Vol. 9, No. 1, 2018
ISSN 2233-4203/ e-ISSN 2093-8950
REVIEW www.msletters.org | Mass Spectrometry Letters

Recent Progress on Microfluidic Electrophoresis Device Application in Mass Spectrometry

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Received February 14, 2018; Revised March 09, 2018; Accepted March 12, 2018
First published on the web March 31, 2018; DOI: 10.5478/MSL.2017.9.1.1

Abstract : Microfluidic technologies hold high promise and emerge as a potential molecular tool to facilitate the progress of fundamental and applied biomedical researches by enabling miniaturization and upgrading current biological research tools. In this review, we summarize the state of the art of existing microfluidic technologies and its' application for characterizing biophysical properties of individual cells. Microfluidic devices offer significant advantages and ability to handle in integrating sample processes, minimizing sample and reagent volumes, and increased analysis speed. Therefore, we first present the basic concepts and summarize several achievements in new coupling between microfluidic devices and mass spectrometers. Secondly, we discuss the recent applications of microfluidic chips in various biological research field including cellular and molecular level. Finally, we present the current challenge of microfluidic technologies and future perspective in this study field.

Keywords : Microfluidic devices, Lap-on-a-chip, Matrix-assisted laser desorption/ionization, Electrospray ionization, Mass spectrometry

1. Introduction

Microfluidics systems coupled with microtechnology, and soft lithography has paved the way in linking technology to biology and life sciences in general. These high throughput techniques are massively explored by microtechnologists and chemists and applied by biochemists and biologists for protein patterning, assay miniaturization, diagnostics, advanced purification, and separation.[1] Microfluidic technology, also called Lab-on-a-chip (LOC), boosts some unique advantages over conventional approaches and has been widely used to different fields of cell research.[2-4] In the last decade, a large number of scientists have been involved in the development of microfluidic systems for a variety of technological applications. Due to the massive developments in the recent years, the microfluidic technology has gradually become a powerful tool for biological analysis.[5-10] In microfluidic systems, small (10^{-9}-10^{-18} L) amounts of fluids are manipulated within channels on the scales of tens to hundreds micrometres.[11,12] Also, the microfluidic system is conceived to be science and technology of systems and miniaturized version of a conventional laboratory.[7]

Since microfluidic device's introduction in the early 1990s, it has appreciably influenced in the field of analytical chemistry. These devices, comprised of a variety of fabrication techniques and materials, offer fabulous advantages over comparable bench-top instruments.[13,14] Microfluidic devices can integrate the entire analytical process, including sample handling, preparation, separation and detection. Therefore, these devices include integration of multiple analytical operations onto a single platform to acquire diverse chemical and biological functions.[1,15,16] However, the dimensions of microfluidic channels are comparable to the sizes of cells, thus facilitating precise cell manipulation. Consequently, the micro-channels can minimize sample consumption, avoid sample dilution, and allow rapid mass and heat transfer.[17]

In the last decade, mass spectrometry (MS) has become the most powerful analytical tool for bioanalysis due to its enhanced sensitivity, and useful for additional structural information of target molecules for qualitative and quantitative determinations.[18,19] Mass spectrometry-based technology with higher throughput, more rapid and convenient analysis, and lower sample consumption are

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still in high demand to meet the new challenges in life science. A miniaturized total analytical system coupled with microfluidic latest interface systems has revived interest in analytical chemistry.[20,21] MS allows the ionization of intact molecules to obtain a highly accurate molecular weight, making identification of molecules easier. The development of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), as well as of sophisticated ion activation/fragmentation techniques represents powerful toolsets that can acquire the capacity to analyze large biomolecules such as proteins.[22,23]

Although MS has become a potential tool for biomolecule analysis, MS detection using microfluidic systems has not been extensively explored regarding other methods due to the difficulty in coupling the studies performed on microfluidic devices to the off-chip mass spectrometer. However, in the last couple of years, published reviews are very limited about the approaches for coupling microfluidic devices to MS,[22,24-26] and their applications including proteomics[18] glycomics,[27] and small molecules.[28] This review presents a thorough overview on recent developments on microfluidic systems for diagnostic applications. The first part focuses on the fundamental information about the microfluidics and the commercial microfluidic interfaces coupled to various mass spectrometers. The second section describes the various applications including the studies of cell biology, microfluidic application in metabolomics and metabolic profiling, and molecular levels.

2. Basic concepts on microfluidics

Over the past decade, microfluidics has emerged as a new analytical tool for many chemical and biological applications. The central idea of microfluidics is the ability to manipulate and control small volumes of fluids at the micrometer scale. In comparison to conventional macroscale techniques, microfluidics offers many advantages such as reduced sample and reagent consumption, faster kinetics and thus decreased analysis time, the capability to integrate multiple functional components, portability of system, and potential for full automation.[29,30] Microfluidic system has shown great potential toward cell culture and cell-based assays.[31-33] Recent decades have witnessed significant advances of microfluidic technologies for biochemical characterization of cells.[12,34] Microfluidics is extending its way into the characterization of single-cell biophysical properties.[35,36] Most microchip-based high performance liquid chromatography (chip-HPLC) devices have been extensively utilized in proteomic researches including on-chip protein pre-concentration and separation as well as enzyme digestion, biomarker screening, and protein identification for proteomic applications.[37-39]

2.1. Recent updates on diverse microfluidics

Although to date there are limited examples of LOC, microfluidic techniques continue to gain popularity as alternatives. Currently, there are two paradigms of microfluidics: channel microfluidics and digital microfluidics.

2.1.1. Channel microfluidics

In channel microfluidics, fluids are manipulated inside micron-dimension channels. Fluid flow in these microfluidic devices can be continuous or discontinuous/segmented. Continuous fluid flow can be achieved by capillary action, external pressure sources (e.g., pumps, syringes, etc.), internal pressure sources (e.g., micro-valves, gas expansion principles, etc.), and various electro-kinetic mechanisms.[40-43]

With decreasing length scale, surface phenomena (e.g., surface tension, capillary forces, etc.) become increasingly dominant over volume phenomena (inertia forces, vortices, etc.). This permits purely passive fluid flow based on capillary action used in popular lateral flow assays that are known as test strips (e.g., the pregnancy test strip).[42] This combination of controllable diffusion mixing and stable phase arrangements has led to the development of hydrodynamic focusing technology, a technique used to align particles or cells in continuous flow for analysis and sorting in flow cytometry.[44]

On the other hand, discontinuous or segmented flow systems are mainly powered through external mechanical pumps. These systems can generate small liquid segments which act as individual microreaction vessels for confinement. This leads to reduced reagent consumption as well as the ability to perform a large number of different experiments within a short period, which makes the platform a promising candidate for high-throughput screening applications.[45,46]

2.1.2. Digital microfluidics

Digital microfluidics is one of the primary application fields of microfluidics. Digital microfluidics has been established as a research field of its own for a decade. Together with continuous-flow droplet microfluidics, digital microfluidics lays the foundation for droplet-based microfluidics. In addition, digital microfluidics (DMF), which is a droplet-based microfluidic system with a planar geometry can be simply fabricated by photolithography.[47,48] In digital microfluidic devices, which are categorized into open and closed configurations, droplets can be manipulated by various technological applications including electrowetting on dielectric (EWOD), dielectrophoresis (DEP), surface acoustic waves (SAW), magnetic force, thermocapillary force, optoelectrowetting and magnetic actuation of liquid marbles.[49-52] Regarding the potential developments of the above mentioned methods, EWOD offers the most flexible and best

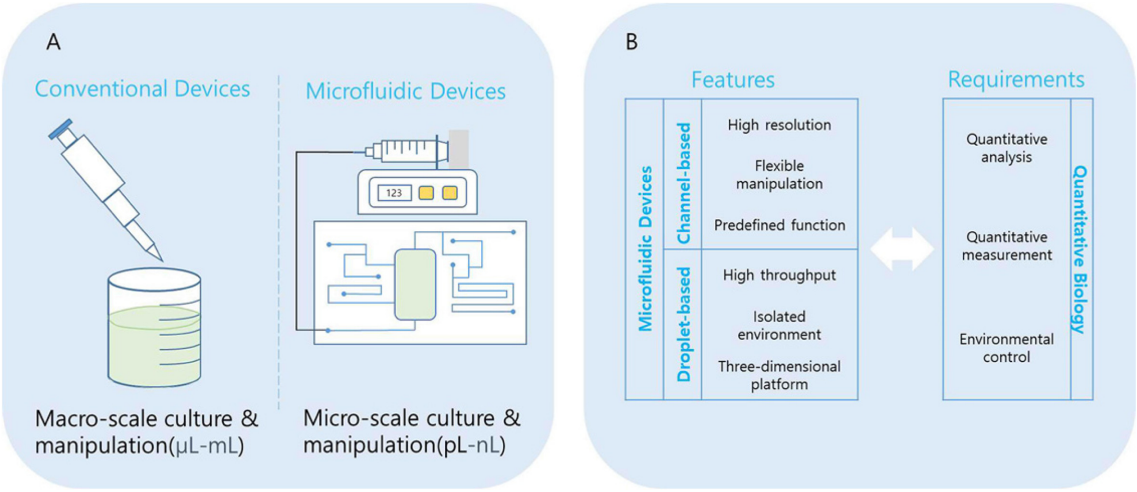

Figure 1. A) Differences between conventional and microfluidic devices for biological researches. In conventional experiments, people usually use flasks, bottles or tubes for macro-scale culture and use pipettes for fluidic manipulation with the volume from microliters to millimeters. Whereas, microfluidic devices only need micro-scale space for cell culture and used micro-pump for fluidic manipulation with the volume from picoliters to nanoliters. B) The way that microfluidics promotes the development of quantitative biology. Microfluidics, which contains two major functional categories (channel-based and droplet-based) provides quantitative biologists a new tool to control, measure and analyze their objectives.

functionality for droplet actuation and has been extensively used for lab-on-a-chip applications.[47,48]

Currently, EWOD is the most popular actuation concept, followed by magnetic and SAW. EWOD is very beneficial for droplet manipulation and offers automated and precise droplet control. Most importantly, EWOD can split and dispense droplets with great ease. Many recent advances have overcome earlier limitations of the EWOD platform. EWOD-based digital microfluidic platforms can now be applied for more complex operations and more intricate bioassays. To date, several reviews have covered various aspects of EWOD-based digital microfluidics.[53,54]

In EWOD-based DMF devices, the electrodes are patterned on a substrate (usually a glass slide or a silicon wafer with thermally grown oxide layer) using photolithography and then covered with a dielectric and a hydrophobic layer. In the closed configuration, the droplet is sandwiched by a top plate which is usually an ITO-coated glass slide used as a transparent electrode, covered with a hydrophobic layer. Droplet manipulation is performed by applying a DC or AC voltage to the system.[52,53,93]

Magnetic actuation is less popular than EWOD, but its unique advantages should not be overlooked. Conventionally, magnetic digital microfluidics manipulates droplets by controlling magnetic particles in the droplet using permanent magnets or electromagnets. The chemical function of magnetic particles makes magnetic digital microfluidics very attractive for droplet-based bioassays. EWOD-based digital microfluidic platforms often employ functional magnetic particles for complex bioassays, thus requiring the introduction of magnetic control or other separation mechanisms in addition to the primary droplet actuation mechanism.[55-57]

Magnetic digital microfluidics provides a much more flexible fluidic control. Compared to conventional channel-based microfluidics where fluids flow in a pre-defined path, the fluidic path in digital microfluidics is virtual and reprogrammable. Therefore, a single digital microfluidic design can be used for multiple purposes. Magnetic digital microfluidics is more flexible than EWOD-based microfluidics (Fig. 1).[58]

3. Microfluidic interfaces coupled to mass spectrometry

In particular, microfluidic devices typically exhibit small footprints, low reagent consumption, multiplexing abilities, and potential for integrated and efficient downstream analysis.[59,60] The miniaturized layout of microfluidic devices can minimize sample losses that result from sample adsorption on the instrumentation surface. Overall, the inherent benefits of analytical process integration, multiplexing, high-speed analysis, and disposability could greatly facilitate proteomic applications that necessitate high-throughput sample processing.[61]

In the past 15 years, there has been great interest in coupling microfluidics with MS. Because ESI and MALDI are the two widely used ionization methods for analyzing biomolecules by MS they have been the most popular methods for coupling microfluidic devices to mass spectrometry. In general, microfluidic chips can be directly coupled to MS via ESI using pressure driven or

Electroosmotic Flow (EOF) driven systems to direct the liquid into the instrument.[62-65] Recently, researchers have introduced the EW-enhanced MALDI-MS sample preparation technique e-MALDI to a variety of small pharmaceutical molecules. This technology leads to substantially smaller and more homogeneous sample deposits on the target plates. In addition, subsequent MALDI-MS analysis displays 2-30 times enhanced signal strength along with a substantially improved lateral homogeneity. Basically, e-MALDI technology is simple to use and compatible with sample preparation protocols and does not require any additives that might interfere with the signal of interest. In particular, the technology is fully compatible with advanced microfluidic sample pretreatment assays based on electrowetting. In this review, we focus the different kinds of microfluidic-MS interfaces reported in the literature and discuss the most promising geometries.

3.1. Microfluidic-ESI-MS interfaces

Due to its ease in accepting low flow rates, ESI is compatible with microfluidic devices. As a result, it has been a widely exploited ionization method for on-line microfluidic-MS analysis. With the continued development of microfabrication technology, the coupling of the main types of microfluidic systems (analog, digital, and droplet microfluidics) to MS via ESI has become more common, and a number of successful examples have been achieved. However, various approaches were performed for forming microfluidic-ESI-MS interfaces. These methods can be broadly classified by how the electrospray is generated, including: (1) analog microfluidics, (2) droplet microfluidics and (3) digital microfluidics.

3.1.1. Analog microfluidics ESI-MS

Analog, or conventional, channel-based microfluidics are often utilized due to their wide versatility in a number of analyses, such as sample preparation, pre-concentration, micro-reactions, and separation. In this section, we describe the most common means of coupling analog microfluidic systems to ESI-MS followed by select applications using these systems.

3.1.1.1. Spray from chip

The simplest approach for interfacing micro-channels with mass spectrometry is to electrospray directly from a channel (i.e., the unmodified edge of a device). Spraying directly from chip would be ideal because connections between a microchip and external parts can increase the dead volume and decrease the efficiency of separations as a consequence. Although MS emitters formed from the unmodified edge of a device are easy to fabricate, their spray performances are limited because of eluent spreading at the edge of the chip resulting from the non-tapered geometry and the hydrophilic nature of the substrate. This limitation can be overcome by integrating hydrophobic coatings on the edges of the devices.[66,67]

3.1.1.2. Spray from a mated emitter

The second strategy for interfacing micro-channels with mass spectrometry is coupling the micro-channels to conventional pulled glass capillary tips. Because of the tapered geometry of pulled glass capillary tip, no spreading of fluid at the exit is observed. Another advantage of this method is the fact that metal coated emitters or even stainless steel emitters can be used to simplify the application of high voltage to the device.[68,69] A major drawback for this strategy, however, is that dead volumes at the junction of the chip and the capillary emitter compromise the resolution of chemical separations within the microchannel. Moreover, adhesives are often used to immobilize the capillary onto the end of the microchannel, which can cause unwanted, contaminating peaks to appear in the mass spectra.[70]

3.1.1.3. Spray from integrated, microfabricated emitter

A third strategy for coupling microfluidic devices to ESI-MS is the use of microfabricated, tapered electrospray tips.[71-73] These emitters exhibit similar tip shape to pulled glass capillary to limit fluid spreading at the tip, and they are fabricated using micromachining processes developed for microelectromechanical systems (MEMS) technologies. These devices are capable of sustaining a stable spray with no dead volume between the channel and tip. The drawback for the devices is the complexity involved in their fabrication (i.e., multilayer patterning/developing), requiring many sequential photolithography steps in a clean room.[71]

3.1.1.4. External emitters

Despite the robustness of integrated emitters, fabrication can be a challenge, and those new to microfluidics may prefer to utilize conventional emitters. As a result, there are multiple applications and approaches described in the literature that utilize external emitters placed within the microfluidic substrate. There have been several applications of external emitters used in microfluidic ESI-MS platforms toward proteomics. In one example reported by the Wilson group, a protein digestion micro-reactor in a microfluidic device was coupled before ESI-MS.[74]

3.1.2. Droplet microfluidics ESI-MS

Droplet-based microfluidics (also called plug-based microfluidics, segmented-flow microfluidics, and multiphase microfluidics) is the science and technology for manipulating and processing small (10^{-6} to 10^{-15}L) amounts of droplets or plugs carried by their immiscible phase.[46,75,76] Droplet microfluidics has emerged as a powerful technique in which highly monodispersed droplets in the picoliter to nanoliter volume range can be generated and manipulated with high frequency. Droplets

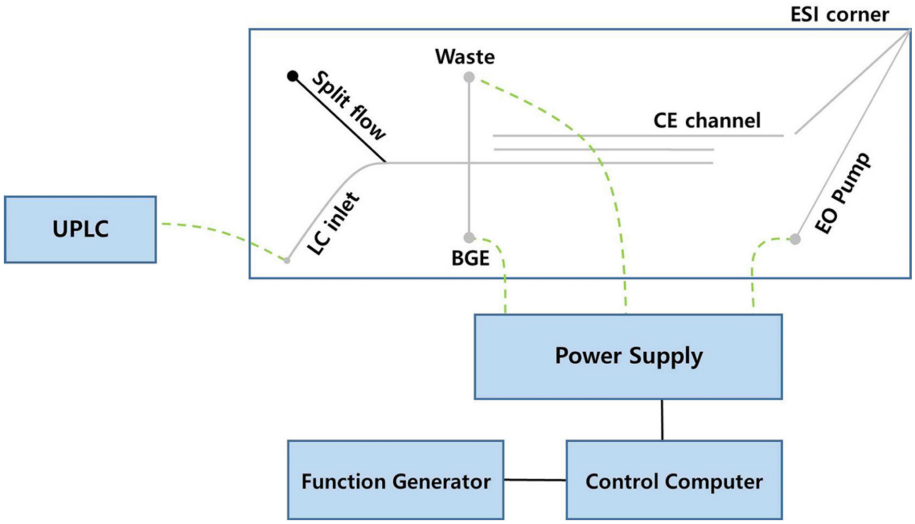

Figure 2. Hybrid capillary LC microfluidic CE-ESI. A capillary LC system is connected to the microfluidic device as shown by the green line. The electrical connections to control the microfluidic CE-ESI system is indicated by the green lines. To compensate for flow rate differences between the LC and CE system, the majority of the LC eluent was split to waste via the split flow channel.

are formed in a multi-phase environment, and due to their independent nature, each droplet can be considered as a micro-reactor with no cross-contamination between droplets.[77] Mass spectrometry analysis of droplet composition has attracted considerable attention due to the possibility of high throughput detection in a label-free and online manner.

Digital or droplet-based microfluidics involves the generation and manipulation of discrete droplets inside micro-devices.[78,79] Droplet microfluidics also offers enormous potential for increased throughput and scalability than continuous flow systems. In the past five years, several groups have used droplet microfluidics to form irregular particles, double emulsions, hollow microcapsules, and microbubbles.[80-82] One of the challenges lies in the ability to analyze droplet content qualitatively and quantitatively. The analytical detection techniques for droplets play critical roles in the development and application of droplet-based microfluidic systems (Fig. 2).

3.1.3. Digital microfluidics ESI-MS

A different way to manipulate droplets is through the use of digital microfluidics (DMF).[83] This technique is based on the manipulation of discrete droplets using an array of patterned electrodes. Wheeler's group has pioneered the coupling of DMF to ESI-MS. In one of their earlier designs, a hybrid DMF-microchannel device was developed.[84] The benefits of DMF are similar to droplet microfluidics, discrete droplets enable minimal crosstalk between analyses, while an additional advantage is a particular microfluidic channel layout does not need to be designed before experiments because the electrode array allows any number of user-designed analyses to be performed on a single device. To couple DMF devices to ESI-MS, two main problems need to be addressed: transferring droplets from DMF to an ESI emitter and dissociating the voltages used to perform droplet manipulations and the ESI spray voltage.

3.2. Microfluidic-MALDI-MS interfaces

MALDI is an alternative to ESI for an interface between microfluidic platforms and MS. The geometry of conventional MALDI detection features arrays of crystallized sample spots on an open surface, and the process is (in general) performed under vacuum. MALDI is another common soft ionization method, which was initially proposed in the late 1980s.[85] MALDI uses for substantial molecules analysis, such as proteins, peptides, and other biomolecules.[86,87]

MALDI is another favorite technique for coupling microfluidics to mass spectrometry. The use of microfluidics with MALDI has revolved primarily around the integration of sample preparation steps, such as fraction collection from separations or protein digestion steps. Additionally, automation of MALDI spotting can minimize user variability, which is a desirable feature given that matrix mixing and spot application can strongly influence sample analysis.[88]

These microchip interfaces can be broken down into two categories: offline or online coupling. Offline coupling with micro-channels can be accomplished by spotting, spraying, centrifuging, or stamping (specific for DMF platforms). Online coupling with microfluidics is quite challenging but can be achieved using continuous flow or other mechanical interfaces.[89]

3.2.1. Offline coupling of microfluidics and MALDI-MS

In offline coupling with MALDI, samples are directly deposited onto a target plate under atmospheric pressure; the plate is then transferred to a MALDI instrument for analysis under vacuum. The most commonly used method is spotting, for example, with a robotic target spotter. Automated digestion was reported in a previous study whereas deposition system formed by mounting a microfluidic chip inside a commercial MALDI target spotter.[90] The chip contains an immobilized enzyme micro-reactor in which proteins were digested and then the products were merged with a coaxial matrix flow. The chip comprises an immobilized enzyme micro-reactor in which proteins were digested and then the products were merged with a coaxial matrix flow. The resulting mixture was eluted off into an infused stainless steel tube and spotted onto a MALDI target plate. As a proof of principle, tryptic digestion of cytochrome c using the microchip was performed.

DMF has been used to process proteomic samples and form arrays of spots for analysis by MALDI-MS. These spots can be transferred onto MALDI target plates through a stamping process.[91] For instance, the previous investigation implemented such a system by moving the sample droplet to the stamping site that consisted of a loading hole defined in the DMF top plate. The droplet was then passively transferred onto the MALDI target positioned above the top plate. Using this technique, MS spectra of protein calibration solution were collected, and all proteins in the stamping sample were correctly identified.[92]

Recently, the purification and extraction of five chemical warfare agent (CWA) stimulants, dimethyl methyl phosphonate, di(propylene glycol) methyl ether, methyl salicylate, triethyl phosphate, and diethyl phthalate, on a DMF device have been demonstrated. However, using this technique, the CWA analyses can be automated on the DMF-MS platform, thereby minimizing human involvement.[93]

3.2.2. Online coupling of microfluidics and MALDI-MS

Online coupling of the MALDI technique with microfluidic devices is difficult because both sample deposition and ionization processes usually take place in a vacuum. Moreover, enclosed micro-channels are by definition not accessible to laser desorption/ionization, which requires an open surface from which analytes can be sampled into the spectrometer. Therefore, integration of microfluidic chips with MALDI for online analysis requires special precautions. Several strategies, however, have been adopted to circumvent these challenges.[89,94,95] This approach has the advantage of decoupling the ionization process from the separation step without compromising the overall system performance. However, the online coupling is a challenge because a MALDI target is under vacuum while the operations on microfluidics are at atmospheric pressure (Fig. 3).

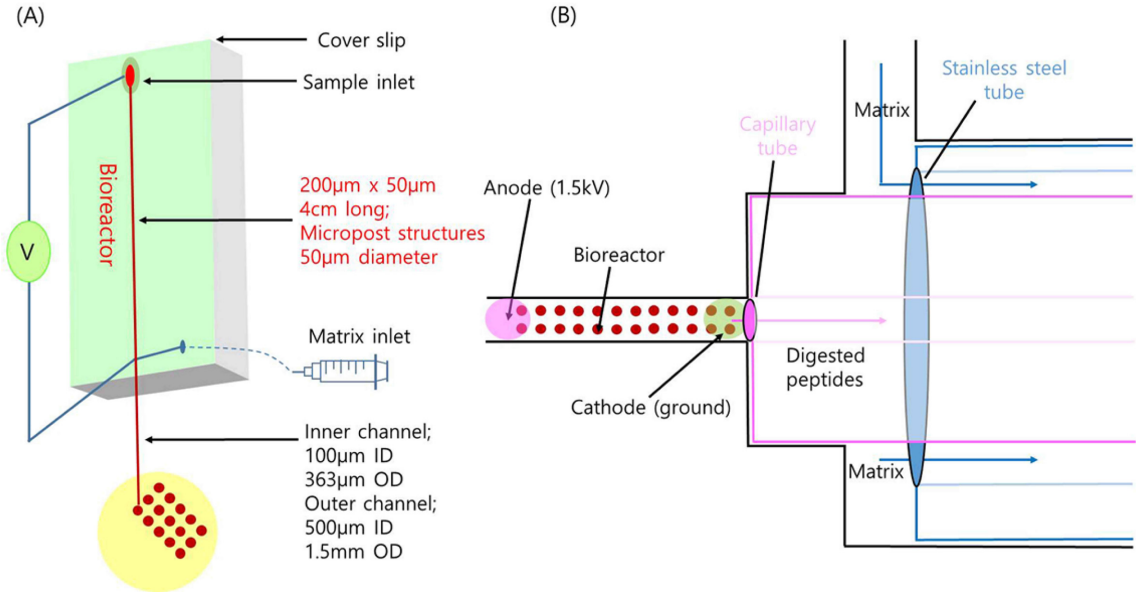

Figure 3. (a) Assembled tryptic digestion microfluidic chip; chip components including the PMMA substrate and coverslip, inlet and outlet connectors, capillary and stainless steel tubes. The sample solution was electrokinetically infused through the bioreactor, and the matrix solution was loaded hydrodynamically with a syringe pump. Coaxial tubes mixed the bioreactor output with a matrix solution for deposition on a MALDI target. (b) Schematic top view of the fluid connection between the micropost bioreactor and the capillary tube interface to the deposition system.

4. Applications of microfluidics systems

The goal of this part is to summarize the use of microfluidics as a novel toolset for metabolomics, metabolic profiling, and other metabolite related biological studies. In the last decade, the applications for microfluidic (MF) devices have proliferated at an explosive rate similar to the revolution brought in the field of microelectronics by the invention of the integrated circuit. Advances in microfluidics technology are revolutionizing molecular biology procedures in diverse fields of biological applications, including cell sorting, enzymatic assays, immuno-hybridization reactions, and polymer chain reaction (PCR), cell-cell communication, phosphoproteins, metabolomics, and proteomics.

4.1. Microfluidics in cell biology

Microfluidics technologies are capable of manipulating and mixing small volumes of solutions and reagents using networks of channels and reaction chambers. These features of microfluidic devices also make them well suited for the analysis and/or growth of cells and tissues. Cells are the building blocks of all living organisms. The knowledge of cells and their functions is thus crucial in several areas including cell biology, human physiology, and tissue engineering. The fundamental cellular study involves the three most crucial steps-isolation, culture, and analysis.[96,97] In the next section, we will discuss on the advancement made by LoC technologies in stem cell research, neurology, drug discovery, cell sorting, patterning and immobilization, and cell-cell communication analysis.

4.1.1. Stem cell research

Stem cells are the cells that are capable of continued self-renewal through replication and becoming precursor cells of specific tissue types. It offers a steady supply of physiologically relevant cells from pathogen-free sources that both in vivo and in vitro can differentiate into mature somatic cells.[98] On the other hand, LoC platforms can much better mimic the complexity of in vivo tissue and provide more precise control of different parameters. This capability can be beneficial for understanding the biology and improving the clinical potential of stem cell-based therapies.[98,99]

A lot of research has been conducted, where stem cells have been used to study various life processes. Klein et al. developed a high-throughput droplet-MF approach for barcoding the RNA from thousands of individual cells for subsequent analysis by next-generation sequencing and analyzed mouse embryonic stem cells.[100] Jung et al. performed flow-based sorting of human mesenchymal cells by using optimally designed MF chips based on the principle of hydrodynamic filtration (HMD).[101] Kang et al. developed an efficient on-chip cell culture MF device capable of repeated, temporal delivery of molecules into a population of cells tool.[102] Thus, significant attention is given to stem cell research owing to high therapeutic potential and their use as in vitro models for drug screening and understanding the developmental mechanisms.

4.1.2. Neurology research

Neurology is another field which is being explored for MF applications. MF is employed for both in vivo deliveries of drug solutions from on-chip reservoirs situated on neural implants as well as in vitro studies of neuronal cells via highly precise delivery growth and inhibitory factors by the use of gradient-generating devices.[103] It is tough to probe the complex interactions that occur among neural cells using conventional methods of analysis. In this context, MF has proved to be the most suitable technique for neurology experiments. For instance, rapid, highly sensitive determination of trans-membrane potential has been possible in MF devices utilizing charged membrane-permeable, potential- sensitive dyes, with minimal use of reagents.[104] NMR (nuclear magnetic resonance) micro coils have been efficiently used to study single non-perfused neurons,[105] where NMR probes have been micro-fabricated on the glass substrate MF platforms and sucrose solutions are used for their testing.[106] Besides this MF principle and techniques have been applied in the isolation of brain tissue culture studies. The separation of brain tissue specimens under in vitro conditions is a very complicated task as it requires exquisite control over experimental conditions and access to neural networks and synapses.[107] Mauleon et al. developed an MF system that allows diffusion of oxygen throughout a thin membrane and directly to the brain slice via MF gas channels.[108] Although a lot of research is being conducted for exploring the neurological processes seeking the help of MF technologies, still efforts are in their initial stage and scope of MF in neuroscience is endless.

4.1.3. Drug development

The drug development process comprises of two phases i.e., drug discovery and drug testing. The first phase includes the target selection, lead identification, and preclinical studies, while the development stage includes clinical trials, manufacturing, and product lifecycle management.[109] Miniaturized MF devices being small sized in nature are emerging as useful tools for the study of target selection, lead identification and optimization and preclinical test and dosage development.[110] Caviglia et al. developed an MF cytotoxicity assay for studying the impact of anticancer drugs doxorubicin and oxaliplatin.[111] Sung et al. developed a device using hydrogel cell culture for pharmacokinetics-pharmacodynamics (PBPK) studies of the three cell lines that represent the liver, tumor, and marrow for testing drug toxicity.[112] Psaltis et al. designed a synthetic tumor to examine in vivo delivery efficiencies of the drug vehicles.[113]

4.1.4. Cell sorting

Cell sorting is a preliminary step for cellular level investigation of biology. Microfluidic chip-based cell sorters that take advantages of system miniaturization have been widely studied in recent years. A large variety of active or passive sorting mechanisms have been employed for microfluidic cell sorters, such as electric, magnetic, hydrodynamic and optical mechanisms.[114] The electric sorting mechanism is the most popular method employed by microfluidic cell sorters, which have advantages of flexibility, integration, and potential for automation. Cell sorting based on electro-kinetic switching is a common approach.[115-117] However, electro-kinetic forces are relatively weak for the manipulation of large cells or particles. Recently, Yao et al. combined gravity and electro-kinetic force for flow cytometry and fluorescence-activated cell sorting. This system was applied to estimate the necrotic and apoptotic effects of ultraviolet light on HeLa cells.[118] Inglis et al. recently reported a microfluidic device that can isolate cells selectively tagged with magnetic nanoparticles.[119]

Hydrodynamic sorting is the most traditional method employed by microfluidic sorters. Cell sorting was realized by active switching of flow directions upon detection of fluorescent signals from cells.[120,121] Recently, Chabert and Viovy reported a high-throughput hydrodynamic cell sorting approach for the high throughput encapsulation of single cells into pico-liter droplets, and spontaneous self-sorting of these droplets.[122] Due to its non-physical contact and minimal contamination during manipulation, optical sorting mechanisms such as optical tweezers and optical traps are another attractive type of method for microfluidic sorters.[123-125] Wang et al. demonstrated optical force switching for the separation of fluorescent cells on microfluidic chips.[61]

4.1.5. Patterning and immobilization

Immobilization of cells is usually necessary before analysis can be effectively conducted on microfluidic chips. One commonly used method is cell patterning in microfluidic channels by soft lithographic techniques, such as micro-contact printing, patterning using microfluidic channels, and laminar flow patterning.[126] Recently, Kaji et al. demonstrated patterning of electrically conjugating cardiomyocytes by micro-contact printing.[127] Other cell immobilization methods have also been reported for microfluidic systems in recent years, such as cell docking,[128,129] acoustic trap[130] and optical trap.[131]

4.1.6. Cell-cell communication

Cell-cell signaling has also been investigated using microfluidic chip-based methods. Klauke et al. recently studied cell-cell signaling between longitudinally linked primary heart cells in a microfluidic system.[132] Wei et al. fabricated a microfluidic co-culture system for investigating cell-cell interaction in the distance.[133] Song et al reported another microfluidic co-culture system. for distant cell-cell interaction studies, in which the effect of human embryonic germ (hEG) cells on SKOV3 cells was investigated.[134]

4.2. Microfluidic application in metabolomics and metabolic profiling

In this section, attention was given to those microfluidic applications of cellular analysis that measured general metabolic activity, or that monitored targeted metabolic products. Research articles in this category are classified as bacterial monitoring, cell stimulus and exposure, toxicity screening, and clinical diagnostics.

4.2.1. Bacterial monitoring

Microfluidic devices that can isolate or immobilize bacterial cells can be used for toxicity measurements, monitoring the presence or absence of bacteria, and investigating the metabolic activity of specific bacterial strains. A silicon-based microfluidic chip was used to concentrate bacterial cells from dilute samples and measure metabolic activity in small volumes using impedance monitoring techniques.[135] A hydrogel bacterial microchip was presented for biosensing and monitoring of intracellular metabolism.[136] A poly(dimethylsiloxane) (PDMS) microfluidic device was presented that isolates single bacterial cells into nanoliter droplets using plug-based microfluidics.[45] Another example of bacterial monitoring using microfluidics included a PDMS microfluidic bioassay chip containing immobilized bacterial strains for bioluminescent detection. A 5×5 well array was used to stimulate bacterial strains using varying concentrations of mitomycin C.[137] Also, as an example of targeted metabolic profiling, a microfluidic flow-injection system fabricated from silicon was introduced to measure glucose and ethanol secretion from immobilized Saccharomyces cerevisiae cells using chemiluminescence detection.[138]

4.2.2. Cell stimulus and exposure

The second class of cellular analysis using microfluidics involves the stimulus or exposure of single isolated cells, tissues, or cultured cell populations. Fully integrated μTAS devices are capable of both exposing or stimulating the cells and measuring the inherent metabolic response. A microfluidic device for monitoring ammonia metabolism from hepatocytes cultured on-chip was presented by Satoh et al..[139] The Kennedy group reports a microfluidic device for monitoring glycerol secretion and metabolism in cultured adipocytes using a continuous flow enzyme assay.[140] A microfluidic system was developed to determine the amounts of lactate produced from single cardiomyocytes and also monitor changes in extracellular pH and concentrations of Ca^{2+}.[141]

4.2.3. Toxicity screening

Microfluidic cell culture devices can also be used to investigate the toxicity of drugs and assess the changes in metabolic activity under various conditions. A microfluidic microreactor was introduced for evaluating metabolism and other pharmacological properties of drug candidates by exposing microsomes and hepatocytes entrapped in polyethylene glycol hydrogels.[142] Baudoin et al. report a PDMS microchip device for cell culture application and toxicity studies.[143] A bio-MEMS chip was developed for general toxicity monitoring using bioluminescent bacterial cells that respond to the presence of reactive oxygen species.[144]

4.2.4. Clinical diagnostics

Several examples of the use of microfluidic devices for cell analysis have been applied to distinguish clinical samples. For instance, a parallel channel PDMS device was used to quantitate levels of glutathione and C-peptide in red blood cells and distinguish diabetic samples from control.[145] Carraro et al. describe a novel microfluidic device for hepatocyte culture that contained a microfluidic network capable of providing a low shear stress physiological environment for cell growth and monitoring.[146] Biomedical diagnostics has been an important application area of MF technologies. The unique features of MF technology make it naturally suitable for the fabrication of Point of Care (PoC) testing devices. Till date, some prior DNA separation techniques and diagnostics have been successfully miniaturized.[147,148]

4.3. Biological applications at molecular level

In general, these applications can be categorized as clinical diagnostics applications, enzymatic assays, tissue engineering and cell-based applications, DNA-based applications, immunoassays and proteomics.

4.3.1. Genomics

Numerous MF systems have been used for the analysis of various molecules including DNA, RNA and other chemicals for general purpose as well as for disease detection. A large number of devices used for diagnosis of disease including pathogen detection have reported MF as the general theme of fabrication.[149] Malhotra et al. has fabricated impedimentary microfluidic-based nucleic acid sensor for quantification of DNA sequences specific to cancer. The MF chip was prepared by patterning an indium-tin-oxide coated glass substrate followed by sealing with PDMS microchannel.[150] An integrated MF device was used by Dimov et al. for tmRNA purification and nucleic acid sequence-based amplification.[151]

Microfluidic chip-based methods certainly play an essential role in high-throughput genomic studies.

4.3.1.1. Sequence analysis

Capillary array electrophoresis (CAE) has been the golden standard for genome sequencing purposes, where multiple capillaries are used in parallel for high-throughput sequencing of target DNAs.[152] Micro-fabricated CAE device (μCAE) was first introduced by Woolley and Mathies for DNA sequencing using microfluidic channel arrays.[153] Liu et al. reported automated DNA sequencing in 16-channel microchips with an integrated four-color confocal fluorescent detector, which yielded more than 450 bases in 15 min with 99% accuracy.[154] In addition to μCAE, many other miniaturized systems have also been reported. A disposable plastic electrophoresis system was demonstrated earlier by Shi and Anderson for high-resolution single-stranded DNA analysis.[155] Kartalov and Quake reported a fully integrated PDMS (polydimethylsiloxane) microfluidic system for DNA sequencing-by-synthesis, using a heterogeneous assay that combined active plumbing, specific surface chemistry, and parallelism.[156] Applications of miniaturized systems for DNA sequencing are still growing; several reviews[157-159] on this fast progressing field have also been reported lately.

4.3.1.2. PCR and other applications

Besides DNA sequencing, other implementations of microfluidic chips include DNA separation, analysis and polymerase chain reaction (PCR). Wang et al. developed a microfluidic chip capable of quantitative detection of low-abundance nucleic acids by incorporating confocal fluorescence spectroscopy, molecular beacons and a molecular-confinement microfluidic reactor.[61] Lagally et al. earlier demonstrated an integrated microfluidic device, combining submicroliter PCR chambers with microfabricated capillary electrophoresis (CE) system for the stochastic PCR amplification of single DNA template molecules and subsequent CE analysis.[160] Chen et al. fabricated a polycarbonate microchip. for the PCR amplification of DNA templates using an electro-kinetically driven synchronized continuous flow PCR configuration.[49]

4.3.2. Proteomics

The proteome is directly derived from the genome and it, in turn, regulates both the gene expression and cellular metabolism. Thus, quantitative investigation of the proteome is essential for understanding biology at the systems-level. Here, we discuss the recent applications of microfluidic chips in proteomics with an aspect of preconcentration, on-chip separation, single-cell proteomics and mass spectrometry coupling.

4.3.2.1. Preconcentration

Preparation of proteins is usually a prerequisite step in proteomic studies. When working with natural low-abundance proteins, preconcentration is necessary to bring target proteins into detectable range. Various methods have been reported for protein preconcentration on microfluidic chips. Song et al. reported laser-patterned nano-porous

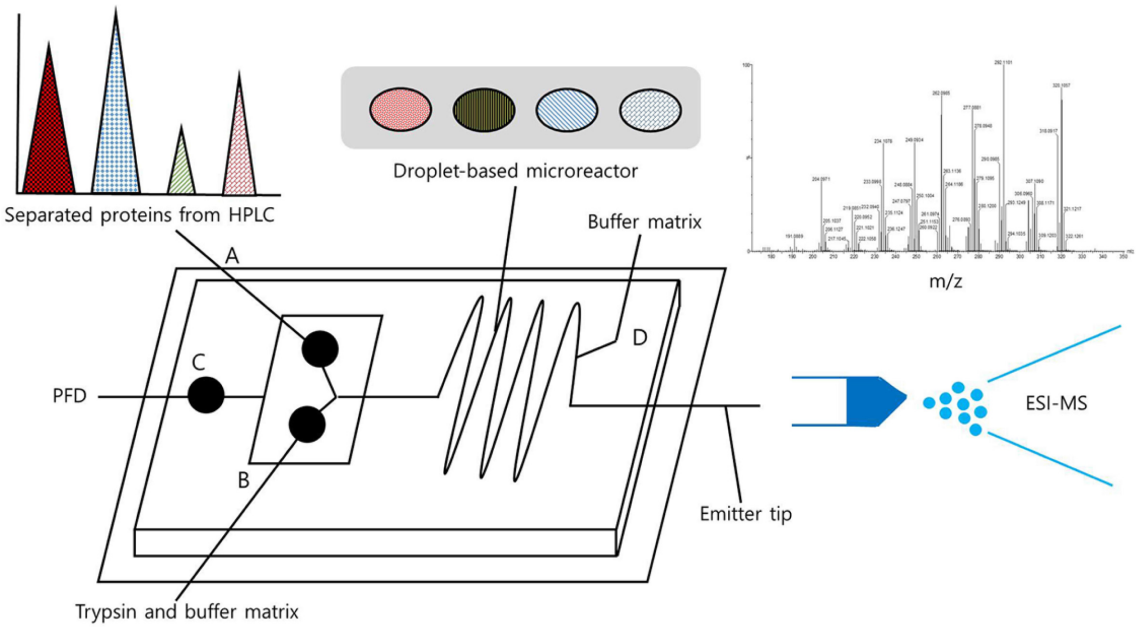

Figure 4. Schematic presentation of the integrated platform for protein analysis, combining protein separation by HPLC, on-line digestion by a droplet-based microfluidic reactor, and protein identification by ESI-MS/MS. The separated proteins were infused directly into the channel from inlet A, trypsin was infused from the inlet B, and the oil (PFD) was infused from inlet C. The pH adjuster (50% ACN/49% water/1% FA) was infused through the inlet.

membrane at the junction of a cross-channel on a microfluidic chip.[161] Upon application of voltage, the linear electrophoretic concentration of charged proteins can be achieved at the membrane surface with a concentration increase of 2-4 orders of magnitude. Using phase-changing sacrificial layers, Kelley and co-workers[162] demonstrated miniaturization of electric field gradient focusing on microfluidic chips for protein preconcentration.

Recently, Wang et al.[163] reported a microfluidic sample preconcentration device based on electro-kinetic trapping. A flat nano-fluidic filter was fabricated within a microchannel, functioning as an ion-selective membrane for electro-kinetic trapping of charged biomolecules. As a result, the electro-kinetic trapping of proteins can be maintained at the nano-fluidic filter region for several hours with a concentration factor as high as 10^6-10^8. A PDMS microfluidic chip was reported by Kim et al.[164] for the enrichment of proteins. A thin-walled PDMS section (~20 μm) was fabricated between two micro-channels. Under an electric field, preconcentration of negatively charged proteins was readily achieved on the anode side of the section with a 10^3-10^6-fold increase.

4.3.2.2. Protein analysis

The application of mass spectrometry in proteomics still faces technical challenges due to the complex sample matrixes and the ion suppression in the MS ion source. Purification of target proteins from complex biological samples is a vital step for proteomics research, which needs a series of purification procedures including desalting, matrix removal, target enrichment, and separation. The development of microfluidic-based technologies for proteomics by integrating necessary analytical processes into a platform has dramatically accelerated the identification speed of proteins and various post-translational modifications.[165,166] Ji et al. developed a droplet-based proteolysis reactor for online tryptic digestion of proteins separated by HPLC, and the generated peptides were directly identified by ESI-MS/MS.[167]

Phosphorylation of proteins plays a vital role in regulating many signaling pathways and controlling of enzymatic functions.[168,169] Despite the vast abundance and importance of protein phosphorylation, their analysis is one of the most critical challenges in proteome analysis. To surmount these issues, great efforts have been made in the development of enrichment technologies for phosphopeptides to aid their detection, such as titanium dioxide (TiO_2)-based affinity enrichment, hydrophilic interaction chromatography, and immobilized metal affinity chromatography.[170-174] Heck's group developed a TiO_2-based phosphochip-Q-TOF for global screening of phosphoproteome of primary human leukocytes.[175]

Membrane proteins are important drug targets that numerous studies have attempted to analyze. However, they are difficult to solubilize and are liable to aggregate

which makes them more difficult to analyze. Detergents are often used to solubilize them, but they cause significant interference in their subsequent analysis, such as by MS. Several conventional strategies have been used to separate proteins from interfering detergents, such as dialysis, ion exchange chromatography, and hydrophobic absorption. Recently, a microfluidic electrocapture method has been developed for preconcentration and separation of analytes from interferents, and then detection by ESI-MS.[37,176,177] Mok et al. developed a DMF platform for protein biomarker detection for quantifying protein abundance and activity[178] (Fig. 4).

4.3.2.3. Single cell proteomics

Single-cell proteomics allows the investigation of protein expression in individual cells, providing a valuable insight of that expression within a heterogeneous cellular population. Different methods have been reported for realizing single-cell proteomic studies. For example, the research group of Ewing and co-workers[179-181] demonstrated mapping of cellular and subcellular contents from individual cells by time-of-flight secondary ion mass spectrometry. Recently, microfluidic systems have drawn attention of researchers as an attractive platform for single-cell proteomics,[182,183] due to their compatible sizes with cells and potential for automation. The research group of Ramsey and co-workers[184] demonstrated a microfluidic device, integrating cell handling, cell lysis, electrophoretic separation and fluorescent detection. The loaded cells were hydrodynamically transported from the cell-containing reservoir to a region where they were focused and rapidly lysed using an electric pulse field. A microfluidic device was fabricated by Huang et al., in which they can manipulate, lyse, label, separate and quantify the protein contents of a single cell using single-molecule fluorescence counting.[185]

4.3.2.4. Glycan analysis

Glycosylation, the most common protein modification in the human proteome, has a close relationship with many critical biological functions, such as cell development, cellular differentiation and adhesion, immune responses, and even host-pathogen interactions. Glycans have been proved to have a great impact on the properties of proteins, such as solubility, folding, secretion, immunogenicity, thermal stability, and so on.[186] Mass spectrometry is an efficient tool for label-free glycans or oligosaccharides detection and identification. For the overall analysis of glycans or oligosaccharides, the competitive ionization in the ion source of a mass spectrometer is a significant barrier to the observation of many trace-level analytes. The use of a separation technique before MS analysis is perhaps the most efficient approach to minimize the adverse effects from competitive ionization. The most reported separation methods are graphitized carbon chromatography, hydrophilic interaction chromatography, high-performance anion-exchange chromatography, chip-based reverse-phase LC-MS.[187-190] Lebrilla's group first used an Agilent HPLC-Chip/Time-of-Flight MS system for the separation of serum glycans, and then online detection by nano ESI-MS.[188,190]

4.3.2.5. Protein-protein interaction analysis

Protein-protein interaction networks play an important role in regulating many cellular and physiological processes. Abnormal protein aggregation might induce several diseases, such as Alzheimer's disease and Parkinson's disease. Thus, disruption of protein-protein interactions is a novel therapeutic strategy for these diseases.[191,192] The commonly used methods for protein-protein interaction analysis are yeast two-hybrid system, pull-down MS approaches. Recent developments of surface plasmon resonance (SPR) and MS have provided the feasibility of better integration that could be dedicated to the identification and characterization of protein-protein interactions.[193,194] The incorporation of the SPR technique was used to detect proteins interacting with specific peptides or proteins immobilized on a gold sensor chip. The MS/MS method setup ensures the protein identification more confidently by providing not only peptide mass data but also the amino-acid sequence information.

4.3.2.6. Mass spectrometry coupling

Mass spectrometry (MS) based methods have been extensively used for proteomic studies,[195,196] such as peptide mapping, posttranslational modification, and protein-protein interactions. With the rapid progress of microfabrication technology, microfluidic chips have been coupled to MS analysis to address issues like speed, throughput and cost efficiency. Recently, Gustafsson et al. developed a high-throughput microfluidic compact disk (CD) for protein preparation and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.[197] Electro-wetting-on-dielectric-based technique for digital microfluidics was used by Wheeler et al. to perform inline sample purification for proteomic analysis with MALDI-MS.[198] A PDMS microfluidic device was demonstrated by Dodge et al., which combined online protein electrophoretic separation, selection and protein digestion for subsequent MALDI MS analysis.[199]

5. Present challenges and future perspectives

After the recognition of MF potential in diagnosis, the realization of this field has been very slow. In fact, thousands of research publications are there, but the outcome as successful devices is very less. Some commercially available LoC products for DNA analysis, protein crystallization, and performing simple chemical

reactions are available but still there is need for so-called "killer application" in the field of clinical diagnostics.[200] The PoC diagnostics have not yet lived up to their forecasted potential. One of the reasons may be the complexity of the systems. Many complex biochemical processes have been demonstrated on-chip for diagnostic application. The other challenge may be the reluctance to the adoption of new technology. As the market is user-driven and not technology driven i.e. the users are habitual to the traditional methods of analysis any new technology introduced has to be simple and must be easily operated by non-experts also. Most of the PoC diagnostics used in hospitals or laboratories are not suitable to be used by common people. User-friendly diagnostic concepts should be employed in the devices such as the simple indicator symbols to indicate the presence of antigens, antibodies, viruses, or other biological targets to be analyzed.

Analysis of real samples like blood and saliva in an MF device, however, is more complicated and problematic than the purified samples usually used in general laboratories. Therefore, new devices need to be designed which are operable in the virtual environments rather than in laboratory conditions. Lack of funds may be another reason for the slow pace of transformation of academic research to practical devices. Manufacturing of MF devices is quite expensive and uses costly instruments that are not available in all laboratories. These expensive MF platforms that are being used for research are not suited for mass production of practical devices. The majority of manufacturing methods published on the LoC devices have been micromachining on glass or silicon, and soft lithography on PDMS, which is again expensive.[201]

However, recently, one research group has introduced microfluidic CE devices that are coated by CVD of aminopropylsilane (APS) reagents. They describe a microfluidic CE method for rigorously evaluating the performance of the surface coatings about EOF, separation efficiency, and stability.[202]

To justify the switch for the consumers from current products the MF technology must significantly outperform or cost less than the present products. An emphasis on global health has increased the demand for low cost, high through output and integrated PoC devices which are likely to become common in the years ahead.

MF is an emerging technology in the field of commercial diagnostics as far as the realization of technology from the laboratory to the real world is concerned, and its future holds enormous potential. The MF devices are destined to replace conventional techniques, and the inherent advantages of the technology are too hard to ignore. The continuous development of MF applications in manufacturing methods, including platform technologies that can be customized easily for each diagnostic test, will be the drivers of success. Commercial success will result in the expansion of the field from biological domain to other areas also.

Proteomics is one of the significant scientific challenges in the post-genome era. The most basic form of proteomics is proteome profiling, identifying all the proteins expressed in each sample which is a demanding task. Considering the above circumstances, 908devices (https://908devices.com/) introduced a novel microfluidic CE-ESI separation device, ZipChip with unprecedented speed and separation capabilities. The applications benefiting from ZipChip CE include biotherapeutics, metabolomics, proteomics, and clinical analyses (qualitative and quantitative). ZipChip CE-MS experiments are typically less than three minutes with high-resolution separations, resulting in high throughput information-rich analytical data. Sample preparation is routine, and run-to-run carryover is minimal. Back-end data collection is achieved through high-powered commercial mass spectrometers, such as the Orbitrap MS product line from Thermo Fisher Scientific. In all, ZipChip CE-MS is the first microfluidic CE solution integrated to a mass spectrometer, and ZipChip CE represents an excellent orthogonal separation capability for MS analyses.

6. Conclusions

With the advent of fabrication approaches, integrated emitters, and rapid prototyping techniques, microfluidic devices are becoming more popular and more accessible in both industry and research settings. A systems-level understanding of biology requires extensive information of individual components and their correlations within complex biological systems. Microfluidic chip-based method certainly plays as one of the essential roles in such demanding investigations, revolutionizing the means for biological research fields due to advantages such as high-throughput, small sample consumption and reduced analysis time. In this review, we present the basic concepts and the state of the art of microfluidic technologies. ESI and MALDI are thought to be two widely used ionization methods for analyzing biomolecules by MS, and they also have been achieved great interest for coupling microfluidic devices to mass spectrometry. Therefore, this review emphasizes the recent advances of various methods in coupling microfluidics with MS. In the last decade, significant progress has been made in developing advanced physical approaches and biological affinity strategy of microfluidic devices. While the majority of bioanalytical microfluidics applications are focused towards genomics, proteomics, metabolomics, and targeted metabolic profiling applications. The microfluidics modified procedures are leading to discoveries in the laboratory, and new devices fabricated based on these innovations are changing the landscape of biological systems. A lot of work has been done in this direction, but still, there is enormous scope for future development.

Acknowledgments

This research was supported by the National Research Council of Science & Technology (NST) grant by the Korea government (MSIP) (No. CCL-17-21-KBSI) and Korea Basic Science Institute (KBSI) (Grant No. C38914).

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