Recent Advances in Microfluidic Sample Preparation and Separation Techniques for Molecular Biomarker Analysis: A Critical Review

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Abstract

Microfluidics is a vibrant and expanding field that has the potential for solving many analytical challenges. Microfluidics show promise to provide rapid, inexpensive, efficient, and portable diagnostic solutions that can be used in resource-limited settings. Researchers have recently reported various microfluidic platforms for biomarker analysis applications. Sample preparation processes like purification, preconcentration and labeling have been characterized on-chip. Additionally, improvements in microfluidic separation techniques have been reported for cellular and molecular biomarkers. This review critically evaluates microfluidic sample preparation platforms and separation methods for biomarker analysis reported in the last two years. Key advances in device operation and ability to process different sample matrices in a variety of device materials are highlighted. Finally, current needs and potential future directions for microfluidic device development to realize its full diagnostic potential are discussed.

Graphical Abstract
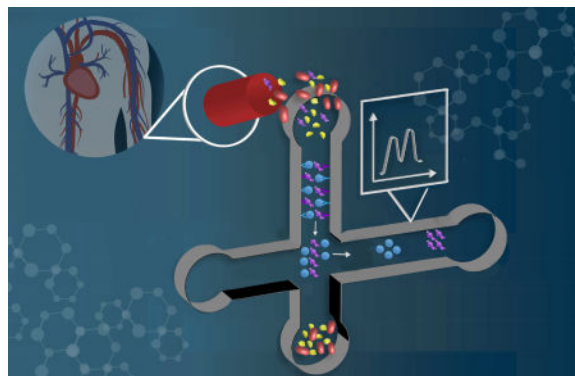
Keywords

Biomarkers; Capillary electrophoresis; Solid-phase extraction; Microfluidics

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1. Introduction

Disease diagnostics are important for improving human health, and the effective treatment of many life-threatening conditions is dependent upon the accuracy and speed of the diagnosis, which can result in improved human life expectancy. Technologies currently used in healthcare diagnostics often require expensive instrumentation or a modern testing laboratory, neither of which are feasible in many developing nations or in remote locations. Hence, low-cost, rapid, portable, and easy to use tools are desirable to advance clinical diagnostics, especially in developing countries or remote areas that lack appropriate infrastructure.

Analyses of biomarkers, biomolecular indicators of medical conditions, hold excellent potential for clinical diagnosis of various diseases. These biomarkers are frequently found in complex biological matrices or bodily fluids, which almost always require sample preparation prior to analysis. Sample preparation steps often need large volumes (> mL) and experienced personnel, which further increase the analysis time and cost. Thus, fast and effective sample preparation techniques are necessary to facilitate early diagnosis.

Microfluidic systems offering advantages like low cost per device, rapid analysis, and small sample requirements [1] have potential to transform diagnostics, especially in developing countries or remote locations due to amenability to point-of-care testing. Biomarker analysis has been one of the most actively pursued applications in miniaturization of chemical analyzers. A number of microfluidic systems have been reported recently that can perform sample preparation steps like purification, preconcentration and labeling on a chip prior to quantitation [1–3]. Separation techniques have also advanced for the analysis of molecular biomarkers in a microfluidic setup.

This manuscript critically reviews microfluidic techniques reported for biomarker sample preparation and separation over the last two years. Developments in microfluidics for biomarker analysis prior to 2015 have been reviewed previously [4, 5]. Herein, we specifically focus on microfluidic sample preparation methods, such as biomarker purification from biological matrices and preconcentration of trace components, and approaches that are used for biomarker separation. Furthermore, novel aspects of device design and analysis methods are highlighted.

2. On-chip sample preparation methods

Microfluidics can be used to miniaturize and integrate sample preparation processes on a microchip platform. Typically, building these systems requires innovations in device design and manufacturing; fluid transport, automation and control; preparation of samples before analysis; separation; multiplexing; and detection [6]. Often sample specimens are limited in volume, contain matrix-related interferences, require multiplex analysis and have low target analyte concentrations [7]; therefore, sample preparation is a key part of analysis. Commonly used sample preparation processes include analyte purification, enrichment and labeling. In this section we focus on select techniques for microfluidic sample preparation.

2.1 Molecular affinity extraction

On-chip sample preparation can be used to selectively extract, preconcentrate and label selected analytes in an automated fashion. The ability to extract trace amounts of desired analytes from a complex sample matrix such as blood significantly simplifies the analysis [8]. Such on-chip sample preparation could replace laborious benchtop processes, and thus decrease analysis time and potentially allow point-of-care usage. Affinity approaches using antibodies or aptamers on a solid support can purify target species in blood from undesired matrix components that complicate analysis. A summary of key information related to molecular affinity extraction work discussed in this section is given in Table I.

2.1.1 Antibody-based extraction—An antibody (Ab) offers high selectivity and specificity towards its target antigen, and can be used in microfluidic systems for the selective capture of desired molecules [9, 10]. Antibodies can be placed in a microfluidic setup by device surface modification [11, 12] or through a solid support like porous polymer monoliths [13], beads [14, 15] or nanoparticles [16, 17] introduced into microchannels.

An immunosensor was developed on a PDMS treated glass microfluidic device using Ab-conjugated polyvinyl alcohol covered zinc oxide nanoparticles for the extraction of epithelial cell adhesion molecule (EpCAM), a biomarker for epithelial cancers [18]. Whole blood was centrifuged and lysed off-chip to prepare the supernatant that was introduced into the microfluidic devices. Bound EpCAM interacted with anti-EpCAM conjugated to horseradish peroxidase, which catalyzed the oxidation of non-fluorescent 10-acetyl-3,7-dihydroxyphenoxazine to fluorescent resorufin, as shown in Fig. 1a. Extraction results from blood samples obtained from cancer patients and healthy volunteers were compared to a commercially available test, and a linear correlation was obtained from 2–2000 pg mL^{-1} EpCAM with a detection limit of 1.3 pg mL^{-1}. Future work in correlation of measured EpCAM levels with cancer incidence with minimum off-chip sample preparation would be impactful.

In a different study a paper microfluidic device was reported that utilized an antibody-based sandwich assay for detection of tumor necrosis factor alpha (TNFα), an inflammation biomarker [19]. Carbon electrodes were printed on the paper device and anti-TNFα immobilization through both covalent binding and physical adsorption was tested for immunocapture and electrochemical detection, as shown in Fig. 1b. The limit of detection for TNFα was 4 ng mL^{-1}, and diluted human serum samples spiked with TNFα were analyzed down to 20 ng mL^{-1}, although further improvements in detection will be needed to analyze TNFα at native levels in blood. With further work in miniaturizing the electrochemical detection instrumentation, such disposable devices may show promise for detection of diseases, potentially in a point-of-care setting in developing countries. Ali et al. [20] also reported a microfluidic device for electrochemical detection of a breast cancer biomarker, epidermal growth factor receptor 2 protein family (ErbB2). Using carbodiimide linkage methods Anti-ErbB2 was covalently attached to graphene foam modified with titanium dioxide nanofibers, which served as an immuno-electrode inside a polydimethylsiloxane (PDMS)-glass microfluidic device. Detection by differential pulse voltammetry and electrochemical impedance spectroscopy worked for ErbB2 from 100 nM

to 1 fM in the presence of interfering antigens. Experimental results were obtained in buffer solutions, so studies with cell lysate or serum samples as a next step would provide a greater impact.

With the advent of smartphones, diagnosis based on ubiquitous camera capabilities offers great potential for point-of-care [21] and microfluidics applications. One recent example used an on-chip complement fixation test for the detection of carcinoembryonic antigen and recombinant avian influenza A virus using a PDMS-glass microchip [22]. Fluidic-based and agar gel-based complement fixation tests were developed to indicate the presence of a specific antibody or antigen. Colorimetric changes for concentrations in the range of 1–100 pg/mL were easily imaged and analyzed using a smartphone, indicating strong potential for point-of-care application. Another study reported a smartphone-operated microfluidic colorimetric immunoassay for detection of influenza infection [23]. The PDMS microfluidic device contained nitrocellulose paper with influenza virus epitope spots to capture the corresponding primary antibodies present in the diluted sample. These primary antibodies were then detected using alkaline phosphatase conjugated secondary antibodies that induced a color change for a concentration range of 1–100 μg/mL in diluted serum. The device was battery operated and portable, and the total analysis time was 18 min. The results in both these studies involved colorimetric changes that were imaged and analyzed using a smartphone, which as shown previously [21] can be advantageous for point-of-care applications. Improved microfluidic devices that can effectively detect clinically relevant concentration of biomarkers in typical biological matrices with little to no sample preparation are needed to further advance smartphone-enabled diagnostics.

Yang et al. [24] reported a PDMS microfluidic device containing wells separated by pneumatic valves for affinity capture, tryptic digestion and isotopic labeling leading to mass spectrometric analysis of an apoptosis-related protein, Bcl-2. Anti-Bcl-2 antibodies were covalently linked to indium tin oxide on the bottom of the wells, and sequence coverage of 50% was reported for mass spectrometric analysis after tryptic digestion and iTRAQ labeling of captured Bcl-2. Going forward, cell studies and multiplex biomarker capture are important capabilities that should be addressed for these systems to have greater impact in proteomics.

In a different study an electrochemical microfluidic chip for the multiplexed detection of gastric cancer biomarkers was reported [25]. Gold working electrodes were fabricated on glass, antibodies were covalently bound and the device was formed by bonding a PDMS mold cover. Six biomarkers for gastric cancer (carcinoembryonic antigen, carbohydrate antigen 19–9, H. *pylori* CagA protein, P53 oncoprotein, and pepsinogen I and II) were electrochemically detected from serum samples using these immunosensor chips with a linear correlation from clinically relevant pg/mL to ng/mL concentrations. Future work in developing a concentration-based assay would eventually allow early diagnosis and monitoring of gastric cancer.

PDMS has been widely used for making microfluidic devices but it has disadvantages like hydrophobicity and poor fabrication scalability [26, 27]. Thus, a thiol-acrylate resin that exhibits low background fluorescence was reported for making microfluidic devices at room

temperature [28]. Simple electrostatic interaction between the channel walls and an Ab to E. *coli* was used for immobilization. Using these devices, 10^5 cfu mL^{-1} of fluorescently labeled E. *coli* were detected. Although this resin shows promise as a material for microfluidic devices, improvement in the detection limit, immobilization method and ability to detect unlabeled bacteria will be needed for utility in disease diagnosis.

Beads are routine solid supports for antibodies and can be easily manipulated within fluidic networks [14]; thus, microfluidic systems that utilize beads for immuno-capture and detection of disease biomarkers are being developed [15]. Gao et al. [29] used antibody-conjugated magnetic beads for the detection of an anthrax biomarker, poly-γ-D-glutamic acid (PGA), in human serum using surface-enhanced Raman scattering (SERS) in a microfluidic setup, shown in Fig. 2. Detection was done using competition between PGA and PGA-conjugated gold nanoparticles for anti-PGA linked magnetic beads in a PDMS microdevice. A linear decrease in SERS signal was observed for clinically relevant PGA concentrations ranging from 100 pg/mL to 100 μg/mL in human serum. Future work should focus on extension of the approach to additional biomarkers and developing multiplexing methods.

Another study reported a magnetic bead-based immunoassay for urinary protein biomarker detection in a microfluidic device [30]. Magnetic beads with epoxy groups were conjugated with antibodies to capture apolipoprotein A1, a bladder cancer biomarker. Addition of negatively charged DNA on the antigen-antibody complex increased charge, resulting in signal enhancement on the semiconductor sensor platform within the microdevice. Apolipoprotein A1 was measured in urine samples within 20% error compared to established methods, with a limit of detection of 10 ng/mL. Although this microfluidic device shows promising results for quantitation of clinically useful levels of a urine biomarker, the error in concentration measurement has room for improvement.

Zhao et al. [31] also used magnetic beads to capture intact exosomes from human serum for the detection of three ovarian cancer biomarkers (see Table I). A microchip containing a serpentine channel with Y-shaped inlets was fabricated in PDMS for mixing beads and capturing exosomes. These beads were then collected in a microchamber using a magnet, incubated with fluorescent antibodies for three ovarian cancer exosomal markers and detected by multi-color fluorescence imaging. Comparable results to a conventional assay for cancer vs. healthy samples were obtained using the microchip in a 40 min analysis time. Future efforts to correlate detected biomarker concentrations with occurrence of cancer will be needed for diagnostic applications.

Another group developed a PDMS microfluidic device that used antibody-coated magnetic beads for selective capture of Aβ peptides, biomarkers of Alzheimer's disease [32]. The device had a nanoporous hydrogel membrane for peptide preconcentration, and a microchip electrophoresis (μCE) channel for separation of Aβ peptides. Using this integrated device 25 ng of Aβ peptide spiked in 100 μL cerebrospinal fluid (CSF) was detected. To further improve this method, mixing of Ab-coated magnetic beads with CSF could be done on-chip and analysis of patient CSF samples instead of spiked ones should be done. Lee et al. [33] used antibody-conjugated magnetic nanoparticle clusters in a 3D printed helical channel

device to capture E. *coli* in milk. The concentration of captured bacteria was determined by UV-Vis absorption, and the limit of detection was 100 cfu/mL in milk. This study indicates the potential of 3D printed fluidic devices for biomarker analysis; however, the channel dimensions are larger than traditional microfluidic dimensions.

2.1.2 Aptamer-based extraction—Aptamers are short oligonucleotides that have high affinity for their target molecule; they have been used for capture and extraction in microfluidics. Compared to antibodies, aptamers offer advantages like easy synthesis, high stability and low cross-reactivity [34, 35], but aptamer research has not been pursued as deeply as the research of antibodies.

Jolly et al. [36] reported aptamer-based microfluidic immunoassays for prostate cancer biomarker measurement. Amine-linked aptamers covalently attached to PDMS channels derivatized with (3-glycidyloxypropyl) trimethoxysilane were used to capture prostate specific antigen (PSA) from buffer solution, as shown in Fig. 3. In one immunoassay, free PSA was measured by introducing secondary antibodies, followed by chemiluminescence detection with a limit of detection of 0.5 ng/mL. Additionally, detection of glycosylated PSA was done using a biotinylated lectin after aptamer capture, with a limit of detection of 3 ng/mL. This device shows a novel aptamer-based assay that can detect clinical levels of free PSA; however, multiplexed experiments using serum samples still need to be evaluated for greater impact. In a different report aptamers were used for sandwich immunoassays in a PDMS microfluidic device for potential diagnosis of diabetes [37]. Two parallel assays were conducted for quantitation of glycated hemoglobin and total hemoglobin. Analytes were captured from pretreated blood samples by incubating with aptamer-coated magnetic beads. After capture, a second labeled aptamer was added for fluorescence detection. This assay showed good correlation in the quantitation of typical human blood hemoglobin levels compared with benchtop HPLC results, in a shorter analysis time. Going forward, hemoglobin concentrations should be assayed on control vs. diabetic blood samples.

Lin et al. [38] reported a PDMS microfluidic device for cell culture and used aptamer-functionalized microchannels for analysis of vascular endothelial growth factor (VEGF). Amine-modified aptamers were attached to the carboxy-silane derivatized surface via carbodiimide coupling. Cell-cell communication was studied under different low oxygen conditions and for various distances between cell cultures. The results showed faster cell migration under oxidative stress, and captured VEGF (indicating tumor development) was detected using fluorescence and UV-Vis absorption. Another group developed an aptamer-based electrochemical microfluidic biosensor for the detection of creatine kinase, a cardiac biomarker [39]. A gold electrode surface was coated with a carboxy-terminated thiol, which was then functionalized with amine-linked aptamers via carbodiimide coupling. Impedance signal for creatine kinase was linear from 10 pg/mL to 100 ng/mL (relevant to clinical concentrations) in both buffer and culture media samples. A heart-on-chip cardiac bioreactor was integrated with this device, and doxorubicin-induced cardiac damage was assessed through changes in creatine kinase concentration. Both these studies demonstrate microfluidic cell culture devices that detect secreted proteins; future work enabling detection of multiple biomarkers for diagnostic purposes would be impactful.

To summarize, a variety of microfluidic platforms have been reported recently for affinity capture of targeted biomarkers using antibodies and aptamers. A key area of improvement needed for many of these systems is the ability to maintain good detection limits with biological matrices. Multiplexing is another pursuit that could improve disease diagnosis in these microfluidic devices. Finally, efforts are needed to design diagnostic assays that are well suited for point-of-care applications.

2.2 Sample preconcentration

To achieve rapid analysis in a point-of-care setup, a system must measure analytes from blood or other specimens with minimal sample preparation. Since biomarkers are often present in trace amounts, a preconcentration step can be desirable to improve detection [40]. Sample preconcentration on a microfluidic platform can be achieved through electrokinetic means, filtration or chromatographic interactions, as summarized in Table II.

Ge et al. [41] developed an approach to concentrate DNA in a PDMS microchannel, where temperature gradient focusing (TGF) occurred at the interface of a channel that expanded rapidly (see Fig. 4), supplementing electrokinetic concentration. A combination of high-frequency AC with DC voltage reduced the backpressure due to electroosmosis and improved efficiency. DNA preconcentration of 500-fold was achieved in 40 s of operation. This device showed a good preconcentration efficiency for model DNA; however, device performance with real samples still needs to be evaluated.

Combining hydrodynamic control with electrokinetic methods can be beneficial. Indeed, Cong et al. [42] developed an electrokinetic sample preconcentration microfluidic device with electrokinetic or hydrodynamic injection, a pneumatic valve for preconcentration and μCE separation. With the valve closed during electrokinetic injection, current flowed but bulk flow was blocked, allowing ion concentration polarization for sample preconcentration at the closed valve interface. A preconcentrated sample was hydrodynamically injected for μCE, showing 450-fold enrichment. This initial demonstration with model analytes in buffer solution could be improved upon with analysis of biomarkers in a complex sample matrix.

Paper microfluidics can provide inexpensive but often low performance devices. Paper microfluidics for electrokinetic preconcentration of model analytes were demonstrated by forming a cation selective Nafion membrane to induce ion concentration polarization [43]. Preconcentration was further enhanced by decreasing channel depth through a two-sided wax-printing process, resulting in an 800-fold increase in the concentration of bovine serum albumin. In future efforts this method should be tested on disease-related biomarkers in blood or other sample matrices.

Similarly, Lee et al. [44] fabricated an ion-selective Nafion membrane inside PDMS microfluidic channels to electrokinetically concentrate lipid vesicles, as illustrated in Fig. 5. Under an applied voltage ion concentration polarization occurred at the interface of the microchannel with the Nafion, causing a 160-fold lipid vesicle enrichment. Further development is needed to achieve greater preconcentration as well as for application to specific biomarkers.

To provide analyte selectivity a PDMS microfluidic device was developed with integrated polycarbonate track etched membranes having different sized nanopores [45]. This double-membrane microfluidic device processed a urine sample, with the 100 nm pore membrane excluding particles and cells in human urine, but passing proteins, small molecules and ions. A second 10 nm pore membrane passed small molecules and ions but excluded albumin, which was then analyzed by μCE. This approach took 2 min to process a urine sample, and yielded a 6–100 μg/mL linear range and 1.5 μg/mL limit of detection for albumin. This work shows promise in analyzing proteins in a real sample matrix; future efforts to detect additional biomarkers in more complex matrices would be desirable.

On-chip pneumatic pumps and valves offer advantages such as reproducibility and precise control of fluids. Our group developed a microfluidic device integrating solid phase extraction (SPE) and μCE to analyze for a preterm birth biomarker [46]. In this device, SPE was performed in a reversed-phase porous polymer monolith made inside cyclic olefin copolymer (COC) material, and the hydrodynamic controls were formed in PDMS. An integrated SPE-μCE analysis was performed on a preterm birth protein biomarker, ferritin, and a 100-fold enrichment factor was achieved relative to μCE without on-chip SPE, which makes this device amenable for detection of serum levels of ferritin. Although a real sample matrix was not tested, this work lays a foundation for the development of pneumatically operated integrated microfluidic systems for biomarker analysis, potentially integrating additional sample preparation steps like analyte labeling. Very recently, Sonker et al. [47] reported an integrated SPE device for electrokinetic preconcentration of preterm birth biomarkers. A reversed-phase monolith inside a COC microchannel was used to perform pH-based SPE of clinically relevant low nanomolar concentrations of preterm birth biomarkers with 50-fold sample preconcentration. Additionally, this SPE method was integrated with μCE for combined enrichment (15-fold) and separation of a peptide preterm birth biomarker, as shown in Fig. 6. This electrokinetically operated microfluidic device for biomarker analysis in diluted serum could be further enhanced with future integration of on-chip analyte labeling and immunoaffinity extraction steps.

2.3 Sample labeling

In addition to preconcentration and purification, analyte labeling is an important sample preparation step that can be performed on a chip to save time. On-chip labeling requires loading, reacting and purifying, and typically uses a support inside the microchannels; fluorescent labeling is the most common approach being explored.

Herzog et al. [48] developed an integrated microfluidic device on a glass substrate for electrokinetic labeling and separation of peptides and proteins. The integrated device had a reactor for fluorescent labeling with Atto 425, a separation compartment for free flow isoelectric focusing, and a pH sensor layer to calibrate pI values. This device allowed the analysis of proteins and peptides in 5 min, offering process integration and speed; however, the resolution of the separated mixture still needs to be improved.

Our group used reversed-phase porous polymer monolith SPE for the preconcentration and fluorescent labeling of model proteins with fluorescein isothiocyanate (FITC) and Alexa Fluor 488 [49]. Different reversed-phase monolith recipes and eluent compositions were

optimized for selective elution of fluorescent dye and labeling of bovine serum albumin and heat shock protein 90. Recently, we advanced this on-chip SPE and fluorescent labeling technique to process preterm birth biomarkers [50]. Octyl methacrylate reversed-phase monoliths were polymerized inside channels in a COC device as the sample preconcentration platform. Successful FITC labeling of three preterm birth biomarkers was accomplished using this device. Both of these studies indicate the potential of microfluidic devices to integrate preconcentration and labeling to minimize off-chip sample preparation time and effort. Future efforts should focus on lowering biomarker concentrations detected, subsequent integration with separation by μCE and on-chip processing of samples in complex matrices.

3. Molecular separation techniques

Multiple biomarkers are often analyzed simultaneously in clinical diagnostic applications; thus, separation is an integral part of biomarker panel analysis. Various separation techniques have been developed and explored in microfluidic systems for analysis of biomarkers [3, 4]. Molecular separation techniques in microfluidics discussed in this section are summarized in Table III.

Shameli and Ren [51] developed a PDMS-glass microfluidic chip for two-dimensional separation of proteins by combining TGF with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Initially a mixture of bovine serum albumin, ovalbumin, trypsin, and parvalbumin was injected and separated by TGF in the first dimension; subsequently, the TGF peaks were analyzed by denaturing gel electrophoresis in a different channel. In an 8 min analysis time a 70% improvement in the peak resolution of a model protein sample was achieved compared to a separation with only TGF. However, further improvement in separation efficiency to achieve baseline resolution is desirable for quantitative applications. Another group reported a gel-based microchip for preconcentration, separation and extraction of DNA fragments [52]. The device was fabricated by thermally bonding 4 PMMA layers, agarose gel electrophoresis was used to separate DNA and a cellulose ester membrane was used for extraction of DNA fragments. Sample was preconcentrated prior to isotachophoresis, and two parallel channels were used for separation with one channel containing a reference DNA ladder. PCR products were analyzed and an extraction efficiency of 50% was reported. With further work in biological matrices, this device could be used for separation of nucleic acid biomarkers in the future. Redman et al. [53] reported an integrated μCE-electrospray ionization device for separation of monoclonal antibodies (mAbs). A T-shaped device had a separation channel with tapered turns and which extended to the edge of the device to produce an electrospray, as shown in Fig. 7a. This microchip was further combined with mass spectrometry for identification of separated mAbs, showing potential for rapid mass spectrometric analysis of biomarkers using microfluidics, provided more complex sample matrices can be analyzed.

In a different study a T-shaped electrophoresis device for separation of D-amino acids (biomarkers of *Vibrio cholerae* infection) followed by electrochemical detection was reported [54]. A graphene electrode at the end of the separation channel allowed the amperometric detection of liberated H_2O_2 when D-amino acids reacted with D-amino acid

oxidase; in contrast L-amino acids showed no amperometric signal. Although this work utilized a novel signal generating mechanism, further improvements in analyte concentration are needed for potential clinical applications. Pagaduan et al. [55] reported a μCE device for determination of thymidine kinase 1 (TK1), a cancer biomarker. A microchip immunoaffinity assay was reported for measuring Ab-TK1 complex after separating it from the unbound mAb. Although this study reported separation of purified TK1 in buffer incubated with mAb off-chip, there is potential for translation to clinical application if the assay can be performed with adequate detection limits in a relevant matrix like blood.

Sahore et al. [56] reported a pressure-actuated microfluidic device for separation of biomarkers associated with preterm birth. A three-layer PDMS device (see Fig. 7b) was fabricated with integrated valves and a peristaltic pump for pressure-actuated injection for μCE of these biomarkers. Injection was optimized for valve spacing and actuation rate, eliminating bias and yielding an increase in signal, resolution and number of theoretical plates compared to electrokinetic injection. Although this device was used to separate off-chip-labeled biomarkers in buffer, further integration with on-chip sample preparation should enable analysis in more complex matrices. Another study reported a two-dimensional electrophoresis microdevice for separation of proteins using pH gradient isoelectric focusing and zone electrophoresis [57]. The device was constructed with glass and used acidic and basic buffers driven through a voltage difference to create a multilayer pH gradient for isoelectric focusing of proteins. Using this setup, a mixture of proteins (myoglobin, carbonic anhydrase, and catalase) was separated at concentrations ranging from 0.1 to 0.3 μg/μL. Although not shown in this work, native proteins could be utilized for separation and studying protein interactions in the future.

4. Conclusions and future trends

Important innovations and developments continue to be made in sample preparation and separation of biomarkers in microfluidics. Significant advances have been achieved in affinity capture, preconcentration and sample labeling processes on-chip, as well as integrating these together. Antibody- or aptamer-conjugated channels, monoliths and particles have been employed for extraction of biomarkers. Additionally, analyte preconcentration and labeling have been achieved on-chip using various solid support and stacking approaches. Some progress has been seen in combining multiple sample preparation steps within the same platform to provide integrated analysis. Clearly, further research that leads to improvements in limits of detection, sample purification, multiplexing and ability to rapidly analyze samples directly from biological matrices is still needed.

Separation is a key microfluidic technology that continues to advance; for example, through innovative strategies for μCE of biomarkers. Integration of sample preparation with separation and other analyses has moved forward, but at a much slower pace, and advances in such integration would be highly beneficial for development of sample-to-answer diagnostic devices. One area of focus would be to perform whole blood processing on chip to fractionate cells from serum [58, 59], followed by other integrated microfluidic sample preparation and analysis steps like affinity extraction, preconcentration and separation to provide automated quantitation of target biomarkers. An attractive characteristic of

microfluidics is portability, which is amenable to point-of-care applications, and substantial ongoing efforts focus on developing portable diagnostic microfluidic devices with easily accessible detection like smartphone enabled sensing [60]. Additionally, the supporting instrumentation for microfluidics including electrical, optical and data analysis systems should be integrated within the device or miniaturized to facilitate point-of-care diagnosis [61–63].

Microfluidics has made progress on potential biomarker analysis applications in the last few years. However, microfluidic platforms still require significant improvements in analysis time, clinically relevant limits of detection, accuracy and cost. Further work in transitioning academic "chip in the lab" research assays toward allowing minimally instrumented microfluidic biomarker diagnostics is required to make point-of-care applications a reality. One promising future direction is the development of 3D printing to create microfluidic devices. Many papers claim to have 3D printed microfluidic devices [64], but most have fluidic features at the mm or sub-mm scale, while very few publications have achieved the ~100 μm size range of 3D printed fluidic features [65–67]. 3D printing could also increase the rate at which new microfluidic designs are tested, which should hasten innovation. However, to achieve this promising potential, diagnostic applications for biomarker detection using 3D printed microfluidics [68] will need to be expanded and improved upon significantly in the future. We believe that with these developments, microfluidics will continue to have a major impact on biomarker analysis and point-of-care diagnostics in future years.

Acknowledgments

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Biographies

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Vishal Sahore is a postdoctoral fellow in the Department of Chemistry and Biochemistry at Brigham Young University in Provo, Utah, USA. Currently, he is developing an integrated microfluidic technology for the assessment of preterm birth risk. He did his Ph.D. at the University of Arkansas, Fayetteville, developing redox-magnetohydrodynamics microfluidics technology for lab-on-a-chip applications.

Highlights

- We critically review recent advances in using microfluidic devices for biomarker sample preparation and separation.

- We discuss improvements in on-chip sample preparation techniques, including affinity extraction, preconcentration and labeling.

- We highlight developments in molecular biomarker separations.

- We provide a critical evaluation of papers, detailing promising directions for additional work.

- We discuss possible future trends that will help to increase the impact of microfluidics in improving human health.
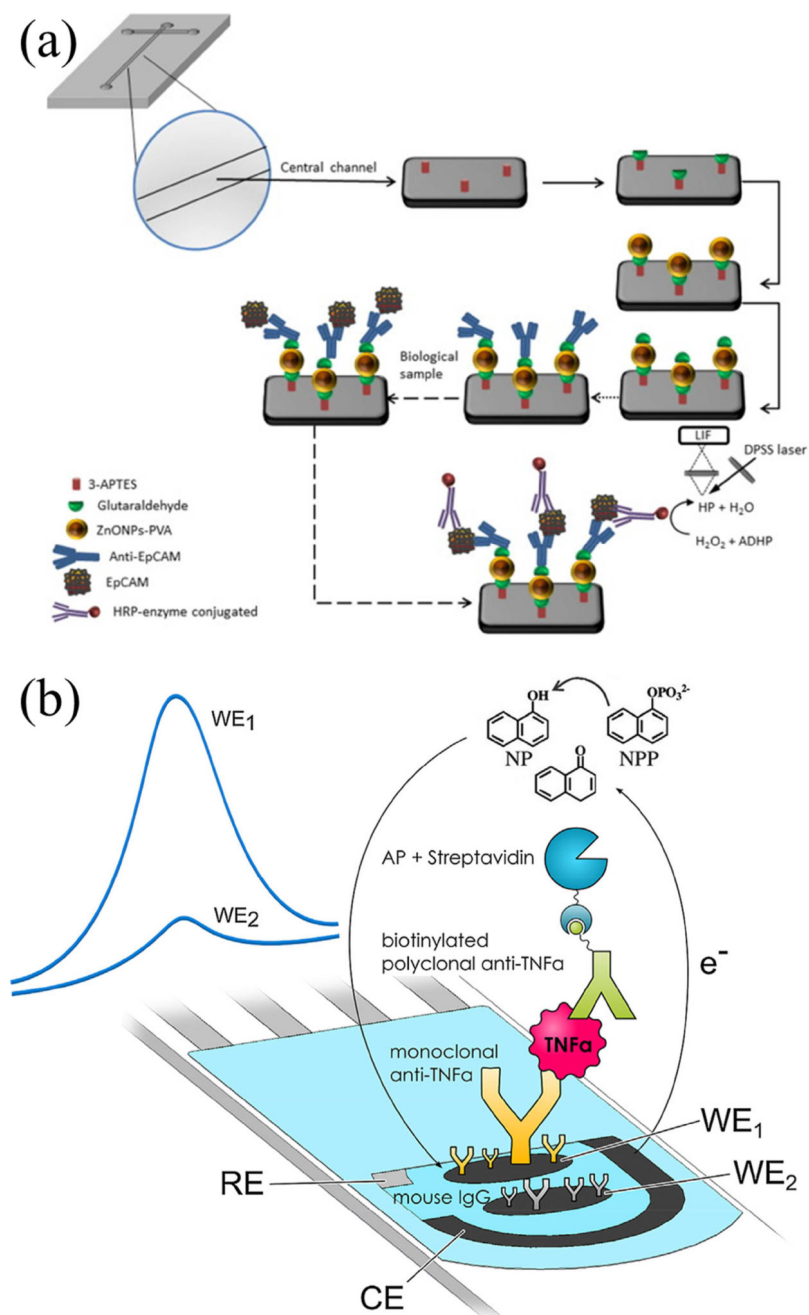
Figure 1.
Immunocapture for biomarker detection. (a) Surface modification scheme for immobilization of anti-EpCAM for detection of EpCAM. Adapted with permission from Fernández-Baldo *et al.* Microchem. J., 128 (2016) 18–25. (b) Sandwich immunoassay for electrochemical detection of TNF-α. Adapted with permission from Eletxigerra *et al.* Sens. Actuators, B, 221 (2015) 1406–1411.
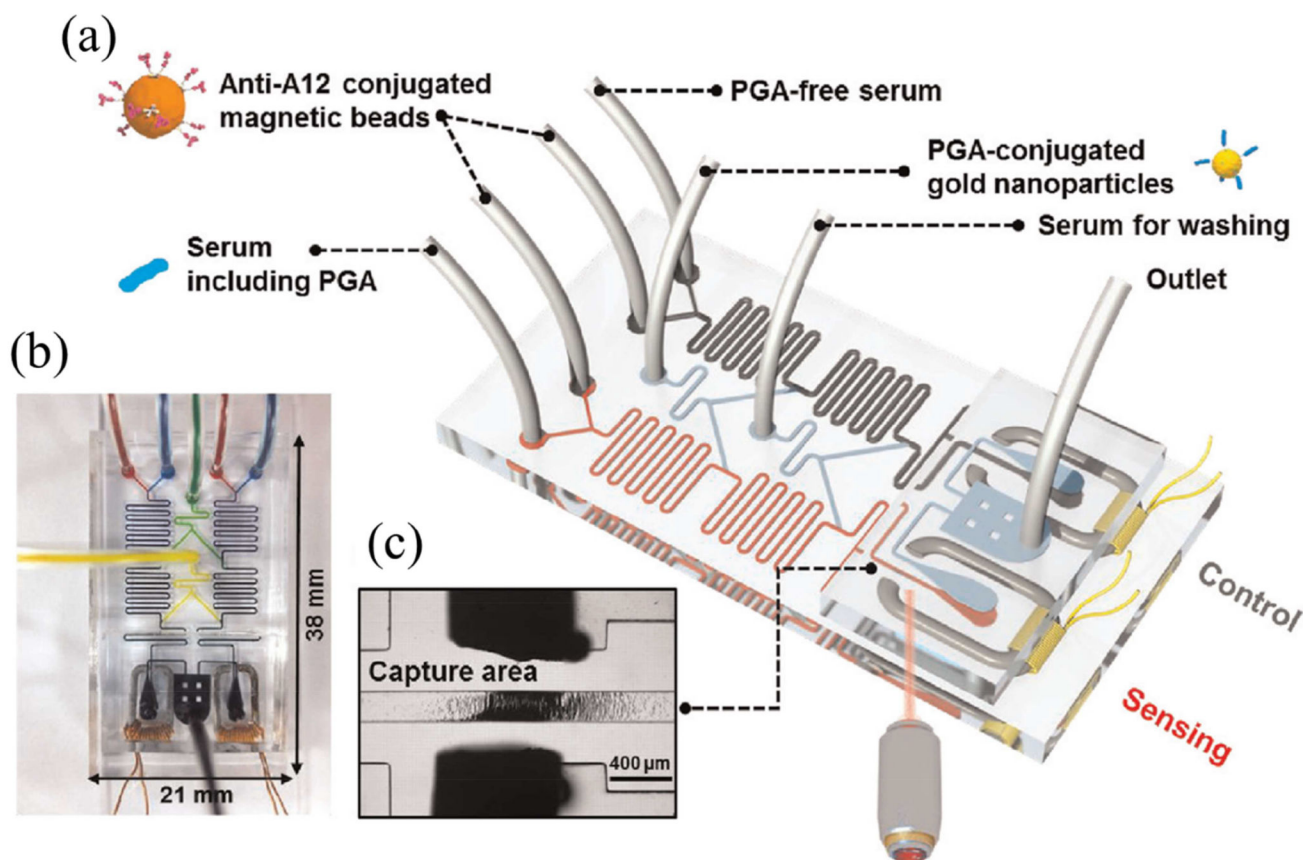
Figure 2.
Microfluidic SERS immunoassay for detection of PGA. (a) Assay overview, (b) image of device and (c) image showing captured magnetic beads. Adapted with permission from Gao *et al.* Biosens. Bioelectron. 72 (2015) 230–236.
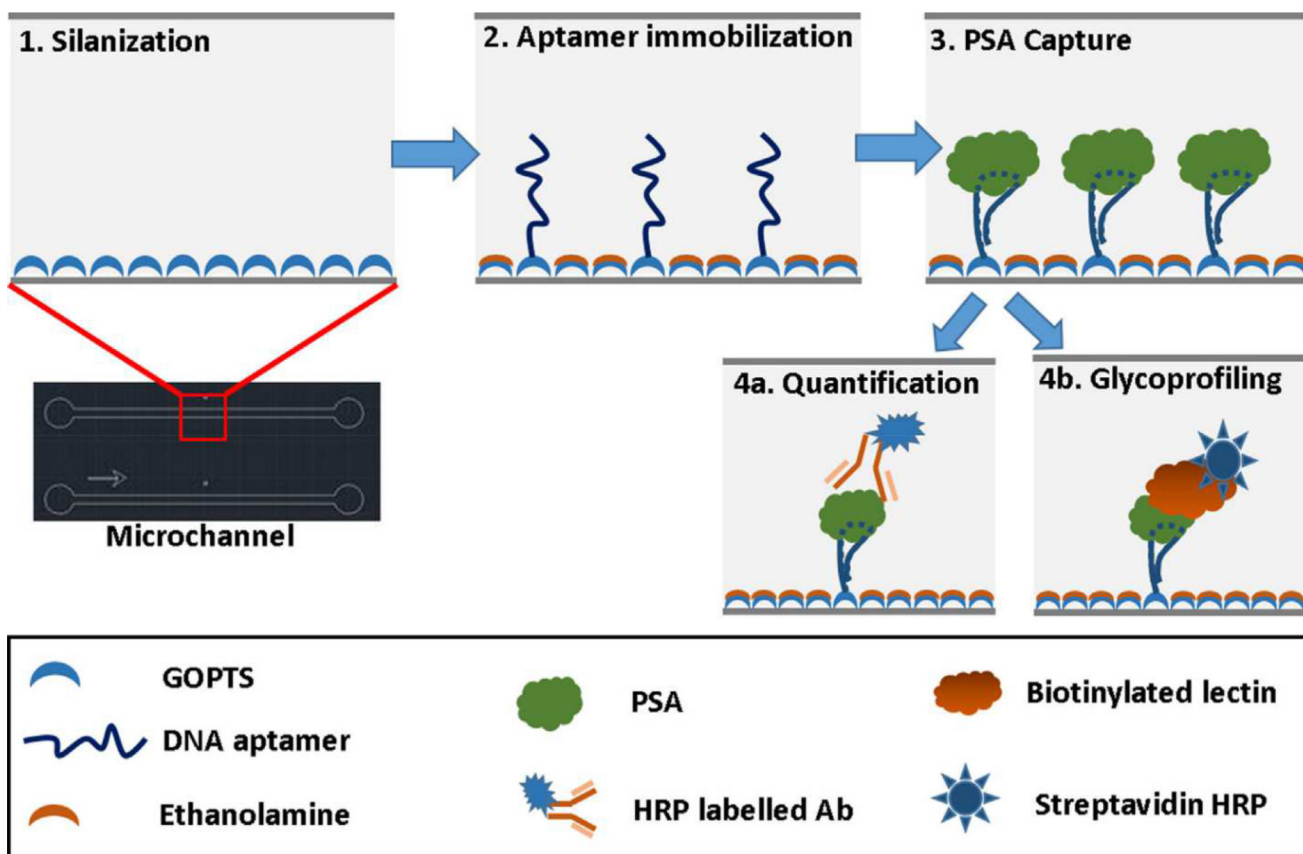
Figure 3.
Channel surface modification scheme for aptamer-based detection of PSA. Adapted from
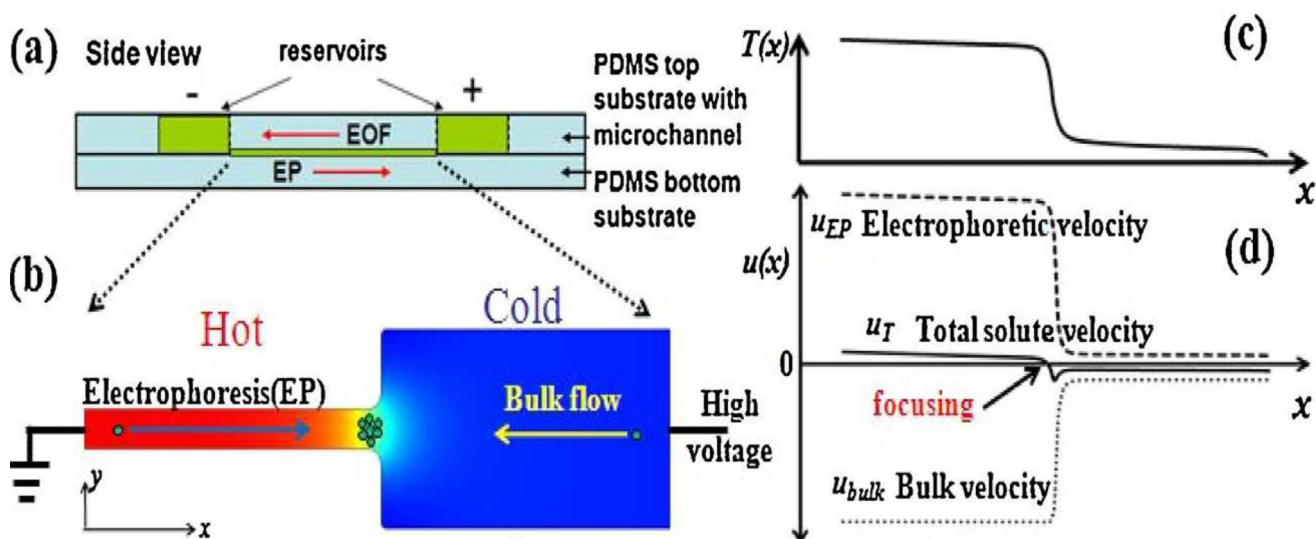Jolly *et al.* Biosens. Bioelectron. 79 (2016) 313–319.

Figure 4.
Working principle of Joule heating induced temperature gradient focusing. (a) Device schematic. (b) Numerical simulation showing temperature profile within the device. (c) Temperature along the microfluidic channel. (d) Velocity profiles along the microchannel under the combined AC and DC fields. Adapted with permission from Ge et al. Anal. Chim. Acta, 858 (2015) 91–97.
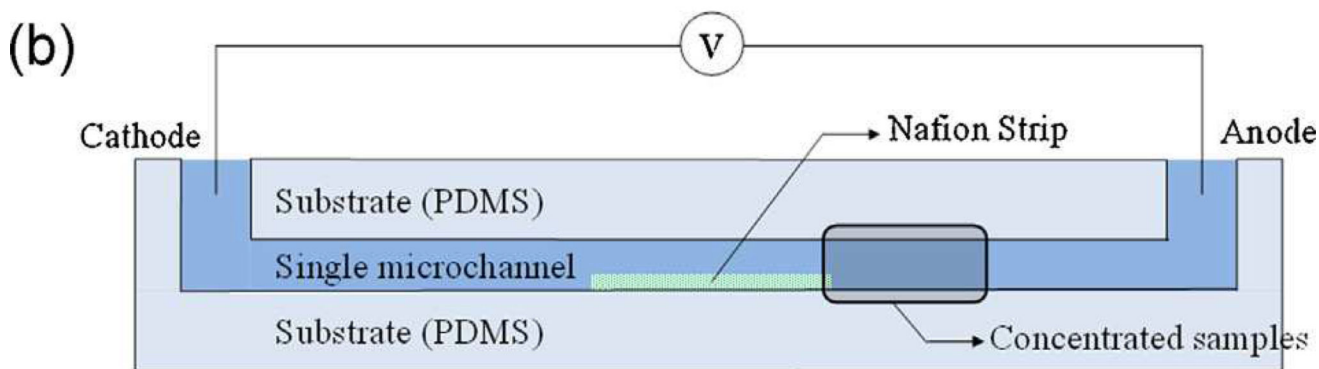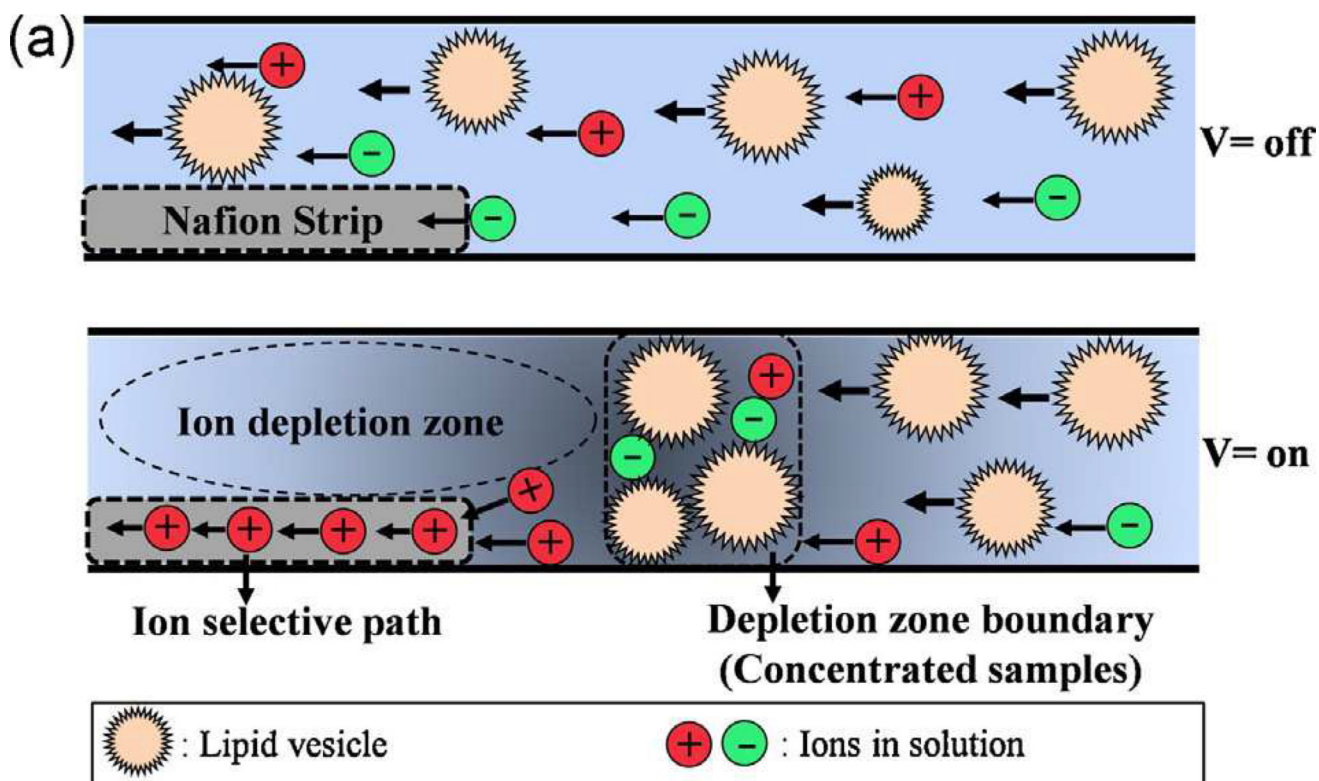
Figure 5.
Nafion-based enrichment of lipid vesicles in a microfluidic device. (a) Working mechanism for the lipid vesicle preconcentrators. (b) Side schematic view of the microfluidic device. Adapted with permission from Lee *et al.* Sens. Actuators, B, 229 (2016) 276–280.
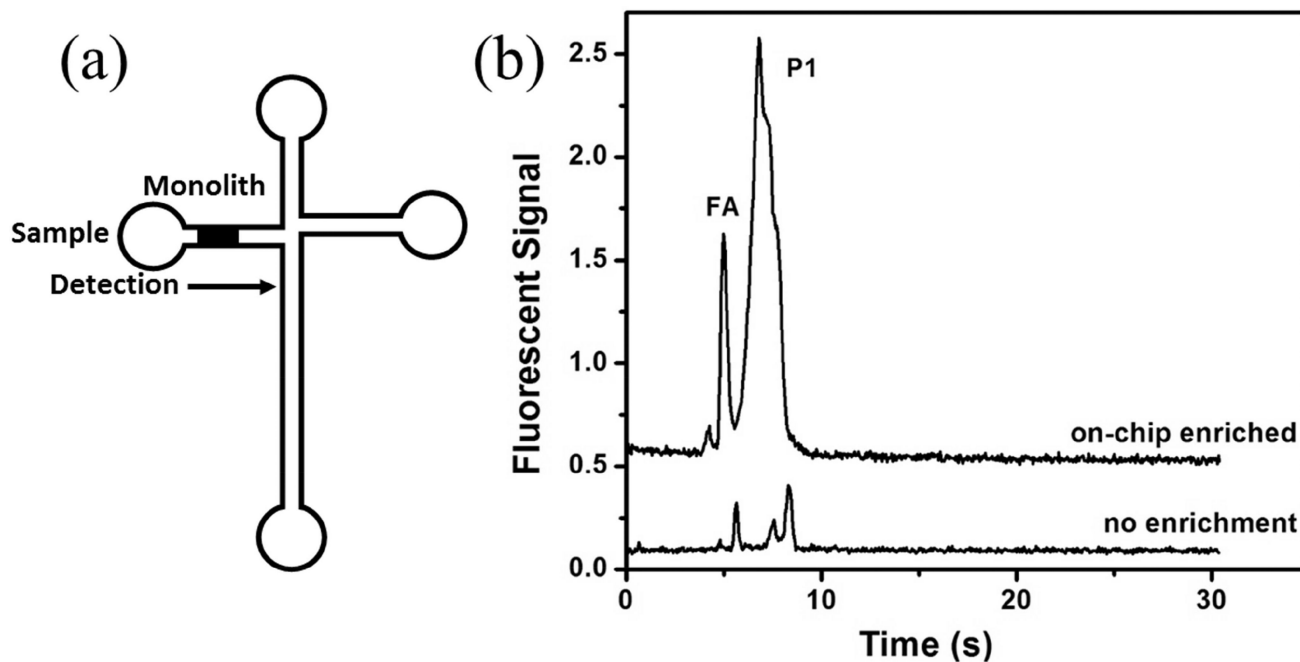
Figure 6.
Microfluidic device for pH-mediated SPE and μCE of preterm birth biomarkers. (a) Integrated device layout. (b) Electropherogram of an on-chip preconcentrated preterm birth biomarker peptide (P1) and a model peptide (FA) compared to μCE without preconcentration on a monolith. Adapted with permission from Sonker *et al.* Electrophoresis, DOI: 10.1002/elps.201700054 (2017).
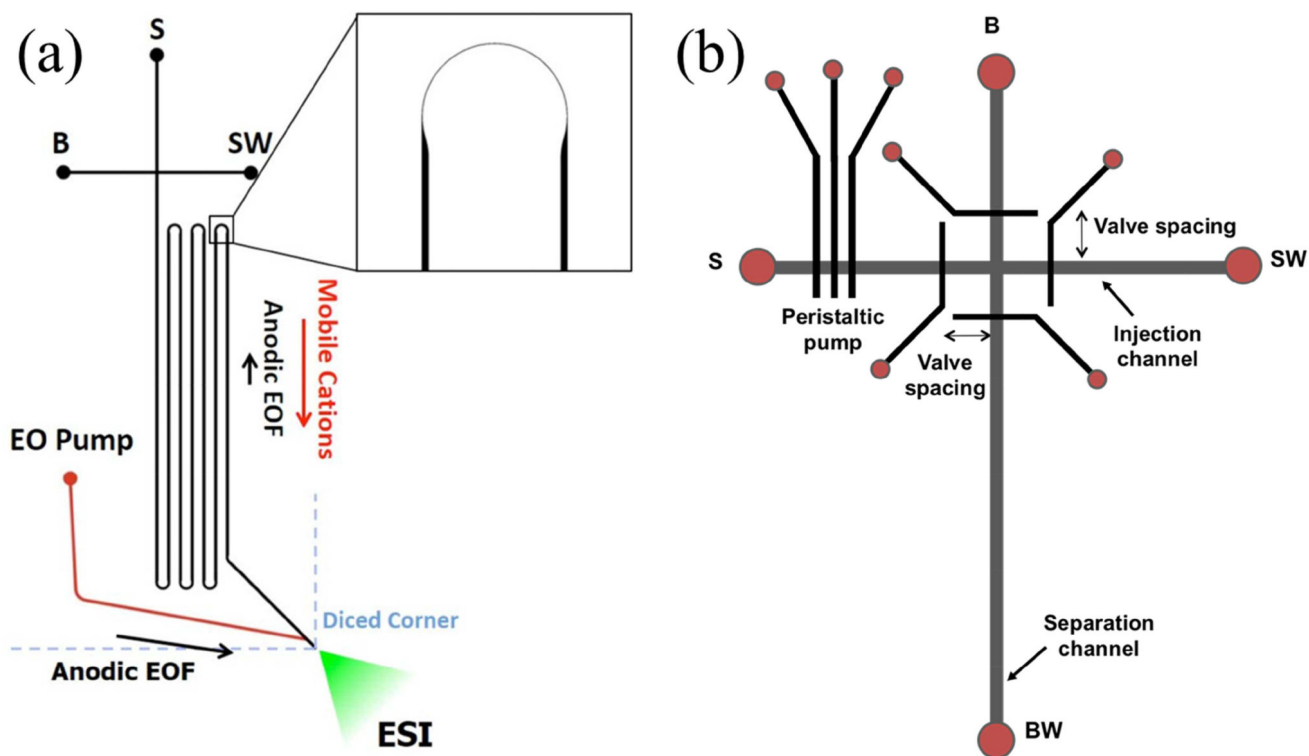
Figure 7.
Innovations in μCE device designs. (a) A μCE-electrospray ionization device. Adapted with permission from Redman *et al.* Anal. Chem. 87 (2015) 2264–2272. (b) A pressure-actuated device for μCE of preterm birth biomarkers. Adapted with permission from Sahore *et al.* Anal. Bioanal. Chem., 408 (2016) 599–607.

Table I

Microfluidic devices for affinity-based analysis. Additional abbreviations used: Carcinoembryonic antigen (CEA), recombinant avian influenza A virus (rH7N9), cancer antigen-125 (CA-125), cluster of differentiation 24 (CD24).

Affinity method	Biomarkers	Disease/condition	Immobilization	Detection	Concentration	Reference
	EpCAM	Epithelial cancer	Channel surface	Fluorescence	2–2000 pg/mL	18
	TNFα	Inflammation	Channel surface	Electrochemical	4–50 ng/mL	19
	ErbB2	Breast cancer	Covalent to TiO_2 nanofibers	Electrochemical	1 fM – 0.1 μM	20
	CEA, rH7N9	Influenza A	in-solution and in-gel	Colorimetric	1–100 pg/mL	22
	Influenza virus epitope	Influenza	in-solution	Colorimetric	1–100 μg/mL	23
	Bcl-2	Apoptosis	Covalent to tin oxide	Mass spectrometry	140 nM	24
Antibody	Gastric cancer biomarker panel	Gastric cancer	Channel surface	Electrochemical	pg/mL–ng/mL	25
	E. coli	E. coli infection	Channel surface	Fluorescence	10^5–10^7 cfu/mL	28
	PGA	Anthrax	Magnetic beads	SERS	100 pg/mL-100 μg/mL	29
	Apolipoprotein A1	Bladder cancer	Magnetic beads	Semiconductor based ion-sensitivity	12.5–1000 ng/mL	30
	CA-125, EpCAM, CD24	Ovarian cancer	Magnetic beads	Fluorescence	$7.5×10^5 – 1×10^7$ particles/mL	31
	Aβ peptides	Alzheimer's disease	Magnetic beads	Fluorescence	25–100 ng	32
	E. coli	Pathogens	Nanoparticle clusters	UV-Vis	100–10^5 cfu/mL	33
	PSA	Prostate cancer	Channel surface	Chemiluminescence	3–50 ng/mL	36
Aptamer	Hemoglobin	Diabetes	Magnetic beads	Fluorescence	0.7–14.8 g/dL	37
	VEGF	Cervical cancer	Channel surface	UV-Vis, Fluorescence	2–40 ng/mL	38
	Creatine kinase	Cardiac damage	Covalent to gold electrode	Electrochemical	10 pg/mL-100 ng/mL	39

Table II

On-chip sample preconcentration.

Mechanism	Medium	Device material	Analytes	Enrichment	References
Electrokinetic	Liquid phase	PDMS	DNA	500	41
		PDMS	Angiotensin	450	42
	Nanoporous membrane	Paper	Bovine serum albumin	800	43
		PDMS	Lipid vesicles	160	44
	Size selective membrane	PDMS	Albumin	500	45
SPE		PDMS-COC	Ferritin	100	46
	Porous polymer monolith	COC	Preterm birth peptide biomarker Ferritin	50	47

Table III

Molecular separation methods for biomarker analysis.

Technique	Analytes	Device material	Concentration	Reference
Gel electrophoresis	Bovine serum albumin, ovalbumin, trypsin inhibitor, parvalbumin	PDMS-glass	0.05 mg/mL	51
	DNA fragments	PMMA	10–20 ng/μL	52
	mAbs	Glass	0.5–1 mg/mL	53
	D-amino acids	Glass	0.3–0.5 mM	54
Electrophoresis	TK1	PMMA	2–25 μg/mL	55
	Ferritin, preterm birth peptide biomarker	PDMS-COC	100–500 nM	56
	Myoglobin, carbonic anhydrase, catalase	Glass	0.1–0.3 μg/μL	57