3.5 kV is applied to the sheath flow inlet, a fine spray is generated and orientated straightforward from the tip orifice. In the MCE–ESI-MS analysis of alkylammonium salts, drug substances and peptides, very narrow peak width (down to 2–3 s), plate numbers in the order of 10^5 m^{-1} and high resolution can be obtained. It should be noted that the developed process enables highly reproducible fabrication of multiple chips with precisely controlled dimensions.

Recently, Ramsey and co-workers have developed a microfabricated device in which ESI is performed directly from the corner of a rectangular glass microchip to eliminate the droplet formation at the tip [50,51]. The distal end of a separation channel is fabricated at the corner to provide a sharp two-dimensional feature for the ESI tip (Fig. 5). The ESI tip is sharpened in the third dimension by reducing the chip thickness to 300 μm. The detection sensitivity in the developed device is similar with that in a conventional MCE–MS device with a tapered capillary. Furthermore, the baseline noise is about twice as high for the capillary emitter. By the surface coating of PolyE-323, the MCE–MS analysis of peptides, proteins and cell lysates is successfully achieved with the efficiencies over 10^6 m^{-1}. This device operates at about twice the efficiency of the most efficient devices previously reported. Such efficient separation of peptides and proteins opens up the possibility of using the MCE–MS device not only for further advanced proteom studies but system biology.

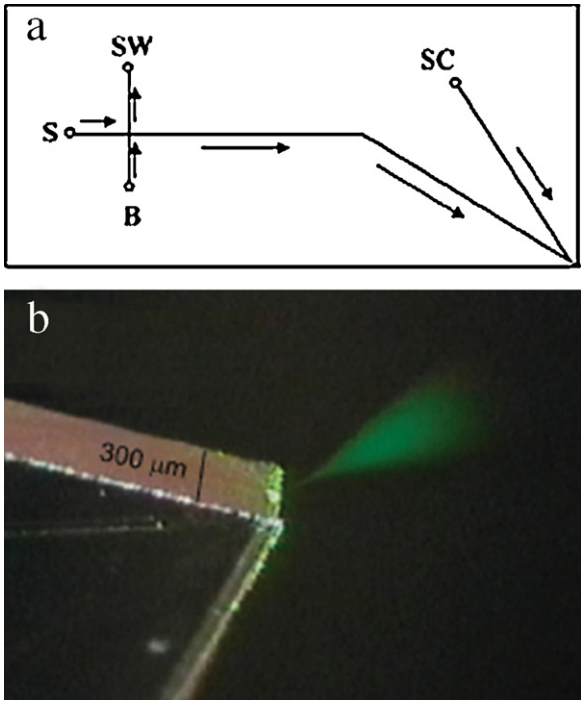

Fig. 5. (A) Schematic of a microfabricated device in which ESI is performed directly from the corner of a rectangular glass microchip. (B) Image of the electrospray plume generated from the corner of a MCE–ESI-MS chip.
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3.6. LDI interface

In the MCE–MS analysis, several buffer systems such as phosphate and borate are incompatible due to low volatility of electrolytes. Especially in ESI-MS, the nonvolatile additives cause low ionization efficiency and lower detectability of analytes. To overcome this limitation, the applications of LDI to MCE–MS have been investigated [52–57]. In the first MCE–LDI-MS report, the MCE separation with a BGS containing conventional matrix component (2,5-dihydroxybenzoic acid) in MALDI is performed in an open-access channel fabricated on a glass microchip, and the chips are transferred to a MALDI source after the solvents is evaporated, i.e., the channel yields a MALDI sample complete with matrix and ready for analysis [52]. Although the separation of several test analytes such as sugars and peptides can be obtained in the open-channel with 150 μm width and 16 mm separation length, the peaks are broader ranging from 2 mm to 3 mm. In this device, the separation voltage is limited to 400 V due to bubble formation, which may cause a band broadening in the separation channel. On the other hand, a combination of a gel electrophoresis chip with matrix-free IR-LDI has been developed [53]. The chip is fabricated from

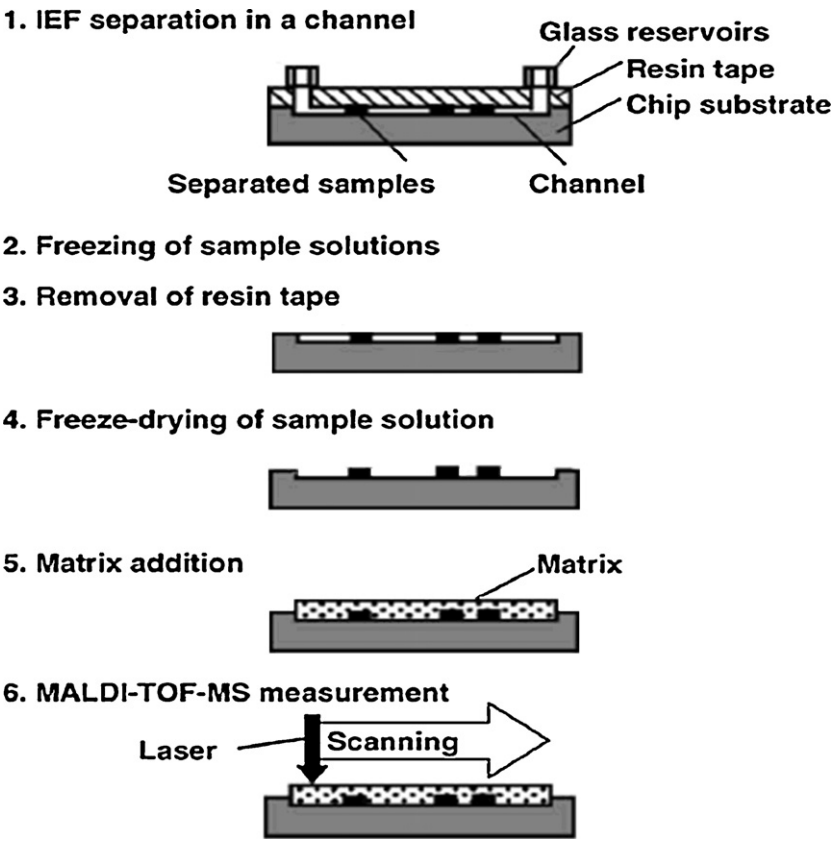

Fig. 6. Schematic representation of experimental procedure in MCIEF–MALDI-MS. Illustrations show cross-sectional views of chip during each process. Reprinted with permission from Ref. [56] (Copyright 2005 Elsevier B.V.).

PMMA with a PDMS cover. After sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) separation of peptides and proteins is carried out in the microchannel, the PDMS cover is removed. The gel attached either to the channel or cover plates is inserted into vacuum and ablated with a pulsed IR laser beam. By using the gel microchip, the separation and MS detection of bradykinin and bovine insulin can be attained in an automated fashion in the mass spectrometer. The chip is driven by the motor at its rate of 1.3 mm min^{-1} and mass spectra are recorded continuously by a computer control, which provides two-dimensional contour plot like slab gel electrophoresis. However, broad peaks are observed in the off-line automated analysis, suggesting that the analytes are sticking to the polymer as it migrates through the microchip. Thus, the reduction of analyte sticking by surface coating and minimization of the dead volume formed between the gel and cover plate should be desired to achieve high efficiencies in MCE–LDI-MS. Similar approaches using a channel substrate and a removable cover have been applied to microchip isoelectric focusing (MCIEF)–MALDI by Mok et al. [54,55] and Fujita et al. [56]. In the Mok's device, the MCIEF of proteins is carried out in pseudo-closed channels [54]. After the cover plate is removed, a matrix solution is applied for MALDI-MS. The authors have found that the pseudo-closed channel is very important since the solvent drying is serious in an open-channel format. Fujita et al. have also developed the MCIEF–MALDI technique using a resin tape cover and freeze-drying process as shown in Fig. 6 [56]. It should be emphasized that the band widths of separated proteins remain constant before and after the fixation by freeze-drying. Thus, no band broadening during the transfer process from MCIEF to MALDI results in good resolution of proteins. The whole process of the 2D-mapping of proteins takes less than 70 min, more than 10 times faster than with conventional 2D-PAGE.

As a unique MALDI interface, on-line coupling of PMMA microchip with MALDI-MS using a rotating ball interface has been developed [57]. In the rotating ball interface, samples are introduced at atmospheric pressure and transported into a high vacuum of a mass spectrometer without breaking an ion source vacuum. The exit of the separation channel is machined to allow the direct contact deposition of effluent onto the rotating ball interface. After a matrix is added onto the surface of the ball, a UV laser is irradiated for MALDI-MS detection. In the microdevice, three peptides are well separated and detected by MCE–MALDI-MS. The tryptic digest of cytochrome *c* can be also analyzed to identify the intense peaks above *m/z* 700 in the contour plot. Thus, the robust coupling of MCE with MALDI-MS is attained with high separation efficiencies by the rotating boll interface. In the present stage, the number of reports on the interface for MCE–MALDI-MS is still limited. The progress in the microfabrication techniques will accelerate the development of novel interfaces and enhance the analytical performance of MCE–MALDI-MS.

4. Coupling of MCE–MS with packed beds

Microfluidic devices fabricated on planar substrates are suitable for a low dead volume connection between several sample handling sites, e.g., combining a sample pretreatment site with an electrophoretic separation channel. In MCE–MS, packed beds for protein digestion, desalting and on-line SPE for sample enrichment have been combined with a separation channel. To combine protein digestion with MCE–MS, a microchip consisting of immobilized trypsin bead beds has been fabricated [58]. The TPCK-treated trypsin immobilized agarose beads with 40–60 μm diameter are packed into the bed channel with 150 μm depth and 800 μm width.

Table 1
Applications in MCE–MS.

Sample	Chip substrate	Channel configuration	Channel coating	Ionization interface	Mass spectrometer	LOD	Reference #
Ammonium salts	Glass	Cross + side channel	LPA on side channel	Sheath liquid-assisted ESI from flat channel opening on chip edge	Ion-trap MS		[22]
Rhodamine dyes	Glass	Cross + side channel		Tapered capillary-type ESI with sheath liquid through glass membrane	TOF-MS		[30]
Carnitines	Glass	Double-T + side channel	BCQ	Sheath-liquid and gas assisted microsprayer ESI	QTOF-MS	fmol	[39]
Carnitines	COP	Double-T		Sheath-liquid and gas assisted microsprayer ESI	API triple–quadrupole MS		[40]
Carnitines/amines	Glass	Double-T	BCQ	Sheath-liquid and gas assisted microsprayer ESI	API triple–quadrupole MS	1 ppm	[41]
Amino acid/caffeine	COP	Cross		Sheathless ESI from sharp-pointed emitter structure	Quadrupole MS		[49]
Glycopeptides	Glass	Cross		Tapered capillary-type ESI with sheath-liquid and gas through two tee-units	QTOF-MS	20 pmol	[42]
Peptides	Glass	Cross		Tapered capillary-type ESI with sheath-liquid and gas through two tee-unit	QTOF-MS		[43]
Peptides	PMMA	Cross		Sheathless ESI from conductive rubber-coated tapered-capillary	ion-trap MS	100 nM	[45]
Peptides	PDMS	Double-T		Sheathless ESI from graphite powder-coated sharp-pointed emitter structure	TOF-MS	1–10 ppm	[47]
Peptides/sugars	Glass	Cross		MALDI on open-access channel	Fourier-transform MS		[52]
Tryptic digests of melittin, BSA and cytochrome *c*	Glass	Double-T + side channel	BCQ	Sheath liquid-assisted ESI from gold-coated tapered-capillary	QTOF-MS		[58]
Pharmaceutical compounds/tryptic digests of BSA	Glass	Cross	HPMC	Monolithically integrated nanospray-type sheathless ESI	Quadrupole MS	~10 ppm	[34,35]
Pharmaceutical compounds/peptides	Glass	Cross		Monolithically integrated nanospray-type sheathless ESI	TOF-MS		[36]
Basic drugs/peptides/tryptic digests of cytochrome *c*	Quartz	Cross		Tapered capillary-type sheathless ESI with liquid junction	Quadrupole MS		[31]
Basic drugs/amino acids/peptides/tryptic digests of cytochrome *c*	Quartz	Cross	SMIL[a]	Tapered capillary-type sheathless ESI with liquid junction	Quadrupole MS		[32]
Basic drug components/peptides/tryptic digests of hemoglobin	Glass	Double-T + side channel		Sheath liquid-assisted ESI from tapered capillary	TOF-MS	Sub-fmol	[60]
Angiotensin peptides/tryptic digests of cytochrome *c*	Glass	Double-T		Sub-atmospheric chamber-type ESI	Quadrupole ion-trap MS	10 nM[c]	[23]
Angiotensin peptides/tryptic digests of BSA	Glass	Triple-T	LPA	Sub-atmospheric chamber-type ESI	Quadrupole ion-trap MS		[25]

Table 1 (*Continued*)

Sample	Chip substrate	Channel configuration	Channel coating	Ionization interface	Mass spectrometer	LOD	Reference #
Peptides/tryptic digests of RNase	Glass	T	APTS	Tapered capillary-type ESI with sheath liquid through a tee-unit	Quadrupole ion-trap MS	fmol	[26]
Peptides/tryptic digests of lectin	Glass	Triple-T + side channel	BCQ	Sheath-liquid and gas assisted microsprayer ESI	Triple–quadrupole MS	45 nM[c]	[37]
Peptides/tryptic digests of phosphorylase B	Glass	Double-T + side channel	BCQ	Sheath liquid-assisted ESI from gold-coated tapered-capillary	QTOF-MS	Sub-nM	[44]
Peptides/tryptic digests of α-casein	Glass	Double-T + side channel	BCQ	Sheath liquid-assisted ESI from gold-coated tapered-capillary	QTOF-MS	5 nM[c]	[59]
Angiotensin peptides/proteins/tryptic digests of BSA	Glass	Quartet-T	LPA PVA[b]	Sub-atmospheric chamber-type ESI	Quadrupole ion-trap MS	Sub-μM	[24]
Peptides/proteins/tryptic digests of BSA/cell lysates	Glass	Cross + side channel	PolyE-323	Sheath liquid-assisted ESI from corner of rectangular chip	QTOF-MS		[50,51]
Peptides/tryptic digests of cytochrome *c*	PMMA	Double-T		MALDI on rotating ball interface	TOF-MS		[57]
Ammonium salts/drug components/peptides/proteins	SU-8	Cross + side channel	PMA[d]	Sheath liquid-assisted ESI from sharp-pointed emitter structure	API triple–quadrupole MS	100 nM[	[20]
Peptides/proteins	Glass	Cross		Tapered capillary-type sheathless ESI	TOF-MS	~100 nM	[28]
Peptides/proteins	PMMA/PDMS	Cross		LDI on gel filled channel chip with removable cover plate	TOF-MS	pmol	[53]
Basic proteins	Quartz	Cross	PolyE-323	Tapered capillary-type sheathless ESI with liquid junction	TOF-MS		[33]
Proteins	Polymeric substrates	Straight		MALDI on IEF chip with removable cover substrate	TOF-MS		[54,55]
Proteins	Glass/resin tape	Meander		MALDI on IEF chip with removable resin tape	TOF-MS		[56]

[a] successive multiple ionic-polymer layers.
[b] poly(vinylalcohol).
[c] obtained with on-line sample preconcentration.
[d] dynamic coating.

For the ESI, a gold coated nanospray is connected to the end of a separation channel. Sample proteins (BSA and cytochrome *c*) pumped through the packed bed are adequately consumed at a flow rate of 0.5–1.0 μL min^{-1}, corresponding to a digestion time of 3–6 min. The ESI-MS analyses of sample digests are performed with the coverage of the amino acid sequence ranging from 71% to 92%. The total time required for the sample injection, digestion, MCE–MS, and cleaning the bed and channels is 11–14 min. The same microchip format packed with C_{18} RP-packings has been employed to the preconcentration of tryptic digests, resulting in the LOD of 5 nM [59]. Tryptic phosphopetides are captured selectively on the bed packed with metal affinity chromatography beads, and subsequent separation and characterization can be obtained by MCE–MS. These chip designs for coupling MCE–MS with sample pretreatment sites provides a convenient platform for automated analytical processing in proteomics applications.

A microdevice which integrates a methacrylate-based polymer monolithic column, an injector, an ESI interface based on a tapered capillary tip has been developed for microchip electrochromatography (MCEC)–MS [60]. The polymer monolithic structure is prepared by filling all microchip channels with acrylate monomer solution containing photoinitiator and exposing the chip to UV irradiation. Although a complete separation of all peptide components is not achieved in the analysis of standard protein digests (800 amol), sequence coverage of 70–80% is obtained in MCEC–MS. Such coupling of MCEC with MS is highly advantageous for enhancing the selectivities. For example, the introduction of a monolithic HILIC [61] channel is expected to analyze polar biogenic compounds in MCEC–MS.

5. Applications

While this review is concerned mainly with advances in methodology and instrumentation, it is also relevant to mention advances in MCE–MS applications. The advances in MCE–MS have been applied to the analysis of amino acids, peptides, tryptic digests, proteins, amines, sugars, and pharmaceutical compounds. Table 1 details the applications for the MCE "separation"–MS in this

review. Apparently, the separation and identification of peptides toward proteomic research is major application in MCE–MS. In the analysis of biogenic compounds including peptides and proteins, adsorption of the analytes onto the inner surface of microchannels is serious problem to reduce the efficiencies. In MCE–MS using glass microchips, several coatings such as BCQ [37,39,41,44,58,59], PolyE-323 [33,50,51], LPA [22,24,25], poly(vinyl alcohol) (PVA) [24], successive multiple ionic-polymer layers (SMIL) [32], HPMC [35] and APTS [26] have been employed to prevent the surface adsorption. However, these modification techniques can not be applied to polymer microchips since they have been developed originally in fused silica capillary-based electrophoresis. In the MCE analysis, dynamic coating using appropriate polymers, e.g., celluloses and poly(ethylene glycol), is generally carried out for not only glass but polymeric substrates. When these coating polymers are applied to MCE–MS, their penetrations to mass spectrometer reduce the MS sensitivity due to their involatilities. Considering recent advances of polymer-based microdevices, therefore, novel coating methods for polymer surfaces, especially for PDMS and PMMA, should be introduced in MCE–MS.

As a novel approach in the dynamic coating of a polymer microchannel, adding a base of commercial SU-8 developer, i.e., 1-methoxy-2-propyl acetate (PMA), to BGS has been reported to suppress adsorption of proteins onto a SU-8 microchip [20]. Comparing with common surfactants used as the dynamic modifiers such as SDS and Tween 20, PMA is more appropriate for the MS analysis due to its lesser impact on the electrospray process. When PMA is added to a BGS to a final concentration of 5% in the MCE–MS analysis on the SU-8 microdevice, a protein sample detected as a symmetric peak. In addition of such volatile dynamic coatings, covalent bonding of appropriate modifiers onto polymeric surfaces is desired for MCE–MS. However, the number of reports on covalent immobilization techniques for polymer microchips is still limited. Hence, further progresses in the surface modification for suppressing sample adsorption are challenging task to expand the application area in MCE–MS.

So far, real sample analyses have rarely been found in the reports on MCE–MS. The difficulty in the surface modification on microchannels discussed above is one of the reasons of this drawback in application studies. Although Deng and Henion have reported the application of MCE–MS to the analysis of carnitines and amines in human urine [39] and plasma [41], respectively, these analytes would show less adsorptivity onto polymeric surfaces. The applications of MCE–MS technologies will be explored in not only bioanalysis and pharmaceutical fields but various areas such as clinical, food, forensic, and environmental researches through the advances in the development of novel ionization interface and channel coating techniques.

6. Conclusion

It is apparent that MCE–MS based analytical systems have a great potential in bioanalysis. The high-speed analyses in seconds and high efficiencies ranging from 10^5 to 10^6 m^{-1} are obtained by MCE. For MS detection, two types of ionization interfaces, ESI and LDI, have been combined with the separation microchips. In the ESI interface, the development of a fine tip structure coupled with a separation channel without dead volume is a key factor to realize high efficiencies and high sensitivities. Although a tapered capillary connected to the separation channel via an access hole fabricated on the edge of a microchip provides a stable ESI with or without the introduction of a sheath liquid, dead volume produced at the connection is a serious problem to reduce the efficiencies. In terms of the separation performance in MCE, the spray tip directly connected with the microchannel is superior to the removable spray. Actually, a microchip containing a monolithically integrated tapered ESI emitter or a microdevice in which ESI is performed directly from the corner of a rectangular chip provides the efficiencies over 10^6 m^{-1}. Regarding the analytical operation, on the other hand, the removable spray tip is convenient because a clogging in the fine ESI tip and/or narrow separation channel is troublesome during replicate measurements. In the LDI interfaces, several techniques which allow a sample transfer from an electrophoresis chip to an LDI target have been developed. The LDI interfaces coupled with an MCIEF or PAGE separation in the microchannel are suitable for analyzing biopolymers.

Since multiple separation channels can be combined on microchips without any connector, various sample pretreatment such as sample preconcentration, desalting, precolumn reaction, and assay can be carried out prior to MCE–MS. Recently, two-dimensional (2D) separation microchips have been developed by utilizing the integration ability in MCE [62–64]. The coupling of the 2D separation chip with MCE–MS is promising for realizing a high-throughput, high-peak capacity, and high-qualitativity analysis system. To attain further high-throughput analyses, automated system with multiplexed separation channels should be introduced in MCE–MS. On the other hand, insufficient selectivity in MCE–MS may be sometimes problematic because a BGS is limited to be volatile. This limitation disturbs the application of several effective separation modes such as micellar electrokinetic chromatography (MEKC) and affinity electrophoresis to MS detection. In CE–MS, successful applications of MEKC have been achieved by using a partial filling (PF) technique [65]. Thus, the application of the PF technique to MCE–MS is expected by developing a novel microchip for PF [66] to obtain highly selective analyses of biogenic compounds. Combining various separation modes including MEKC, MCEC, GE, MCIEF, HILIC, affinity electrophoresis and isotachophoresis with MS should extend the application area in MCE–MS. Through the incorporation of these methodologies into MCE–MS, further high-throughput and high-performance analysis systems will be realized in the near future.

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