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REVIEW

Interfacing microfluidics with information-rich detection systems for cells, bioparticles, and molecules

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Abstract

The development of elegant and numerous microfluidic manipulations has enabled significant advances in the processing of small volume samples and the detection of minute amounts of biomaterials. Effective isolation of single cells in a defined volume as well as manipulations of complex bioparticle or biomolecule mixtures allows for the utilization of information-rich detection methods including mass spectrometry, electron microscopy imaging, and amplification/sequencing. The art and science of translating biosamples from microfluidic platforms to highly advanced, information-rich detection system is the focus of this review, where we term the translation between the microfluidics elements to the external world "off-chipping." When presented with the challenge of presenting sub-nanoliter volumes of manipulated sample to a detection scheme, several delivery techniques have been developed for effective analysis. These techniques include spraying (electrospray, nano-electrospray, pneumatic), meniscus-defined volumes (droplets, plugs), constrained volumes (narrow channels, containers), and phase changes (deposition, freezing). Each technique has been proven effective in delivering highly defined samples from microfluidic systems to the detection elements. This review organizes and presents selective publications that illustrate the advancements of these delivery techniques with respect to the type of sample analyzed, while introducing each strategy and providing historical perspective. The publications highlighted in this review were chosen due to their significance and relevance in the development of their respective off-chip technique.

Keywords Separations · Dielectrophoresis · Electrophoresis · Protein · Microfluidics · Electrokinetics

Abbreviations

DEP	Dielectrophoresis
iDEP	Insulator-based dielectrophoresis
eDEP	Electrode-based dielectrophoresis
nDEP	Negative dielectrophoresis
pDEP	Positive dielectrophoresis
EP	Electrophoresis
IgG	Immunoglobulin G
CM*f*	Clausius-Mossotti factor
BSA	Bovine serum albumin
g-iDEP	Gradient insulator-based dielectrophoresis
EKMr	Electrokinetic mobility ratio
ROI	Region of interest
F_{EP}	Electrophoretic force
E	Electric field strength
q	Total charge
σ^2	Variance
D	Diffusion constant
a	Acceleration
μ_{EK}	Electrokinetic mobility
μ_{DEP}	Dielectrophoretic mobility
σ	Standard deviation
MDA	Multiple displacement amplification
WGA	Whole genome amplification
MALBAC	Multiple annealing and looping-based amplification cycles
MALDI	Matrix-assisted laser desorption/ionization

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Introduction

The greatly enhanced capabilities generated by microfluidic manipulations allow for processing of biological samples in unprecedented ways. Very effective means of isolating single cells in a defined fluid volume in large numbers has been achieved using numerous strategies. Complex samples, including heterogeneous cell populations and mixtures of

complex bioparticles (exosomes, organelles, viruses, among others) or biomolecules, all have been manipulated such that information-rich detection elements may be utilized, such as mass spectrometry, nucleotide amplification, and electron microscopy imaging (along with, of course, other detection modes including fluorescence imaging, vibrational spectroscopy, and cellular mechanical properties). Within microfluidics, it is routine to manipulate sub-nanoliter volumes; in fact, meso- and macroscale volumes higher than a few microliters range can be quite difficult to process. And this is the focus on this review, the effective and accurate off-chipping of precise volume containing the valuable targeted sample without alteration. Nearly all techniques of interest have their sample introduction and manipulation systems optimized to benchtop meso/macroscale volumes, and for companies and labs that have already invested in these detection systems, the re-development of any so-called on-chip or integrated detection systems is not feasible or practical. Several strategies have emerged across many fields to adapt the microfluidic samples such that they may be effectively transferred and interfaced. These include four categories: sprays (electrospray, nano-electrospray, pneumatic), meniscus-defined volumes (droplets, plugs), constrained volumes (narrow channels, containers), and phase changes (deposition, freezing) (Fig. 1). Each of these strategies has specific advantages and disadvantages and is better suited from some detection schemes rather than others. Here, we chronicle and categorize the off-chipping works across several fields focusing on the specific methods used to remove highly defined and valuable samples accurately, precisely, and reproducibly

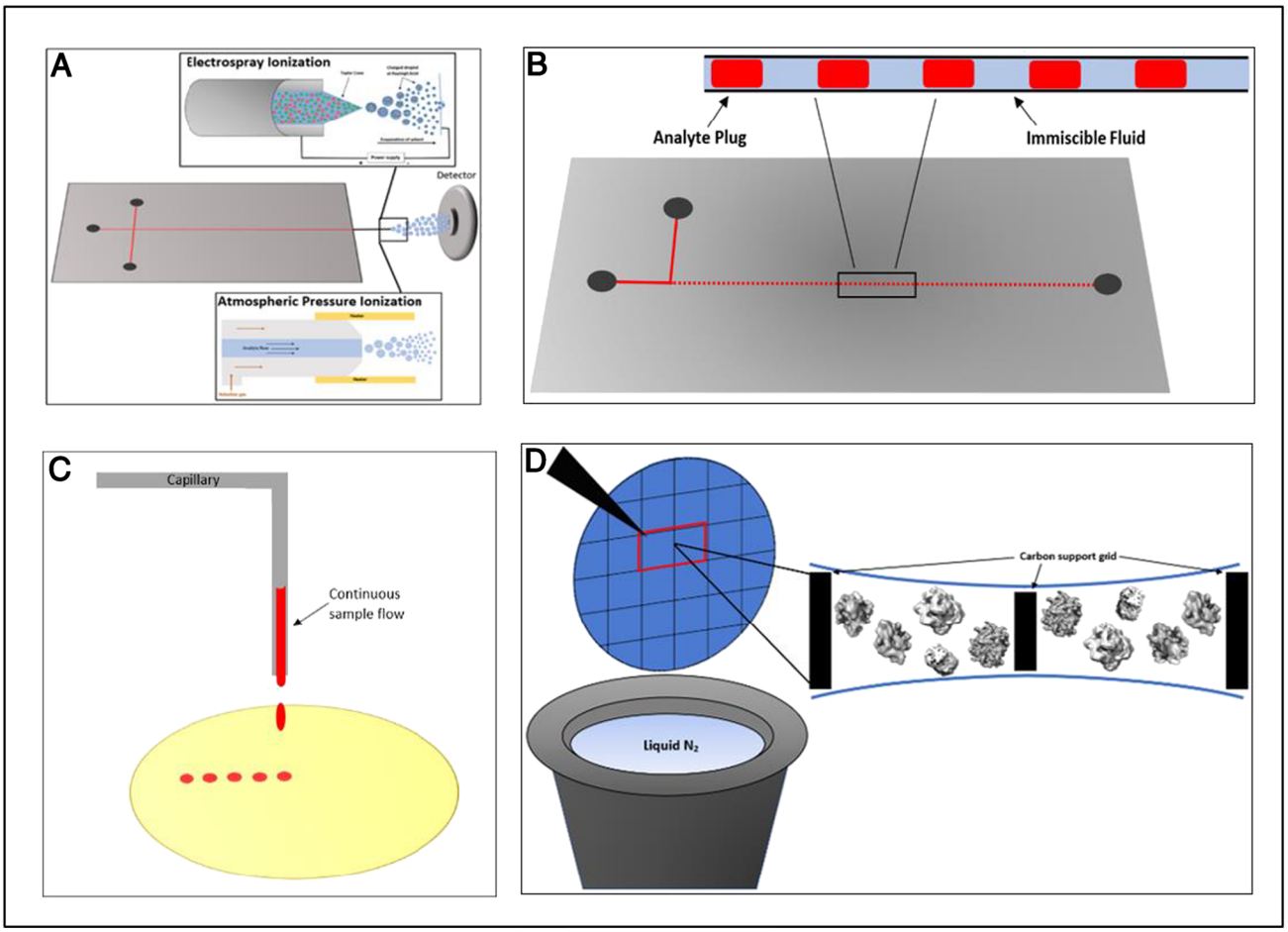

Fig. 1 Representative categories of off-chipping strategies for microfluidic systems. **A** Micro-spraying, two sub-categories: electrospray ionization uses an electric field to form a spray and charge droplets and after evaporation, produces a stream of charged particles. Atmospheric pressure ionization utilizes a nebulizer gas in the spray tip to assist in dispersing the analyte. **B** Droplets: can be made on- or off-chip and spaced within a device using an immiscible fluid. The meniscus-bound fluid element reduces sample volume requirements and maintains concentration. **C** Direct deposition: a capillary is used to deposit minute continuous flow (unbroken meniscus during transfer) sample volumes on to targets like MALDI plates, TEM grids, and microarrays. The deposition location can be highly specific and can dispense in the low picoliter range. **D** Phase change: an integrated phase change can be an integral step to off-chipping. MALDI (1C) is one example and placing proteins to be frozen on a prepared EM grid after off-chipping is another

from microfluidic systems and deliver them to information-rich detection schemes. Conversely and explicitly, we avoid on-chip detection systems in this review, except where they are integral adaptations allowing for, and enabling, off-chipping schemes. Samples include human/mammalian, bacterial and other cells, bioparticles including exosomes, organelles, and viruses, and biomolecules (Table S1, Supplemental Materials). Detection schemes include mass spectrometry, nucleotide amplification, and electron microscopy.

Off-chipping strategies

Spray

Sample deposition by spraying has become a common technique for introducing minute-volume samples to information-rich detection methods such as mass spectrometry and electron microscopy. Advantages for this method include very low and continuous flow, opportunity for evaporation and ionization, and ease of positioning for the deposition. This strategy has been used for cells and their content, bioparticles, and molecules. An important variant of spraying is nano-electrospray which is very popular and has been successfully incorporated on microfabricated devices. Early works used syringe or electroosmotic pumps for precise spraying at low nL/min flow rates. Design modifications like transfer capillaries, new spray tip designs, and sheath flow improved the detection limits and reduced dead volume. Evolving works with surface acoustic waves have also been utilized to generate femtoliter droplets off a piezoelectric substrate without the use of pressure flow. These capabilities were shown for sample deposition on EM grids and decreasing sample input for mass spectrometry.

Meniscus bound

Meniscus-defined volumes including droplets and plugs have been used to manipulate samples and deliver them off-chip. Advantages for this strategy, also termed digital microfluidics, are well documented, including keeping the sample in a meniscus-bound volume allows for maintenance of concentration as well as more control over movement of the target for off-chip collection. Sensitivity is increased as well as a higher throughput with very little sample volume because of the droplet or plugs ability to be isolated, prepared, and transferred off-chip digitally. Plugs are common for DNA or RNA extraction of cells within a microfabricated device. Modifications like elastic valves allow for cells to be sequestered in nanoliter-sized volumes for preparation steps. Samples can then be transferred to open wells where manual and autonomous removal using pipettes or capillaries is performed. Other nanodroplet processing strategies include nanoPOTS, which can enhance proteomic analysis in a 200-nL droplet confined in a glass chip.

Constrained volumes

When a solid surface defines the boundary, this is classified as a constrained volume, and includes narrow channels and/or small containers. They are a simple and effective means for handling small volume samples by precise sample deposition into various detection schemes. Advantages include simple operation and very flexible design options. This technique is proven effective in non-contact deposition of low volume samples on an MALDI-MS target by either a flow force or an electrically mediated force. Other contact systems utilize a transfer capillary to deposit prepared samples on target plates by microspotting. Constrained volume deposition has also changed the way EM grid preparations are performed. Various robotic spotting systems have been developed to deliver samples onto an EM grid at adequate thicknesses without the use of traditional blotting techniques. With the ability to deliver pico-nanoliter-sized droplets onto an EM grid, structural resolution is increased along with the deposition precision down to individual grid squares. Even EM grid advancements have allowed for the ability to deposit up to three different samples onto one grid with negligible cross-contamination using constrained volumes. These micro-deposition strategies proved to be much more advantageous than previous benchtop blotting techniques with high sample loss.

Phase change

For the purposes of this manuscript with its effort to organize various off-chipping strategies, phase change includes evaporation and freezing. These give options that are simple not addressed by other techniques, and their advantages include volume reduction, sample stabilization, and preserving structures. Some examples are used in concert with other off-chipping methods, such as MALDI and EM preparations where the off-chipping step is followed by the phase change. While these are separate categories, successful systems must fully integrate the strategies to allow success.

Summary of samples and targeted analytes

With the numerous off-chipping strategies that have been implemented on a chip, a wide range of sample types have been successfully explored. Over the last few decades, samples anywhere from cells to biomolecules to even simple molecules have been addressed with various manipulations on a chip, as well as efficiently delivered off-chip to a secondary detection scheme. These samples are divided

into three categories: cells, bioparticles, and molecules. Not all published samples and applications have been addressed, but instead, a highlight of the most representative or impactful publications is presented and discussed.

A large number of cells have been manipulated and then analyzed by an information-rich detection scheme [1–34] (see Electronic Supplementary Material: Table S1, first section). Among these are human and mammalian cells [1–20], bacterial cells [21–27, 29–33, 35], and other cells [28, 34]. Several works simply isolated the cells for single-cell assessments [1, 2, 9–15, 17–25, 27–33], while others isolated sub-populations by some differentiating factors including chromatography and fluorescence-activated cell sorting [3–8, 16]. The isolated cells or cellular contents were off-chipped by electrospray/nano-electrospray [1–3, 30], use of droplets or plug flow [6–12, 14, 15, 17, 21, 23, 24, 26–28], or held in a constrained volume or deposited [13, 16, 20, 22, 34]. In parallel and sometimes in concert with the microfluidic developments, a host of molecular biology tools that may be adapted to these processed samples have been demonstrated. These include whole genome sequencing from limited numbers of cells [13] and evolving techniques for amplification [16, 19]. Single-cell amplification has been demonstrated [25] and RNA sequencing along with full transcriptomes have been generated from limited number of cells including single cells [18, 26, 31–33].

Bioparticles have been manipulated and analyzed by detection schemes at a microscale [36–48] (Table S1, second section). These includes viruses [40–42], ribosomes [43–46, 48], DNA [37], and other particles [36, 38]. Pretreatments include various staining modules [36, 40, 41] or droplet partitioning [37]. Sample collections are off-chipped to a detection scheme by electrospray/nano-electrospray [43–45, 48], by the use of droplets/plug flow [37, 47], or by constrained volumes or deposited [38–42, 46].

A wide variety of molecules have also been subjected to microscale manipulation and analysis by detection schemes off-chip [49–106] (Table S1, last section). Samples are introduced into the system by continuous flow sampling [49–57, 59–66, 68–70, 72–76, 80–83, 89, 91, 93–96, 98–102, 104, 106] or droplets or injection plugs [67, 71, 77–79, 84–88, 90, 92, 97, 105] and subject to on-line manipulations such as separation [52–55, 57, 58, 60–63, 65–67, 72, 74, 75, 80–83, 85, 87–92, 94, 99, 100, 102, 104, 105] and digestion [49, 50, 68, 72, 73, 83, 94]. Samples can be transferred off-chip by electrospray/nano-electrospray [49, 51, 54–57, 59–66, 68, 70, 72, 74, 77, 82–92, 95, 101, 103–106], by the use of droplets/plug flow [47, 71, 78, 79, 97], or by constrained volumes or deposited [50, 52, 53, 67, 73, 75, 76, 80, 81, 93, 94, 96, 98, 99, 102].

Sample types described with respect to off-chipping method

Cells

Spray

The Ramsey group first reported MS detection of single-cell lysate proteins from a microchip by continuously lysing individual cells and separating its contents by electrophoretic separation [1]. This system did not require the use of detergents and was able to analyze twelve cells per minute using a high-voltage lysis zone coupled with a nano-electrospray off-chipping strategy. On-chip cell lysis coupled with a nano-electrospray off-chipping technique for MS detection has also been achieved through a double nano-electrode cell lysis technique [2]. Urban et al. presented a MALDI-MS analysis of single cells where a combined spray and deposition strategy on a high-density microarray was done [30]. Picoliter-sized sample volume depositions combined with matrix loading by ultrasonic spray allow for aliquots to align with the dimensions of the MALDI laser beam diameter and decreases the sample mass requirements by three orders of magnitude.

Droplets or plug flow

Various droplet formation techniques have been used with microfluidics to improve detection performance. Many of these strategies have been termed “digital microfluidics.” Meniscus-bound systems involving cells include early work from the Ismagilov group, where they sensed bacteria on a microfluidic chip and assessed their susceptibility to antibiotics in a plug (droplets in a confined flow stream) of nanoliter volumes [29]. The system promised faster and more information-rich analysis for bacterial detection and characterization. Zhang et al. incorporated a passive cell-trapping system that collects a single cell from an aqueous medium and transfers it to a picoliter-sized hydrogel droplet [12]. This minimized the reaction volume transferred off the chip for more efficient RNA sequencing (Fig. 2). Using a clever “hook” design in a pipette tip, the Qin group performed a rachet capture of single cells in sub-microliter volumes and processed them further using suitable techniques including single-cell PCR and RNA sequencing [28]. The strategy could be integrated rather inexpensively into standard laboratory manipulations. Using a nanoliter injection nozzle system, droplets with single cells were placed in oil-filled containers for a variety of assessments, including single-cell proliferation, drug-resistance testing, PCR, rtPCR, and whole genome

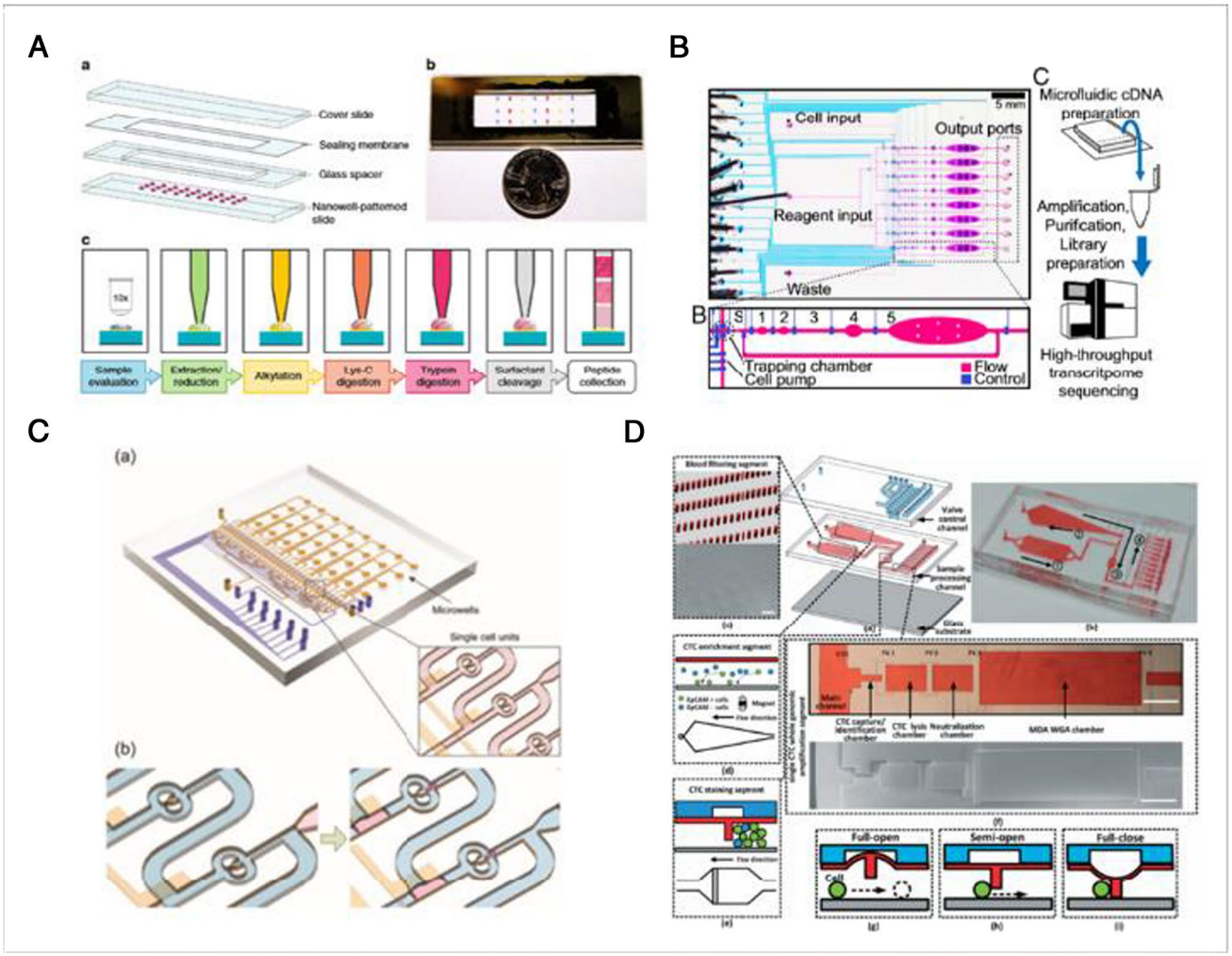

Fig. 2 Schematic representation of various meniscus-bound techniques for sample off-chipping. **A** NanoPOTS: a proteomic sample preparation technique with the use of single droplets where all preparations are performed in a one-pot protocol with each nano-well containing ~ 200 nL of sample [6]. **B** Reaction pipeline: a detailed diagram of a single-reaction pipeline with the use of elastic valves for precise sample trapping. Single cells are isolated in these trapping chambers where cell lysis, reverse transcription, polyA tailing, primer digestion, and cDNA synthesis are performed separately and sequentially. Prepared samples are pushed to an open well, where a meniscus-bound sample is formed for extraction and sequencing off-chip [11]. **C** A single-cell phase switch device is used on-chip to collect a single cell into a picoliter-sized hydrogel droplet. These droplets are directed to open microwells where extraction and further detection are made possible [12]. **D** An improved chip design using trapping chamber for sample preparation was used to isolate and amplify circulating tumor cells from blood samples. An added filtration system and MDA WGA chamber improved detection results of prepared sample extracted from open wells at the end of the system [14]

sequencing (WGS) [24]. High precision and reproducibility were demonstrated with the "interfacial nanoinjection" system, which can be integrated with many common workflows.

Cells must be disrupted to release DNA and RNA for many assessments. This step can present significant issues, including its compatibility with microfluidics, but a variety of solutions have been developed. A microfluidic device involving a micropillar array was used to capture single HeLa cells for genomic DNA purification and amplification [9]. This micropillar array induced DNA extraction and immobilization, allowing for whole genome amplification (WGA) to be performed in a continual fluid flow on the chip while maintaining the ability to be removed from the chip for sequencing. Both gram-negative and gram-positive bacteria lysis and genomic DNA extraction have been done in a single microfluidic system [21]. The lysis procedure incorporated thermal, enzymatic, and chemical lysis methods and was followed by on-chip WGA. This method proved the compatibility of a bacterial single-cell lysis method with microfluidic-based genome amplification.

Kelly and co-workers developed a robotic chip-based platform for enhanced proteomic analysis using nanodroplet processing termed nanoPOTS [6]. Using the nanoPOTS,

a complete processing procedure was performed within a 200-nL or smaller droplet confined in a glass reactor chip, and then sequentially collected by a capillary. They were able to analyze 1500–3000 protein groups from 10 to 140 HeLa cells when coupled with highly sensitive LC–MS. This ultrasensitive nano-LC–MS was also coupled with fluorescence-activated cell sorting for protein analysis of single mammal cells with as low as 15 pg of total protein [7]. This allowed for more precise cell dispensing and proper dilution for reduced background noise. They were able to identify 670 protein groups from a single HeLa cell. To eliminate the use of a robotic pipetting instrument, Kelly's group showed that the nanoPOTS method could be utilized even if volumes were increased tenfold by use of commercial pipettes [8]. A reduction in proteome coverage was observed but over 1000 proteins were still identified from as little as 25 cells.

Improvements in microfluidic devices reduced bias from single-cell amplification for various sample types. A common approach is the use of sorting chambers, separated by valves, before being released to an open microwell. The Quake group developed a complex microfluidic device made of elastomer with embedded valves such that single cells could be sequestered into 60 nL volumes [23]. The individually sequestered cells were introduced to a series of additional chambers for amplification and released for sequencing, which showed improved performance compared to standard 50 μL volumes for single cells. Bontoux et al. further developed this concept of an on-chip single-cell assay by applying a whole transcriptome assay that can profile all genes expressed by an individual cell [17]. The whole transcriptome microfluidic assay solved a major limitation in the number of genes that can be analyzed at a time. Streets et al. investigated a microfluidic approach to a low cell count RNA-seq using single mouse embryonic cells [11]. Individual cells were isolated and lysed on a microdevice and their poly(A) tails reverse transcribed into cDNA, which was then processed with next-generation sequencing after further amplification off-chip. This strategy helped increase mRNA detection sensitivity and improve precision in comparison to conventional techniques because of its reduced volumes used for amplification and its all-inclusive preparation platform that eliminates contamination. Li et al. improved this technique with circulating tumor cells (CTCs) using an added MDA WGA chamber and a blood filtering system such that CTCs can be isolated and amplified from whole blood samples [14]. This scheme is different from other WGA chips because of its inclusion of a cell identification function, which is needed when working with CTCs. The Walther-Antonio group presented a workflow for the isolation, lysis, and SC-WGA of epithelial cells collected from uterus tissue [15]. Over 25 ng of DNA was collected after amplification with no measurable contamination, providing sufficient samples for sequencing off the chip. Single microbial cells have also been manipulated in this device for single-cell WTA using femtograms of the RNA template [27].

Droplets by emulsion have been proven effective in microfluidic systems for improving amplification limitations. By distributing cDNA into 105 droplets (65 pL) using emulsion, random primers can be employed instead of the standard oligo(dT) primers [10]. For eukaryotic cells, this significantly improved the coverage of the 5′-ends and was shown to be applicable to studying nonpoly(A)-tailed mRNA and noncoding RNA. The authors noted the limitations of amplifying small amounts of starting material and using oligo(dT) primers. Some limitations could be addressed by other technologies noted and could be easily integrated with their approach.

Constrained volume or deposition

Microfluidic platforms involving constrained volume and deposition techniques have enabled information-rich detection of various cells by electron microscopy [20, 34] and mass spectrometry [22]. In one study from the Braun group, a single cell is lysed electronically, and the contents aspirated into a capillary and then applied to an EM grid in a lossless method [20]. Several structures were recognizable as being consistent with known components of the cell. The Burg group developed an integrated microfluidic device that enabled cryofixation monitored by light microscopy [34]. Yeast cells were used to demonstrate high temporal resolution as well as continuous live imaging. Lee et al. tested a novel trypsin immobilization technique on a microfluidic chip to demonstrate bacterial fingerprint analysis by MALDI-MS [22]. Tryptic digestion of *E. coli* cells were deposited onto a MALDI target plate directly off the chip using a transfer capillary.

Bioparticles

Spray

A relatively simple interface between microfluidics and EM is to directly deposit materials onto a grid with a nano/micro-spray tip. The Lu group first developed an integrated device of both a micromixer and a 2D in-plane air-assisted microsprayer for cryo-EM analysis [43]. This system allowed for rapid processing times (milliseconds) and produced sufficiently thin films on the grid such that there was no need for blotting. They were able to further reduced their average droplet size while increasing droplet density by incorporating a novel 3D gas-assisted micronozzle sprayer [44]. This improvement made the output levels much more comparable to traditional blotting techniques while drastically reducing consumption levels. Ashtiani et al. were able to achieve femtoliter volume droplets onto a cryo-EM

grid using a surface acoustic wave (SAW)-based atomization [45]. The system was made of a piezoelectric substrate with interdigital transducers patterned to generate surface acoustic waves. The mechanism delivers the sample onto a grid in place of pumping and can be miniaturized further to potentially spray multiple samples on a single grid (Fig 3).

Droplet or plug flow

Combining digital droplets with multiple displacement amplification (MDA) resulted in a dynamic, high-throughput qPCR system presented in Rhee et al. [37]. They achieved a 65-fold increase in coverage in de novo assembly, and more than a 20-fold increase in specificity from 0.1 pg/μL of *E. coli* DNA, compared to conventional tube MDA.

Constrained volumes or deposition

The Tripathi group used two microcapillaries: one for delivery of the sample and one adjacent with precise suction capability [38, 39]. With this flow-based deposition, its fine control of the delivered volume and precise time of vitrification demonstrated subsecond evolution of morphologies. The scheme reduced or eliminated shear-induced artifacts and minimized adsorption of sample species while maintaining high temporal resolution. The Braun group paired

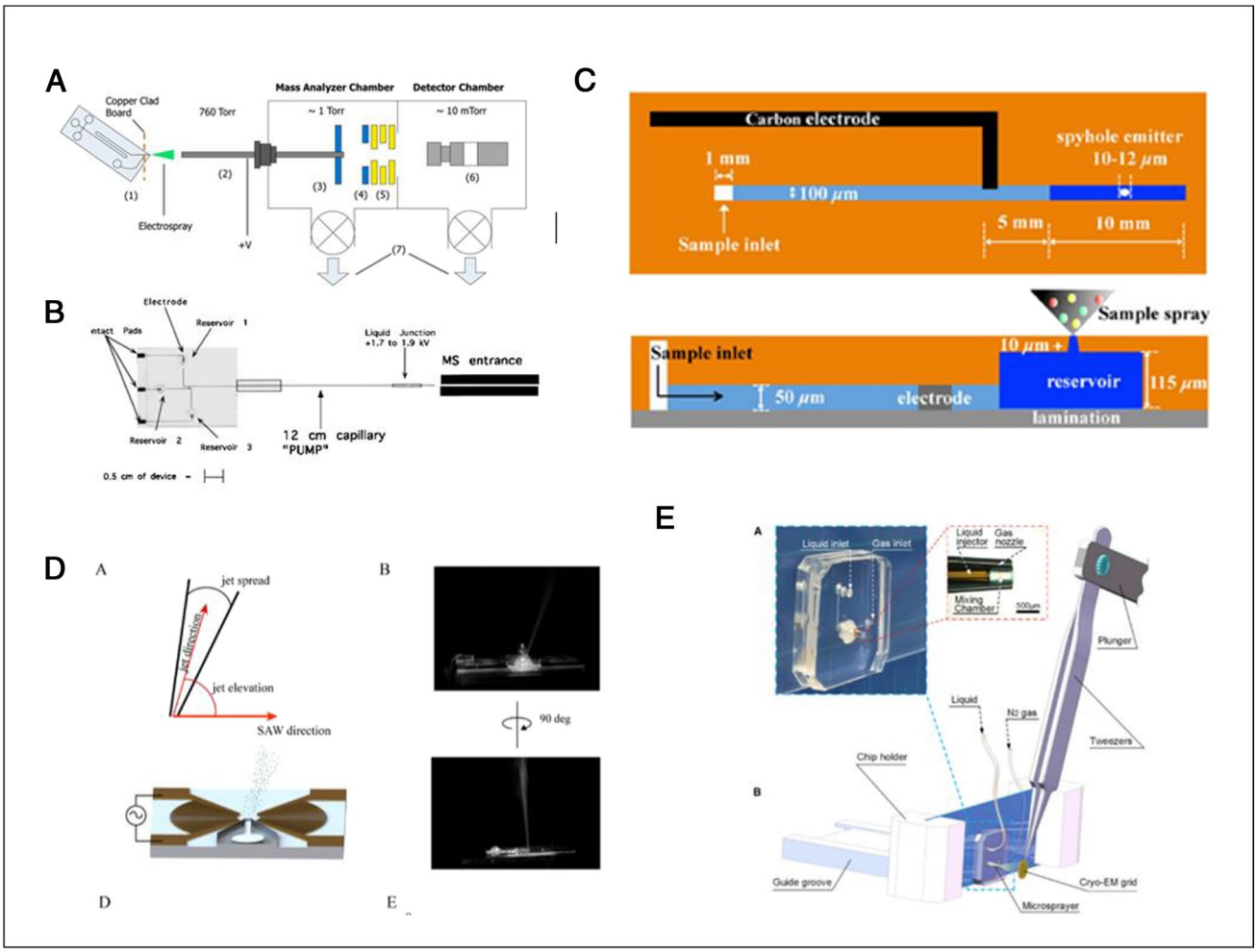

Fig. 3 Schematic representation of off-chipping spray techniques. **A** Microdevice with an ESI spraying module located at the corner of the chip for direct delivery to the mass spectrometer [89]. **B** A microdevice with an added transfer capillary for more precise delivery to a mass spectrometer with very low dead volume [51]. **C** A novel 10–12-μm-diameter spyhole connected to the top of a sample reservoir to deliver sample directly above the chip to a mass spectrometer at low-attomole detection limits [84]. **D** Surface acoustic waves (SAW) were used in place of pumps on a piezoelectric substrate with interdigital transducers to create a spray of femtoliter-sized droplets. The SAW drives atomization instead of the fluid, which decreases the amount of sample required and improves sample delivery onto electron microscopy grids [45]. **E** A PDMS-based chip that includes a micro-spray delivery system for the deposition of a highly consistent layer [95]

microfluidics with electron microscopy by using a microcapillary tube for lossless deposition of conditioned samples [40]. Although the deposition method was effective, the dialysis fiber used for sample conditioning was susceptible to significant sample loss. Arnold et al. were able to eliminate this sample loss by using a microcapillary to collect a 3–5-nL sample plugs and dipping it into a reservoir of negative stain for conditioning [41]. The sample was then deposited onto an EM grid for analysis. A "cryoWriter" used a microcapillary connected to a moving platform as an efficient method for patterning the sample onto the grid prior to vitrification [42]. This modified microcapillary deposition method allowed for more control over the sample thickness suitable for analysis. The Carragher group developed a piezoelectric inkjet dispensing system called a Spotiton for cryo-EM analysis [46]. The device is capable of dispensing volumes in the pico-nanoliter range, and when coupled with a "self-blotting" grid, suitable thicknesses were achieved for vitrification (Fig. 4).

Molecules

Spray

Nano-electrospray ionization (Nano-ESI) has proven to be an effective method for the introduction of small amounts of complex samples to mass spectrometric systems. Various transfer strategies from microfabricated devices to mass

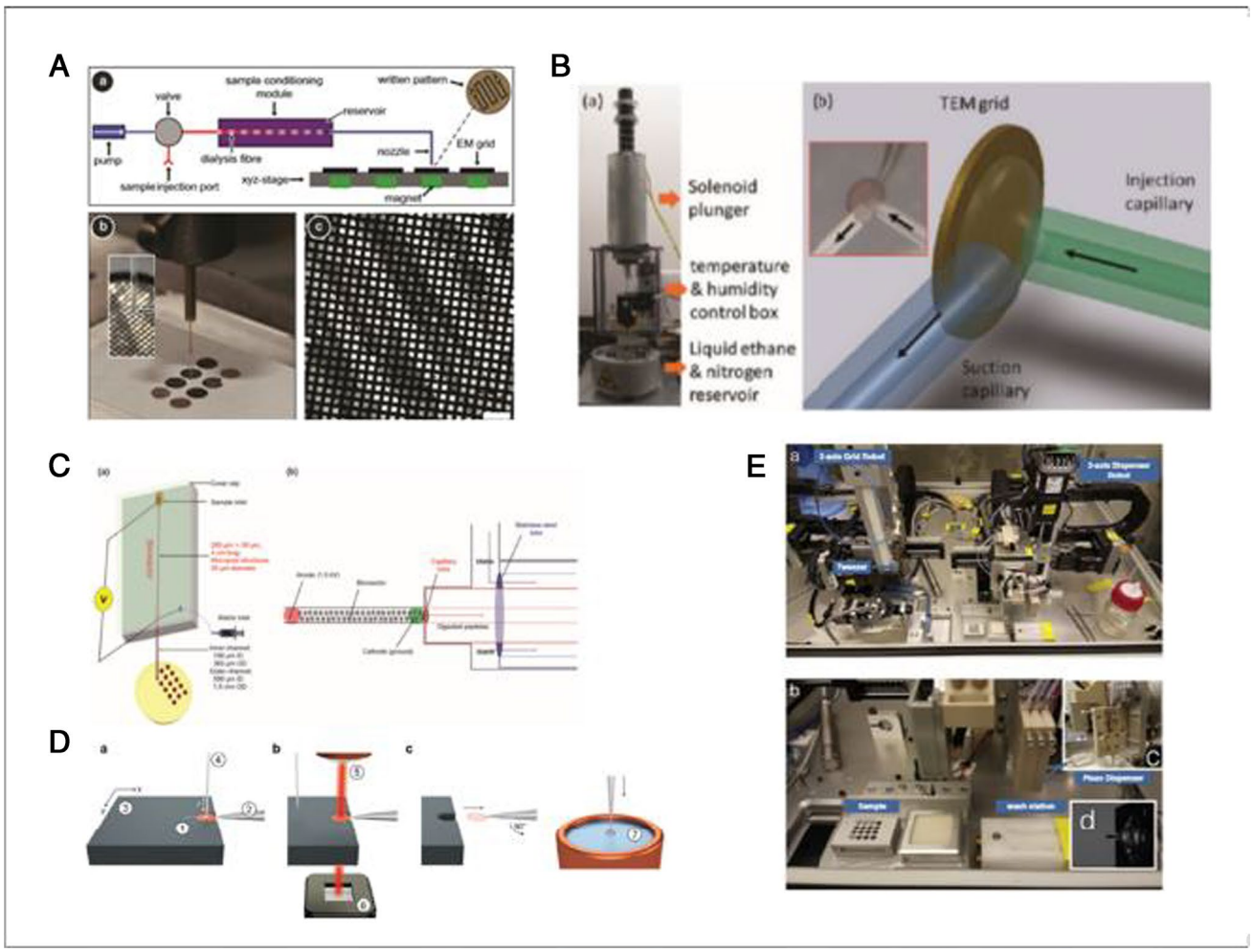

Fig. 4 A schematic representation of various constrained volume off-chipping techniques. **A** An automated deposition module used to deposit 3–5-nL samples to an EM grid. The sample constrained in the capillary tube was dipped in negative stain prior to deposition [40]. **B** An alternative blotless TEM deposition technique involving an injection capillary is placed directly onto the TEM grid while a suction capillary is perpendicular to remove excess liquid [39]. **C** A microchip that includes coaxial tubes to mix the bioreactor output with the matrix solution and deposit sample droplets onto a MALDI target plate for protein identification [22]. **D** A "cryowriter" deposition system that utilizes a sinuous-patterned moving stage to evenly deposit nanoliter volume samples onto an EM grid via a capillary tube [42]. **E** A picture of an automated dispensing system (Spotiton). A piezoelectric inkjet dispensing tip is incorporated to precisely place pico-nanoliter volume sample droplets on an EM grid at suitable thickness for vitrification [46]

spectrometry have been developed using a Nano-ESI interface, including syringe [49] or electroosmotic pumps [51, 56, 57, 59, 64].

The use of syringe pumps in a Nano-ESI/MS microchip system includes early works from the Xue group, which utilized a multichannel glass chip to analyze standard samples of peptides and proteins on a single chip [49]. The sensitivity of this system was in the nanomolar range using either an organic or aqueous solvent. Using the multichannels, they analyzed an on-chip tryptic digestion of the oligopeptide, melittin [49]. The method lends itself to incorporating a separation technique to achieve more precise detection results.

Electroosmotic pumping in Nano-ESI/MS microchip systems has also proven to be effective for small-scale analysis. The Aebersold group utilized a microfabricated device with a coated capillary electroosmotic pump that delivered a micro-spray at volume flow rates of 200 nL/min without the need for sample or s manipulation [51]. Multiple peptide samples were applied to the system at the same time and femtomole level detection limits were achieved with Ion-Trap MS. They also tested this device using a quadrupole time-of-flight MS, which creates the possibility of generating data suitable for both peptide-mass fingerprinting and CID spectra searches on one platform [56]. For more complex peptide mixtures, a solvent gradient was incorporated into the microchip for separation by frontal analysis [57]. Yeast proteins, separated by both 1D and 2D electrophoresis off the chip, were identified at low-femtomole level detection limits. This system was redesigned with a novel computer-controlled high-voltage relay electroosmotic pumping system to direct sample flows [59]. With this automated device, challenges such as contamination and sample loss are addressed, and nine separate samples can be analyzed on the chip through one electrospray outlet. Lazar et al. incorporated an electroosmotic pumping system within a microdevice to control the pressure driven flow on chip in a range of 10–400 nL/min [64]. This pumping system was used for the fluid flow off the chip as well.

Specific physical advancements to the electrospray component for microchips have made significant differences. The Harrison group showed that an external capillary could be attached to a microfluidic chip by a hole drilled into the side of the chip using a flat-tipped drill bit [103]. The connection was much simpler to align and the dead volume was minimized compared to previous works where holes were made with a pointed drill bit [57]. The Ramsey group implemented a nano-spray tip at the end of the capillary which needed no additional pressure assistance for fluid delivery [86]. Using a time-of-flight MS, low attomole quantities of sample was analyzed within milliseconds and at a low fluid delivery rate of 20–30 nL min^{-1}. Another novel modification includes the addition of an on-chip pneumatic nebulizer which minimized the dead volume and controlled the electrospray flow without the use of a transfer capillary [77]. They also fabricated a microchip with the electrospray tip sealed in the miniaturized sub-atmospheric electrospray interface, eliminating the need for glue at the connection [66]. This design was highly efficient in terms of separations science (up to 40,000 plate numbers) and achieved detection limits in the attomole range, making it comparable to previous capillary electrophoresis systems.

Additional creative advancements have been made to the microfluidic/mass spectrometry interface using Nano-ESI. The Foret group made a plastic microchip with sample wells arranged parallel to a standard 96-well plate [70]. Each well was connected to an independent electrospray port, allowing for a high-throughput analysis of potentially 720 samples/h when coupled with quadrupole ion-trap MS. The Ramsey group coupled the ESI with a miniature cylindrical ion-trap (mini-CIT) mass spectrometer [89]. The high pressure of this mass spectrometer eliminates differential pumping regions needed between the inlet and mass spectrometer. Therefore, loss of ion current from the ESI due to pressure change in the transfer region is reduced. A novel "spyhole"-Nano-ESI microchip interface was created by drilling a 10–12-μm-diameter hole above the analyte reservoir [84]. With the spyhole acting as the ESI emitter, low-attomole detection limits were achieved with a dead volume of 100 nL.

The Li group applied the low dead volume connection created by the Harrison group [103] to a CE-ESI–MS-coupled microchip, where the device was able to provide a rapid cleanup and improved resolution [88]. Using a mixture of peptide standards for analysis, the detection limit range was 45–400 nM with a processing time of just seconds. The separation performance was improved by extending the CE transfer capillary and implementing a sheath flow to the electrospray interface. For samples collected from 2D gel electrophoresis that requires higher sensitivity, different preconcentrating methods were evaluated. One included sample stacking, which gave sample loadings greater than 50 nL, while providing detection limits for peptide standards as low as 0.2 nM. The second method used an absorption preconcentration C_{18} membrane for enrichment, providing low nanomolar detection limits but yielded lower peptide recoveries due to incomplete desorption. Preferential enrichment depends on the technique used, as sample stacking preferred hydrophilic peptides and absorptive preconcentration preferred hydrophobic peptides. To increase throughput, an autosampler tray was connected to a sample introduction port [65]. Injections and separations were performed sequentially on peptide standards and tryptic digests for analysis at a rate up to 30 samples per hour will only a 3% sample carryover.

The Yin group presented a microfabricated device that included all components of a nano-LC/MS system onto one

chip [54]. The device used one- and two-dimensional separations for the protein identification of rat plasma samples from either ion-trap or time-of-flight (TOF) MS [62]. The device consists of laser-ablated channels with C_{18}-packed desalting and separation channels, connected to a nano-electrospray. Xie et al. modified this system by integrating a chip-based electrochemical pumping system to eliminate the connection to a conventional LC pump for gradient delivery [55]. The added pumping system was successfully tested with phosphorylated peptides [74].

Creative LC–MS microchip systems have been fabricated to minimize various limitations. Mao et al. presented a silicon-based 24-plex multi-nozzle emitter array chip used for large-scale integration of small volume proteomic analysis [63]. Lotter et al. tested four different ESI emitters on a LC-nano-ESI–MS platform, concluding that all types perform well at nano-HPLC flow rates but a pulled nano-spray emitter was necessary for flow rates below 50 nL/min [85].

A number of electrophoresis methods have been successfully integrated with mass spectrometry on a microchip [58, 61, 72, 107]. A platform using transient capillary isotachophoresis (CITP) and capillary zone electrophoresis (CZE) enabled ESI–MS analysis for peptide concentration and separation [72]. Minor proteins were identified because of the enhanced sensitivity and dynamic range of the system. Isoelectric focusing was used on a polycarbonate microchip with a novel electrospray tip fabricated directly on the side of the chip [58]. A sheath gas or liquid was added to minimize dead volume, and although it did not achieve separation quality comparable to those found in capillaries, because of its simple fabrication, it provides a promising approach for high-speed IEF-MS analyses. A microchip micellar electrokinetic chromatography (MEKC) system with tandem MS compared both a low-sheath flow and sheathless interface for maximizing sensitivity [61]. The sheathless interface had a better sensitivity for surfactant concentrations below 20 mM but proved to be less sensitive at higher concentration compared to low-sheath flow interface. They speculated that this was due to the reduction in the ion suppression effect when the sample was diluted causing offset signal attenuation. The Ramsey group developed a CE-ESI–MS microchip made of glass [107]. This device consisted of a 23-cm-long serpentine separation channel and was able to separate intact proteins with efficiencies between 100,000 and 400,000 theoretical plates. They have commercialized this technology through 908devices and have coined it the "ZipChip."

Capillary-ESI–MS microchips can also be fabricated using various polymers [60, 82, 83, 90–92, 104, 108–111]. A PDMS-based microfluidic device was fabricated and included continuous sampling, peptide separation, and an ESI–MS interface used to analyze a standard mixture of three tryptic peptides [83]. Compatibility with other miniaturized devices such as enzymatic digestion and desalting cartridges was shown in series with the sampling inlet of this device. Dahlin et al. presented a PDMS microchip with fused silica capillaries and a three-dimensional emitter tip that separated peptide mixtures within 2 min [82]. The channel and capillaries were coated with PolyE-323 to prevent unwanted absorption and the emitter tip was dusted with graphite powder to provide conductivity. Liljegren et al. utilized the integration of a graphite-coated ESI emitter tip within a PDMS microchip and successfully coupled on-chip electrochemistry to ESI–MS with subsecond transfer times [111]. ESI emitter tips have also been successfully designed directly out of PDMS. Iannacone et al. developed a multilayered PDMS device and used a low-powered dissecting microscope to cut an angled tip, centered at the end of the spray microchannel [110]. The emitter contributed to enhanced MS signals of standard peptides (bovine insulin) in artificial seawater that have been sequestered into sample plugs on-chip. Svedberg et al. incorporated a more advanced "open" electrospray emitter design made of PDMS involving a groove with parallel walls on each side to form a droplet at the end surface [109]. This emitter was able to deliver 1 μM myoglobin samples sufficiently at flow rates as low as 0.1 μL/min and the mold used for fabrication is robust enough for more rigid thermoset materials. Qian et al. explored a PDMS emitter design where the tip is utilized at the corner of a PDMS substrate [108]. Highly stable ESI–MS performance was demonstrated from rhodamine B detection, as well as high reproducibility from chip-to-chip fabrication. A microchip where all modules were fabricated on the same PDMS substrate was demonstrated by Thorslung [90]. The injection and separations were assisted electrokinetically to provide a sufficient volume flow for stable electrospray and it was established without exceeding a field strength of 270 V/cm. Li et al. attached an ESI interface to a PMMA microchip with a wire-assisted epoxy-fixing method that was low cost and had near-zero dead volume at the connection [92]. They also designed a low-sheath-flow interface consisting of a PMMA reservoir block and platform, along with a replaceable ESI emitter, which provides a number of advantages [91]. The polymer, SU-8, was used by Nordman et al. to obtain detection limits in the nanomolar range with a CE-ESI–MS microchip [104]. Sikanen et al. were also able to fabricate uncoated Ormocomp polymer CE-ESI–MS microchip for the analyses of intact proteins [60].

Electrospray used as a sample deposition method has been proven to be an effective alternative to the dried-droplet method on MALDI target plates. Wang et al. used a polycarbonate microchip with integrated electrospray tips to deposit proteins and peptides onto a MALDI target plate for analysis [68]. This method improved the reproducibility compared to other spotting methods with good spatial control and homogeneity within deposited peptide spots.

Proteins have been analyzed on an EM grid with the assistance of a microsprayer delivery system. Feng et al. created a microsprayer to deposit highly consistent apoferritin sample thicknesses across an EM grid [95]. A PDMS polymer was used as the base, which provides opportunity to couple with other microchip functions. Sub-4 Å reconstructions were obtained on micro- to millisecond timescales for protein conformations using rapid on-chip mixing, delivery onto an EM grid by microspraying, and a vitrification system [101].

Droplets

Exploiting a definitive yet dynamic interface provided by a meniscus, microdroplets have emerged as a major component to microfluidic systems. This allows for increased sensitivity and high-throughput screening with very little analyte of interest because of the ability to isolate, prepare, and deliver these trace analytes to a detection instrument within the enclosed droplet or plug. One microdroplet approach consists of encapsulating the analytes by an immiscible oil and then releasing its contents into a continuous aqueous phase by on-chip de-emulsification for ESI–MS analysis [47, 78, 79]. The Huck group introduced a microchip design capable of using flow-focusing to generate microdroplets and then selectively incorporating the droplets back into a continuous microfluidic stream [47]. An electric field was used to coalesce the microdroplet into the lateral aqueous stream. This design was successfully coupled with a Nano-ESI–MS system [78]. Kelly et al. improved this concept by using cylindrical posts to stabilize the two streams and allow droplets to pass through the gaps into the aqueous stream [79], addressing the dilution problem and high flow rates that limited the sensitivity. Zhu and Fang incorporated a novel droplet extraction interface designed to improve the reliability and controllability of the droplet transfer mechanism [71]. This interface is based on a hydrophilic tongue structure, and it extracts the aqueous contents without leakage of the immiscible oil.

Ji et al. used a droplet-based microfluidic reactor coupled with an ESI–MS to analyze HPLC products and proteolysis buffer in microdroplets [112]. Smith et al. tested surfactant stabilized microdroplets in an ESI–MS microchip for delivery and analysis [97]. The droplets were made off-chip in a flow-focusing droplet generation device and then inserted into the microchip for emulsion before MS detection.

Constrained volume or deposition

MALDI-MS is a useful mechanism of ionization for proteomic research. It uses a relatively simple spotting deposition technique and has a high tolerance to impurities and non-volatile buffers. Wallman et al. presented an automated microchip sample processing method with MALDI-MS detection [75]. Sample cleanup was performed by a solid-phase microextraction array and the enriched sample was autonomously transferred to an off-line MALDI target by microspotting. Automated on-chip protein digestion and sample deposition to MALDI targets have also been proven possible on PMMA-based microchips [22, 50, 73]. The on-chip digestions took only 20 s and yielded sequence coverage up to 97% for protein identification. Tsao et al. utilized a robotic spotting system to interface microfluidic chips with microstructured MALDI target plates for simple, robust, and high-throughput analysis [93]. Other creative spotting systems were designed using droplet microfluidics. Momotenko et al. developed a push–pull scanner to form nanoliter sample droplets at the probe tip that were then deposited off-line to a target plate [105]. Kuster et al. developed a microfluidic T-junction design that formed nanoliter aqueous droplets in perfluorinated oil [67]. The droplets were then transferred to a MALDI plate by a capillary.

Microchip MALDI-MS systems have also been coupled with on-chip separation techniques including LC [52, 53, 80], CE [81, 94], and immunoaffinity [69]. Ericson et al. utilized a novel non-contact liquid deposition system that transferred eluted samples from the outlets of parallel on-chip LC columns to a target plate for MALDI-MS [52]. This was performed by a spatially precise pulsed electric field. This same method was also used for a cyclic olefin copolymer (COC) plate consisting of an array of methacrylate monolithic columns for the analysis of various peptide mixtures [53]. Koh et al. used bead affinity chromatography in a temperature control microsystem to effectively prepare biomarker samples for MALDI-MS analysis [80]. Yang et al. coupled an on-chip immunoassay with MALDI-MS to achieve detection for as few as 300 molecules at the peptide level and 10^6–10^7 at the protein level [69].

Wang et al. presented a modified Si wafer with 250-μm-diameter microspots for on-chip enrichment of phosphopeptide digests and MALDI-MS was performed directly on-chip with sub-femtomole level sensitivity [76]. This method increased the detection sensitivity while minimizing sample handling and reducing sample loss.

Castro-Hartmann et al. used microarray technology to deposit picoliter volume samples onto an EM grid [96]. The use of the ArrayGrid increased the efficiency of grid preparation by one order of magnitude and three different samples were analyzed on a single grid with negligible cross-contamination. The Braun laboratory has produced contributions across several areas with regard to microscale processing and EM, and this area is no exception. They showed EM imaging data for the isolation and concentration of endogenous levels of untagged protein complexes and labeling of specific targeted components [99]. They demonstrated this system could obtain high-quality images of 20S proteasomes from less than 1 μL of cell lysate [102]. Lastly, the Carragher

group developed a piezoelectric inkjet dispensing system called a Spotiton for cryo-EM analysis [98].

Conclusion

The use of spraying (electrospray, nano-electrospray, pneumatic), meniscus-defined volumes (droplets, plugs), constrained volumes (narrow channels, containers), and phase changes (deposition, freezing) are considered powerful tools for microscale processing and analysis. These strategies have enabled the use of detection systems optimized to benchtop-level volumes, where the interface is problematic due to tiny volumes within microfluidic systems. Given these abilities, trace levels of sample can be defined with significantly increasing precision and accuracy. Because of this, further exploration into genomics, proteomics, and metabolomics can be performed with fewer limitations. These techniques were shown to be more adaptive to certain detection schemes, with spraying and constrained volumes more consistently used in accordance with mass spectrometry or electron microscopy imaging and meniscus-defined volumes with sequencing. The technological and design advancements in the last several decades have provided strong promise for clinical optimizations. Processing time and cost can be significantly reduced while also mitigating contamination and processing error, compared to tradition benchtop techniques. This can be crucial when looking to provide effective treatments and therapeutics. The coupling of microscale processing to highly sensitive information-rich detection methods has been established as a robust advancement in analytical chemistry with the potential to be applied to a vast array of sample types.

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Declarations

Conflict of interest MAH declares a conflict of interest with Hayes Diagnostics, Inc. where he is co-founder, COB, CTS, and interim CEO. JPS has no relevant financial or non-financial interests to disclose.

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