

## REVIEW

# Microfluidic devices for high-throughput proteome analyses

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Over the last decades, microfabricated bioanalytical platforms have gained enormous interest due to their potential to revolutionize biological analytics. Their popularity is based on several key properties, such as high flexibility of design, low sample consumption, rapid analysis time, and minimization of manual handling steps, which are of interest for proteomics analyses. An ideal totally integrated chip-based microfluidic device could allow rapid automated workflows starting from cell cultivation and ending with MS-based proteome analysis. By reducing or eliminating sample handling and transfer steps and increasing the throughput of analyses these workflows would dramatically improve the reliability, reproducibility, and throughput of proteomic investigations. While these complete devices do not exist for routine use yet, many improvements have been made in the translation of proteomic sample handling and separation steps into microfluidic formats. In this review, we will focus on recent developments and strategies to enable and integrate proteomic workflows into microfluidic devices.

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## 1 Introduction: The potential of microfluidics to improve proteome analytics

Proteomics, the qualitative and quantitative analysis of complete sets of proteins of a given cell, tissue or organism, has become a standard analytical approach to answer a wide variety of biological and medical questions. Common applications are investigations of cellular physiology in systems biological approaches and in health-related studies as well as discovery of biomarkers for diagnosis and prognosis of diseases [1–5], for clinical monitoring of drug efficiency [6, 7], or for toxicity testing [8].

The two most common approaches for whole-proteome analyses are 2D-GE and shotgun proteomics. 2D-GE is considered to be time intensive, highly skill dependent, and hard to automate, which limits high-throughput performance. Shotgun proteomics has mostly overcome these problems and has gained favor for high-throughput pipelines over the years [9–11]. A typical workflow for shotgun proteomics is outlined in Fig. 1A.

One of the major visions of proteomics is to create a global understanding of cellular activities and responses, by investigating proteomic adaptations of cells under a large variety of conditions [12]. This would require the collection and meta-analysis of proteomic data from a variety of different studies. However, this approach is often hindered by the low reproducibility between individual studies [13, 14]. This issue is also present in the area of proteomics-based biomarker discovery, where validation of proposed protein biomarkers often fails [15–18]. Moreover, an interlaboratory study revealed that even for the analysis of low complexity protein samples, the skill level of the experimenter significantly influences the outcome [19]. In order to highlight how sample handling

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**Abbreviations:** AP, acetaminophen; CTC, circulating tumor cells; DEP, dielectrophoresis; DMF, digital microfluidics; EpCAM, epithelial cell adhesion molecule; ITP, isotachopheresis;  $\mu$ TAS, micro total analysis systems

**Colour Online:** See the article online to view Fig. 1 in colour.

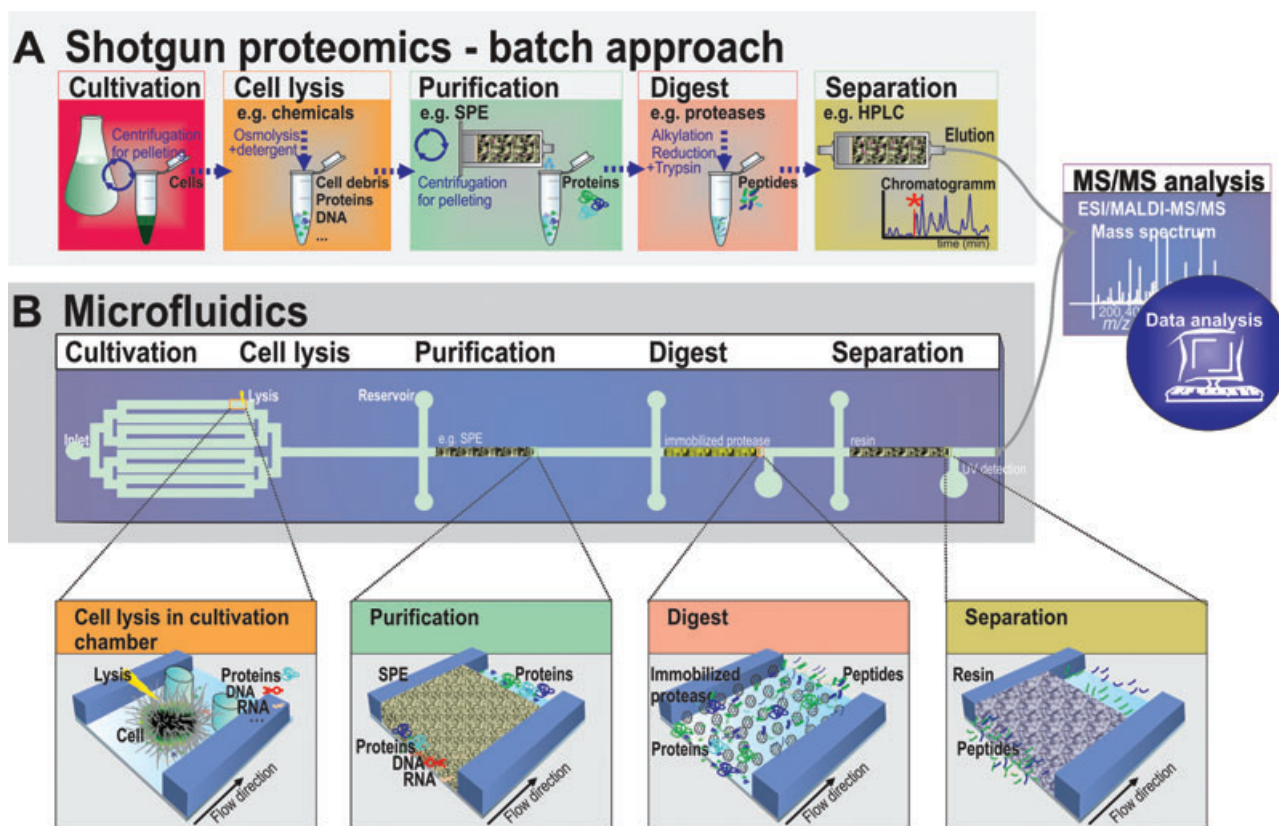


**Figure 1.** Comparison of conventional proteomic workflows and microfluidics-enabled proteomics. (A) Sample preparation for proteomic studies require numerous manual handling steps and sample transfers increasing the risk of introducing variability and contaminations, which can severely affect data analysis. (B) In an idealized microfluidic-based system, all sample handling steps would be integrated into a single platform, eliminating the need of sample transfer steps, and minimizing manual input, thus reducing variability introduced by the experimenter.

and processing may introduce variability, we will shortly describe typical steps in the preparation of proteome samples (see also Fig. 1). First, cells have to be cultivated and treated (e.g. for drug or toxicity assays). Small differences in the culture and harvest conditions can have profound effects on the proteomes of the cells. Subsequent steps include cell lysis and separation of the proteins from cell debris. Often, the protocols are highly adapted to the needs and equipment of the respective laboratory, further introducing variability to the purified sample. In order to identify and quantify the proteins in the mixture, the sample is enzymatically digested (usually with trypsin), separated (mostly via LC), and finally analyzed with ESI or MALDI MS/MS. The resulting peptide MS/MS spectra are then used for protein identification and quantification with or without label-based techniques [20–23]. Efforts for improved standards to achieve higher reproducibility have been proposed and implemented [24, 25], but due to the inherent variability in sample preparation and handling the results are still highly dependent on skill and experience of the experimenter. An additional challenge is the high complexity, plasticity, and dynamic range of proteomes, which may require a large number of analytical runs to avoid un-

dersampling. This is often not possible in cases of samples with limited quantity such as certain body fluids or patient materials. To overcome these challenges an ideal proteomic pipelines would minimize or automate handling steps to reduce variances in the sample preparation and analysis and at the same time allow high-throughput with low sample consumption. It is important to note that the data analysis is also an important factor pertaining to the reliability of the analysis [19], but in this report we will focus on the experimental procedures.

A potential solution to this problem is offered by miniaturized so-called lab-on-a-chip devices that integrate several operations within a microfluidic platform and can be tailored to specific analytical needs [26, 27]. Key attributes of microfluidics are the miniaturization and integration of multiple process operations in a single device enabling (a) handling of nanolitre volumes, (b) different processing steps without sample transfer, (c) short analysis time achieved by reduction of length scales without loss of efficiency, (d) a high degree of parallelization, (e) high-throughput processing, and (f) process automation [28]. Devices like these therefore hold the promise of facilitating highly efficient, reproducible, and

standardized proteomic workflows coupled with low sample consumption. Furthermore, process automation may reduce the requirement of highly trained analytical personnel, which is especially relevant in clinical settings. Moreover, limiting manual input reduces the risk of contaminations, which has a major impact on the reliability of proteome analyses [19]. Finally, the ability of microfluidic devices to manipulate individual cells has opened up the possibilities for protein analytics on the single-cell level [29,30]. However, due to constraints on the detection limits these approaches are only applicable to the mass spectrometric analysis of highly abundant compounds.

In this review, we will report on the most recent developments toward converting proteomic workflows into microfluidic systems and their integration into micro total analysis systems ( $\mu$ TAS) [31].

## 2 Preparation of proteome samples in microfluidic devices

### 2.1 Microfluidic chips

A number of established microfabrication methods are routinely employed for the creation of bioanalytical microfluidic platforms, including combinations of photolithography, wet etching, laser ablation, milling as well as soft lithography for elastomer moulding [32–34]. Depending on the requirements, various materials have been utilized as substrates [35,36], though silica- or polymer-based materials are generally preferred. Glass is an attractive substrate as it exhibits similar properties as fused silica, commonly used for capillary electrophoresis or chromatography [37,38]. Compared with silica-based materials, polymer substrates (mainly PDMS) are more versatile, since they can be easily and inexpensively shaped into the required structures by, e.g. soft lithography or moulding [39,40]. This enables the creation of disposable devices [34,41], which reduces the risk of cross-contamination between samples.

In its most basic form, microfluidic devices consist of an enclosed network of channels with widths in the micrometer range. However, one can generally distinguish between continuous flow and droplet-based approaches. In the more common form liquids are moved in a continuous stream by means of, e.g. electroosmosis or pressure gradients. In droplet-based approaches the analyte of interest is encapsulated within discrete droplets using, e.g. an immiscible fluid phase or bubbles. These droplets are then manipulated within a continuous stream, a method often referred to as segmented microfluidics [42,43]. Digital microfluidics (DMF), an emergent technology, manipulates individual droplets within an electric field via an array of electrodes on a hydrophobic dielectric [44,45]. This method does not require an interconnected fluid network and uses each droplet as an independent reaction compartment. However, sophisticated electrode as-

semblies are required, increasing complexity and cost of the devices.

### 2.2 Cell cultivation and manipulation

One intriguing vision in the use of microfluidics for proteomics is the potential of combining cell cultivation with downstream analysis. An advantage of downscaling cultivation to the microscale is the improved control of the microenvironment surrounding individual cells in an unprecedented manner, especially in comparison to traditional batch cultivation. Devices can for instance be tailored to allow growth within defined gradients under extremely reproducible and homogenous growth conditions [46–48]. This would have a profound impact on the way we can approach the investigation of cellular responses to environmental stimuli on the molecular level. In an idealized microfluidic platform for high-throughput proteomics cells would be cultivated and manipulated directly on the device, replacing traditional batch cultures (Fig. 1B). However, despite the obvious advantages  $\mu$ TAS including cell cultivation for whole-proteome studies has not been realized yet.

A variety of approaches have been described to grow and manipulate cells in microfluidic systems, which could be implemented within a proteomics chip. Groisman et al. [49], described a microfluidic device for chemostatic cell cultivation. Cells were grown in this device in parallel arrays of chambers with constrictions that confined the cells, but allowed constant diffusion of fresh nutrients. Toh et al. [50] created a 3D microenvironment based on similar principles to cultivate liver cells. The bulk of cells were confined within a boundary defined by micropillars, through which medium perfusion was possible. Moreover, the system was multiplexed for eight parallel cell cultivations with independent fluid lines. This device maintained cells better than comparable microwell systems and was successfully used to assess toxicity of model drugs. This could be useful for toxicoproteomic studies. A highly sophisticated design that allowed 96 independent cultivations in parallel was developed by the Quake group [51]. Each chamber with a volume of 60 nL is individually addressable and was used to study proliferation and differentiation of human primary mesenchymal stem cells. Such parallelized cultivation schemes hold the promise to increase the throughput of automated analyses.

Implementing geometries in a similar size regime as cells allows a precise spatial control of individual cells and can further reduce variances in cellular assays. Di Carlo et al. [52] reported the use of U-shaped arrays to trap of HeLa cells in specific regions of a microfluidic channel, while maintaining their viability. Chung et al. [53] implemented hydrodynamic guiding structures in their device to guide prostate cancer PC3 cells into individual microwells, which increased the trapping efficiency. These ordered cell incubation schemes are ideal for cell stimulation assays in which the microenvironment has to be strictly controlled.

Droplet-based microfluidics has the potential to create a new paradigm for cell cultivation. Instead of using micro-wells or interconnected chambers, cells are directly manipulated and/or cultivated in individual nL-compartments. In a recent example, Leung et al. [54] created a device with 95 individually addressable storage chambers in which the flow was controlled by microvalves. Individual *Salmonella enterica* sv. Typhimurium and *Escherichia coli* cells were trapped in droplets, which are suspended in an immiscible oil phase and delivered to specific storage chambers. With this device the authors demonstrated successful growth as well as whole-genome amplification of individually trapped cells.

In addition to cell cultivation, numerous devices have been developed to sort and enrich specific cells from complex mixtures [55], often using immunoaffinity approaches. A prominent example with clinical relevance is the isolation of circulating tumor cells (CTC) from blood. CTCs are important indicators for the progression of cancer as well as the efficiency of cancer treatments [56]; however, they are vastly outnumbered by red blood and other cells within the bloodstream of cancer patients. Since the epithelial cell adhesion molecule (EpCAM) is frequently overexpressed in cancer cells, this feature is often exploited for enrichment strategies. Nagrath et al. [57] for instance integrated microposts functionalized with anti-(EpCAM) antibodies into a microfluidic device and showed successful capture of CTCs from the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast, and colon cancer. In further improvements microposts were replaced with herringbone-patterns enabling enhanced interactions between CTCs and the antibody [58]. Similar capture efficiencies (90%, with flow rates of 10 mL/h) of CTCs from blood were recently reported by Hoshino et al. [59] by labeling the sample with magnetic nanoparticles that are coupled with anti-EpCAM antibodies. CTCs were then successfully captured using an array of magnets. Furthermore, combinations of prelabeling with antibodies and affinity capturing via functionalized posts were also reported for successful enrichment of CTCs [60].

In cases where specific immunoreactions are not feasible other selective parameters have to be explored. Zheng et al. [61] microfabricated a parylene-C filter device with 10- $\mu$ m pore size and sandwiched it between PDMS elements through which fluid lines were inserted. This device was then used to immobilize CTCs whereas erythrocytes passed through the filter. The use of dielectrophoresis (DEP), which is the translational movement of a polarizable object within an inhomogeneous electric field, has also gained wide interest for cell manipulations in microfluidic devices. The dielectrophoretic force acting on a cell is highly dependent on a number of cell properties, including size, membrane capacitance, permittivity, and conductivity [62]. Thus, tailored microfluidic devices can be designed in such a way that the electric field gradients are finely tuned to the properties of the cell and thus affect specific subtypes of a cell within a sample. For example the dielectrophoretic separation of cancer cells from blood cells have been achieved using AC fields with inte-

grated electrodes [63–65]. One disadvantage of this approach is the need to integrate electrode arrays into the separation areas to generate the required electric field gradients. A way to circumvent this is the use of insulating restrictions to create electric field gradients [66,67]. This insulator-based DEP has been shown to be able to manipulate and trap individual cancer cells within a DC field [68]. Yet another approach is the implementation of contactless DEP [69]. Here, the electrodes are placed in conductive microchannels that are physically separated from the actual sample channel by thin membranes. The necessary electric field gradients are created by capacitive coupling. This principle has been used in a number of studies to concentrate different cancer cell types [70] or the separation of cancer and white blood cells [71]. Other microfluidic devices employing similar principles have been developed for a wide range of other cell types, e.g. endothelial progenitor cells, A549 cancer cells [72,73], different organelles, as well as bacteria [74]. Interested readers can find further details in a number of reviews [75–77]. While DEP approaches are very versatile and therefore very attractive for the manipulation of a variety of cells, it is so far unknown to what extent exposure to electric fields will affect cellular physiology and hence, their proteome.

In summary, the above examples demonstrate the flexibility of cell cultivation and manipulation in microfluidic system. However, there are certainly challenges to be overcome before such approaches can be incorporated into MS-based proteomics studies. An obvious issue is the development of an effective strategy to interface cell cultivation and MS-compatible sample preparation and analysis without overly complicating the design and handling of these devices. In addition a higher parallelization is needed to reach the required cell mass for effective proteomic analyses. Nonetheless, the diversity of available strategies is encouraging for the further development of integrated cell cultivation and proteomic analysis platforms.

### 2.3 Cell lysis

After isolating and concentrating the cells of interest, the next step is cell lysis and protein extraction in a controlled and quantitative way. A multitude of lysis methodologies have been developed and realized for microfluidic devices ranging from chemical, thermal, electrical, optical, acoustic to mechanical treatment of the cells (for reviews see Brown and Audet [78] and Kim et al. [79]). For proteomic analysis rapid lysis times and maintenance of protein integrity are important considerations when selecting a lysis methodology.

Chemical lysis, one of the most commonly used methodologies, is based on the disruption of cellular membranes with detergents or in case of sensitive cells by osmotic shock. In microfluidics this can be achieved by adding a lysis buffer line and allowing diffusive mixing of the cell solution with the lysis reagents [80–82]. In order to limit sample dilution the reaction volume can be restricted using, e.g. thermopneumatic

actuator-controlled valves [80]. For a higher throughput, parallel lysis within microwells of a microfluidic device [81] or continuously lysis within a steady stream of lysis solution [83] have proven to be successful. Sethu et al. [83] showed the continuous lysis of erythrocytes within a steady stream in presence of an ammonium chloride solution. However, the main purpose of this device was to destroy the red blood cells whilst maintaining and isolating leukocytes within the same sample. Nonetheless, continuous-flow lysis could prove to be helpful to create protein samples with sufficient abundance for proteomic investigations. Although in certain cases (e.g. *E. coli*, HeLa cells) relatively short lysis times (<1 min) have been achieved, many more stable cells types, such as Gram-positive bacteria, require lengthy pretreatments before lysis occurs.

Mechanical cell lysis methods, such as French press [84] or bead beating [85] are more rigorous and suitable for lysing resilient cell types. However, they are also harder to translate into a miniaturized format. Bead beating relies on a strong agitation of a cell solution in presence of beads. This is usually accomplished using vortexers or other specialized equipment. Nonetheless, Kim et al. [86] presented a bead beating system integrated on a microfluidic compact disc platform. Within this device the cells and spherical particles are first mixed. Then, by spinning the disc, centrifugal forces are exerted, resulting in lysis times between 5–7 min depending on the cell type (CHO-K1, *E. coli*, and Yeast). However, the lysis efficiency only reached 65% of conventional bead beating lysis. An improvement of the microfluidics-based bead beating method was achieved by integrating ferromagnetic discs into lysis chambers. Cell lysis was then accomplished by the relative movement of these discs and beads in an oscillating electric field [87,88]. Subsequently, solids were removed from the sample via spinning of the whole disc platform. Using this principle a similar lysis efficiency as conventional in-tube bead beating of highly stable *Bacillus subtilis* spores was demonstrated [88]. While the results are encouraging, they require specialized chip designs in a disc format and external magnetic actuators. Other groups developed alternative methods for mechanical disruption of cells without these requirements. Di Carlo et al. [89] incorporated, e.g. barbs with a curvature of 25 nm on a silicon chip that facilitated cell lysis by mechanical rupture. The barbs were created by deep reactive ion etching with a distance of 3  $\mu\text{m}$  from each other and arranged in a semicircular array. Lysis efficiencies of about 99% for human promyelocytic leukemia cells were reported at flow rates above 140  $\mu\text{L}/\text{min}$  within this device. Yun et al. [90] constructed a silicon-on-glass chip with a PDMS cover that facilitated cell lysis based on similar principles. Sharp blades distanced 2  $\mu\text{m}$  to each other with a tip size below 10 nm were created by undercutting the silicon during wet etching. EL4 mouse T-lymphoma cells pushed against these nano-blade arrays and were lysed at low flow rates with similar lysis efficiencies as chemical lysis methodologies. Another example of a device utilizing internal structures to facilitate lysis was presented by Wurm and Zeng [91]. The authors

constructed a glass-bonded silicon chip with two consecutive arrays of nozzle structures. The constriction of the nozzles had a width of 3  $\mu\text{m}$  through which Chinese hamster ovary cells were forced. At a flow rate of 120  $\mu\text{L}/\text{min}$  the authors found that 90% of all cells were lysed after two passes.

These mechanical cell lysis strategies are attractive as they do not require external instrumentation. However, due to size limitations bacterial cells may not be easily lysed using these principles. Sonication is another harsh method for cell disruption that has been adapted to microfluidic systems. Tandiono et al. [92] reported a device consisting of a PDMS microfluidic channel and four piezoelectric transducers. Via a T-junction gas can be injected into the system to create gas-liquid interfaces. The high shear stress generated by oscillating cavitation bubbles within the channel via the transducers was shown to rapidly lyse *E. coli* and *Pichia pastoris* cells. Cell lysis via cavitation bubbles can also be induced via short laser pulses in microfluidic channels [93]. A general disadvantage of these methods is the requirement to integrate actuators or add a potentially costly laser setup. Since many microfluidic devices already have electrodes integrated for sample manipulation and flow control, it is only a small step to modify the devices to allow electrical lysis. Restricting the channel width in the cell treatment area results in high electric fields that can be used to electroporate [94] or lyse cells [95] within millisecond time scales. Interestingly, it was also demonstrated that manipulating the strength and timing of the electric fields differential release of cytosolic and nuclear proteins was observed, which opens up the possibility of differential subcellular protein isolation [96].

## 2.4 Purification and preconcentration

After cell lysis the proteins have to be separated from the rest of the cellular contents in order to avoid unspecific signals and ion suppression effects during MS-based protein analyses. In addition, further protein enrichment steps are desirable to gain sufficient material for proteomics investigations. Traditional methods to achieve this include centrifugation, dialysis, protein precipitation, SPE, and liquid-liquid extraction.

In some microfluidic devices an initial separation of intracellular content from cell debris is facilitated due to geometric constrictions at the lysis area, retaining cell debris [97]. However, in many cases additional purification prior to further processing is required. In bulk protein extraction centrifugation is usually the first step to remove cellular debris. However, this is not easily translatable into microfluidics, and so far only disc-based devices were able to incorporate centrifugation as purification step [98]. In order to perform dialysis in a microfluidic format, Xiang et al. [99] sandwiched dialysis membranes between three polycarbonate layers with integrated serpentine flow channels. The upper membrane possessed a high molecular weight cut-off, allowing the protein sample enter the lower channel, whereas the lower

membrane had a low cut-off enabling only salts and other small molecules to pass. This microdevice was used successfully to desalt protein samples from *E. coli*. In a different approach, Lion et al. [100] integrated a PVDF membrane into a microfluidic device between the reservoir and the fluidic connection. This allowed proteins and other hydrophobic molecules to adsorb to the membrane, on which they were washed and finally eluted with an organic solvent. Easier to integrate into microfluidics are SPE materials, e.g. functionalized beads [101, 102] or monolithic polymers that are immobilized within microfluidic channels [103, 104]. The cleanup procedures are very similar to standard SPE (albeit at lower flow rate) and are therefore the most promising candidates for the integration into a complete automated proteomic platform.

The microfluidic format also enables sample purification schemes that are normally not implemented in bulk isolation formats. This includes the use of isotachopheresis (ITP) and DEP. ITP requires only a straightforward electrode setup. However, care must be taken in the selection of the required electrolytes sandwiching the sample [105]. In recent examples, chip-based ITP was used to desalt and pre-concentrate four clinically relevant proteins (HSA, IgG,  $\beta$ 2-microglobulin, and transferrin) [106] from a high salt (urine background). Within minutes a concentration factor of up to 40 was achieved. Using microchips that contained sequential reductions of the channel cross section, concentration factors of up to 10 000 for the biomarker cardiac troponin I were achieved with ITP [107]. Attempts on DEP-based concentration of proteins have also been reported [108–111]. Here, a strong electric field gradient is created to accumulate proteins in specific areas within the device or within streamlines. However, the condition under which concentration occurs is strongly dependent on the buffer as well as the properties of the proteins [110]. Currently, protein DEP is only being explored as a tool for protein analytics with a small number of reference proteins. While promising, the value for selective enrichment or separation of complex protein mixtures with DEP still remains largely unexplored. Hence, DEP may be more powerful for selective enrichment rather than for whole proteome workflows.

In DMF devices cleanup-steps have also been successfully implemented, including procedures like SPE or protein precipitation. Yang et al. [112] reported the desalting of a peptide solution (angiotensin II) as well as the concentration of fluorescein by creation of a monolith for SPE on the chip surface. Jebail and Wheeler [113] created a DMF device containing a double T pattern of electrodes with four buffer reservoirs to precipitate proteins on a specific spot, followed by washing and resolubilization steps.

## 2.5 Digest

After successful protein purification an integrated proteomic chip requires the option to digest the resulting protein

samples. Efficient protein digestion, most commonly with trypsin, is critical for successful MS-based protein identification. In conventional approaches the proteolysis is performed in a homogeneous solution of enzymes and protein sample. However, the digestion times are in the order of hours and can impose a severe bottleneck for the throughput of samples. Increasing the protease concentration in solution leads to higher autolysis rates, which reduces overall digestion efficiency and can impact MS-analyses of the sample [114]. One possibility to decrease digestion time is to confine the reactions to smaller volumes, hence increasing the protease–protein interaction. Another strategy is to immobilize the protease on a solid support that reduces autolysis. Furthermore, digestion with immobilized proteases is compatible with continuous-flow microfluidic chips. Accordingly, a number of studies demonstrated a reduction of digestion times to <25 s using trypsin which is covalently bound to the inner surface of a microchannel without compromising the identification of the source protein by MS [115–117].

For very complex samples such as complete proteomes or for the digestion of low-abundance samples, often a larger surface area is required to ensure optimal digestion. To this end proteases have been immobilized to a number of substrates and integrated into microfluidic channels. One example was the creation of a PET microfluidic chip in which layers of poly(diallyl)dimethylammonium chloride and  $\beta$ -zeolite nanocrystals were electrostatically assembled to form a network into which trypsin was integrated [118]. With a digestion time of less than 5 s the authors were able to subsequently identify proteins with concentrations down to 16 fmol. Alternative strategies include the use of trypsin immobilized on beads [119], porous monoliths [120], or encapsulated in sol-gel [121]. One advantage of bead-based protease reactors is the ability to exchange beads once the enzyme activity is reduced. This has been realized with immobilized trypsin attached to magnetic particles [122, 123], which were kept in place with a magnetic field and could therefore be released and replaced easily. Instead of beads, Fan et al. [124] inserted a trypsin-linked poly(urea-formaldehyde)-coated fiberglass core into a polymethyl methacrylate microchip, which also could be removed, when spent.

DMF systems are very amenable for enzymatic digestion as each droplet serves as an individual reaction chamber. Thus, parallel processing of samples as well as an individual control of the digestion time is possible. The digestion process in DMF is initiated by merging protease droplets with the protein sample and efficient digestion times were found to be in the range of 2–10 min [125, 126]. In an effort to integrate immobilized trypsin into digital microfluidics systems, Luk et al. [127] used hydrogels [128] to which proteases were immobilized. Individual droplets with protein solution were moved over the hydrogels, and allowed to be digested there. An interesting possibility to improve the differentiation of similar proteins, such as splice variants, is to use hydrogels with different proteases. Then, a sample droplet can be split and moved to different protease spots, or the same sample

can be digested consecutively on different spots. This would generate different specific peptide patterns to assist in protein identification. The digital setup also facilitates reduction and alkylation of proteins and peptides by merging the sample with the respective reagents [129].

Overall, numerous strategies exist to fulfill the functions required to extract, purify, concentrate, and digest proteins from cells and other biological samples within a microfluidic format. It is important to note that these examples were created by various groups with very specific applications in mind. Significant modifications of the given examples are likely to be necessary to allow a successful interaction of the individual processing element within a single device for proteome-wide investigations.

### 3 Proteome separation and mass spectrometric coupling

To resolve the complexity of whole proteome digests elaborate separation steps are required prior to mass spectrometric analyses. For protein analysis in microfluidic systems both, established protein separation principles, such as chromatography or isoelectric focusing, as well as novel techniques such as DEP separation have been explored [130]. Subsequently, the analytes have to be delivered in an MS-compatible format, which may require additional sample cleanup and re-buffering. Considerable advances have been made in the creation of MS-compatible  $\mu$ TAS platforms, primarily for the investigation of metabolites, but also for protein analyses. In the following we will therefore highlight a number of recent developments in the microfluidic-MS-based analysis of proteins and cells.

#### 3.1 Microfluidic chips coupled with ESI

The standard ionization sources for biomolecules are ESI and MALDI. In ESI-MS the analytes are delivered continuously in a solvent in order to create a charged aerosol that can be sampled by the MS. The low flow rates of nano-ESI systems [131] is especially compatible with microfluidic devices. The initial microfluidic-ESI interfaces consisted of simple glass chips with open exits at the end of the channels that served as electrospray outlets [132, 133]. Since then different strategies have been employed to improve the capability of chip-based ESI, such as the use of different surface modifications, optimized design of electrospray emitters on the chip, or interfacing the chip to conventional nano-ESI emitters via capillaries [134–136]. In some recent designs the nanospray emitter is directly constructed on polymer microfluidic chips [137]. In order to increase the throughput of analyses, a number of devices with multiple ESI connections have been developed. The highest reported number of connections on a single-automated platform to date was described by Sainiemi et al. [138] who created circular silicon platform with 60 indi-

vidual ESI-chips. By rotating the silicon wafer each chip can be aligned with the MS where the samples are delivered via capillary action.

Work has further focused on integrated devices that possess separation capabilities in addition to an ESI interface. Prime examples are commercially available polyimide chips with integrated preconcentration and separation columns together with a nanospray tip fabricated by laser ablation [139]. These devices require off-chip valves and stators but are able to deliver a similar performance as traditional HPLCs. Sainiemi et al. [140] recently described for example a monolithic silicon/glass LC-microchip design with integrated ESI tip. Separation was facilitated by integrating micropillar arrays within the separation region that were coated with octadecyltrichlorosilane, resulting in a very homogenous separation region for reversed-phase chromatography. Using this device the authors were able to efficiently separate drugs (lidocaine, propranolol, midazolam, and verapamil). However, C18-separation is also suitable for the analysis of proteins.

In further developments, MS-based analysis of cellular responses directly on-chip have been demonstrated. In one device the metabolism of acetaminophen (AP) by human HepG2 liver cells was investigated [141]. For this, the authors immobilized HepG2 cells within a poly(ethylene)glycol hydrogel in a serpentine channel that was connected to a channel filled with SPE beads for desalting. The device was coupled to an ESI-Q-TOF via fused-silica capillaries. The cells were challenged with AP and the metabolism of AP to acetaminophen-glucuronide was subsequently quantified by ESI-MS. A similar device developed by the same group was used for the cultivation and mass spectrometric analysis of MCF-7 breast cancer cells [142]. The PDMS-based device consisted of a 3D microfluidic network that allowed effective mixing of drug and medium, as well as the creation of specific concentration gradients, followed by a cell chamber for the cultivation of MCF-7 cells. Downstream from the cell culture area C-18 beads for SPE were immobilized by the introduction of dams within the channel. Finally, capillaries were attached to the outlets of this device to allow introduction into an ESI source. The chip consists of nine parallel units, allowing others tests such as investigation of cell viability on the same device. Using this integrated device the authors investigated quantitatively the absorption and metabolism of genistein by MCF-7 cells. While this chip was only used for metabolite analysis, it is an interesting approach to integrate cell cultivation with mass spectrometric analyses and may also be adapted to protein and peptide analyses.

Recently, DMF devices that allow electrospray generation from droplets have been described. Baker and Roper [143] have coupled a digital microfluidic device via a transfer capillary to a glass nozzle with an integrated standard ESI-needle. Using  $N_2$ -pulses droplets are pulled from the microfluidic device past the ESI needle to the nozzle exit. The analysis of the sample can therefore be conducted on the complete droplet. However, the performance of this device was only tested with caffeine and theophylline and it remains to be seen how well it

performs with proteins or peptides. DMF was also applied for the analysis of crude dried blood samples [144]. Using DMF techniques the samples were resolubilized and derivatized in order to enable ESI-MS detection of amino acids and succinylacetone, a biomarker for hepatorenal tyrosinemia. A pulled glass capillary with a tapered tip was used for the injection into the ESI orifice.

### 3.2 Microfluidic chips coupled with MALDI

In MALDI-MS the samples have to be deposited on a conductive surface and cocrystallized with a laser absorbent matrix. Subsequently, the sample has to be exposed to the ionizing laser. Thus, coupling normally enclosed continuous-flow microfluidic devices with MALDI is quite challenging. Approaches include the integration of open reservoirs that can be accessed by the laser, depositing droplets using microfluidic piezo-electric dispensers, or using microfabricated MALDI targets that allow sample purification [145–147].

Similar to ESI-coupled systems, newer devices also realize sample processing on the same platform. Lee et al. [148] developed a microfluidic bioreactor allowing proteolytic digestion of protein samples and continuous deposition on a MALDI target that was spin coated with a MALDI matrix. The polymethyl methacrylate device contained microfabricated posts to which trypsin was covalently linked. A PEEK capillary was used as an outlet of the chip and brought into contact with the MALDI target. Deposition of the digested peptide solution onto the MALDI plate was controlled via a XYZ-stage, resulting in either discrete droplets or a continuous pattern with a deposition rate of 3.3 nL/mm. Subsequent MALDI-TOF-MS of cytochrome C, myoglobin, beta-casein, and BSA demonstrated sequence coverages between 35 and 78%.

An interesting approach using microfluidic systems for the absolute quantification of peptides was described by Zhong et al. [149]. The device consisted of a long serpentine channel fabricated in PDMS that is mounted on an octadecyltrichlorosilane functionalized silicone base. The peptides were pumped through the channel and allowed to adsorb to the functionalized silicone during the movement through the channel. After removal of the PDMS manifold the samples were sprayed with matrix solution and subjected to MALDI-MS. Normally, MALDI-MS-based quantification of protein and peptides requires labeling of the analytes. In this case the authors used the MALDI-MS analysis to determine how far the peptide plug has traveled through the channel. By exploiting the fact that the loss of analytes due to adsorption is a function of the distance traveled through the channel, the authors were able to quantify the peptide concentration in a label-free manner after a series of calibration experiments. This device was then used to quantify the release of acidic peptide and  $\alpha$ -bag cell peptide from an *Aplysia* bag cell neuron.

The ability of DMF devices to create discrete sample droplets and allowing it to merge with matrix droplets is

ideal for the creation of sample spots for MALDI-MS analyses [150]. In addition, desalting was conducted by creating a teflon area onto which the sample droplet with the protein or peptides is deposited and allowed to adhere [151]. Subsequently, water droplets were moved over the spot to remove salts from the sample, before a matrix droplet is moved to the spot for MALDI-analyses. This method was found to be more efficient than using C18-packed tips routinely employed to desalt protein and peptide samples. Chatterjee et al. [152] reported a DMF platform that allows the integration of a number of protein processing steps including desalting, reduction, alkylation, enzymatic digestion, and cocrystallization with a MALDI matrix. The reactions were initiated by mixing the sample droplet with droplets of the respective reagent. Mixing was facilitated by diffusion, assisted by repeated movement of the droplet over the electrodes.

Overall, coupling of microfluidic devices to mass spectrometric analyses has undergone significant developments and has shown to be a mature addition to the analytical laboratory. The greatest challenge will be the creation of compatible interfaces between these elements and the cell handling steps, in order to create an automated platform for high-throughput proteomics.

## 4 Conclusions

Microfluidic devices show high potential to overcome current limitations of proteomic analyses, especially in the biomedical field. The flexibility in design and the ability to automate processes make them highly desirable for a wide range of biological and biomedical investigations. Mature microfluidic chromatography devices already exist that can complement or replace traditional MS-compatible LC setups. However, challenges still have to be overcome before fully integrated  $\mu$ TAS systems can be placed into use for routine biological or diagnostic research. Excellent proof-of-principle studies demonstrate approaches for cell cultivation and manipulation as well as protein extraction. However, in many cases significant sample-dependent optimization is still required to achieve the desired results. Moreover, their reliability during routine use in biological laboratories still remains to be proven for the most part. Streamlining device operations would significantly improve the implementation and acceptance of these devices for routine laboratory operations. Other aspects that require improvement include higher parallelization to increase throughput for proteomic investigations as well as successful integration of all the required functions within a single-automated device.

Nonetheless, the strategies presented in this report are encouraging indicators that microfluidic systems have the potential to overcome these issues and enable proteomic analyses with unprecedented throughput and reproducibility. Before this can be achieved more efforts are necessary to move the necessary microfluidic operations beyond proof-of-principle stage. Subsequently, seamless integration of these

operations in a user-friendly manner will be key to the success of  $\mu$ TAS devices for proteomic applications in biological and biomedical research and diagnostics.

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