Microfluidic Sample Preparation for Medical Diagnostics

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Abstract

Fast and reliable diagnoses are invaluable in clinical care. Samples (e.g., blood, urine, and saliva) are collected and analyzed for various biomarkers to quickly and sensitively assess disease progression, monitor response to treatment, and determine a patient's prognosis. Processing conventional samples entails many manual time-consuming steps. Consequently, clinical specimens must be processed by skilled technicians before antigens or nucleic acids are detected, and these are often present at dilute concentrations. Recently, several automated microchip technologies have been developed that potentially offer many advantages over traditional bench-top extraction methods. The smaller length scales and more refined transport mechanisms that characterize these microfluidic devices enable faster and more efficient biomarker enrichment and extraction. Additionally, they can be designed to perform multiple tests or experimental steps on one integrated, automated platform. This review explores the current research on microfluidic methods of sample preparation that are designed to aid diagnosis, and covers a broad spectrum of extraction techniques and designs for various types of samples and analytes.

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1. INTRODUCTION

Diagnostic assays represent an integral step in the treatment and management of a broad spectrum of pathologies. Therefore, a principal aim of contemporary medical science is to refine and decentralize diagnosis by improving the rapidity, fluidity, and sensitivity of the sample-to-result process. Recent years have seen the emergence of micrototal analysis systems, which offer portability, integration, and high-throughput processing, while concurrently lowering reagent use, total cost, and risks of mishandling. Although many of these microchip devices are still in the embryonic stage of development, they hold potential as future point-of-care technologies that may be able to provide quick, reliable, and easy-to-use diagnostic tests at the patient's bedside in virtually any setting.

However, a major obstruction to the development of these technologies is sample preparation—that is, the process of reducing the complexity of a patient's sample by removing or nullifying interferents and extracting target analytes. This foremost step is essential for sensitive detection and successful downstream processing, and presents unique challenges in its translation from conventional to microscale methods. The complexity of the preparation process depends on the sample type [for example, whole blood (WB), serum or saliva], the type of analysis being done, and the analyte being quantitated. A sample may pose difficulties if particles are too large to be transported by micron-sized channels; therefore, whole blood and stool are the most challenging samples to process. WB has an enormous diversity of constituents, including cells that can be as large as 20 μm. In the absence of anticoagulants, WB also can coagulate quickly and block the channels in a microfluidic device. Diluting high-cellularity samples with buffer may alleviate

aggregation problems but it presents another complication by increasing the processing volume and lowering sensitivity due to a decrease in the effective concentrations of analytes. Another concern in microfluidic analysis is nonspecific adsorption of sample components on the walls of the device. Given the high ratios of surface area to volume in microfluidic devices, this may pose a more significant challenge than it does for macroscale techniques. The interference caused by nonspecific coatings can include high nonspecific signals, loss of analytes, changes in flow behavior for separation-based analyses, and in extreme cases, clogging of the channels. This issue becomes more pronounced for low-abundance species. Preliminary treatment of the channel surface has been widely investigated as a means of overcoming this problem, using treatment with bovine serum albumin (1), polyethylene glycol, or various silanes (2).

Despite these technical challenges, microfluidic techniques offer unique advantages over conventional bench-top methods by integrating sample preparation with analysis. By using microscale channels and tubes, microfluidic devices can minimize dead volume and sample waste. To further reduce processing volume, external tubing or pumps can be eliminated. Passive fluid actuation without the use of active pumps has been reported, including capillary driven flow (3–5) and vacuum-driven flow (6, 7), which is ideal for resource-constrained environments at remote sites. Although multiple steps are typically required to prepare samples for biochemical assays, microfluidic devices can automate these steps in a single device, thereby minimizing manual sample handling and preserving sample integrity, as well as improving diagnostic accuracy and reproducibility. However, it is not a trivial task to automate sample preparation and integrate it with detection on a chip, since there is no generic solution for preparing samples for a wide variety of sample matrices. In this review, we discuss a number of sample-preparation techniques that have been reported recently for diagnostic applications, selecting, when possible, those that have demonstrated successful integration with on-chip detection.

2. WHOLE BLOOD FRACTIONATION

In point-of-care and home-use diagnostic applications, WB is preferred over serum because it can be collected easily in small volumes (e.g., from a pin-prick). Nevertheless, it is a complex fluid containing not only disease biomarkers, including proteins, nucleic acids, and metabolites, but also a large number of cells—approximately one billion erythrocytes per milliliter. Thus, despite the ease of collecting a small volume of WB, plasma or serum samples are preferred to avoid clogging, interference [e.g., during polymerase chain reaction (PCR)], and the high level of nonspecific (background) fluorescence associated with WB. Thus, preparing plasma or serum is a requirement, and often the first step, in current medical diagnostics. Microfluidic platforms present many advantages for extracting blood plasma, owing to their microscale processing; these advantages include fast turnaround times, automated operation, reduced sample volumes, reduced cost, and portability. In addition, the microscale enables distinct physical phenomena to become apparent in streamlines and near boundaries, greatly facilitating the separation of smaller molecules, such as proteins and DNA, from larger species, such as cells.

There are numerous technical challenges that need to be overcome when preparing WB samples using microfluidic platforms (8), the first being the high cellularity of WB. Diluting WB with a stabilizing buffer reduces the concentration of cells; however, it also reduces the concentration of target analytes, leading to reduced sensitivity. Another common challenge in WB processing comes from the microfluidic environments: the channel flows typically produce high shear stress or are under high mechanical forces. Red blood cells, although very deformable, are thus exposed to severe conditions where hemolysis may occur. Ruptured red blood cells expel intercellular contents that can hamper subsequent detection or assays. During the past

decade, there has been a growing number of publications on microfluidic plasma extraction (or on the use of similar microscale formats). A thorough review of various plasma-separation techniques is available in Reference 9. In Sections 2.1–2.8, we survey the most recent and promising technologies.

2.1. Centrifugal Microfluidic Separation

Centrifugal microfluidic platforms use a rotating disc to exert centrifugal forces to move fluid, obviating the need for connection to external pumps or power supplies (10). Although diverse applications have been demonstrated, one of the most popular has been the separation of plasma from WB samples (11, 12), and the subsequent diagnostic analyses of the collected plasma (13). The centrifugal microfluidic format is inherently well suited for extracting blood plasma, operating on the same principle as conventional bench-top centrifugation, where the heavy cellular content is forced toward the external edge of the disc, and the lighter plasma is drawn by valve operation to the inner position. Recently, a fully integrated platform has been reported that performs enzyme-linked immunosorbent assays (ELISAs) to detect biomarkers of the hepatitis B virus in WB (13). This system utilized centrifugal forces to extract plasma from WB, and the plasma was transferred by automated valves to the reaction chamber, which contained polystyrene beads functionalized to capture antigens and antibodies. Finally, the incubated fluid was moved to a detection chamber for absorbance measurements (**Figure 1**).

This method of centrifugal microfluidic separation requires numerous valves to control the transfer of fluid between multiple locations. Thus, the performance relies heavily on proper actuation of the valves and optimal rotation speed. Additionally, parallelization is possible but allowed only within the size of a single disc, which is often insufficient for desired throughput. Despite these limitations, the technology is based on solid working principles, and it is easy to fabricate and integrate with diagnostic units for sample-to-answer systems (14).

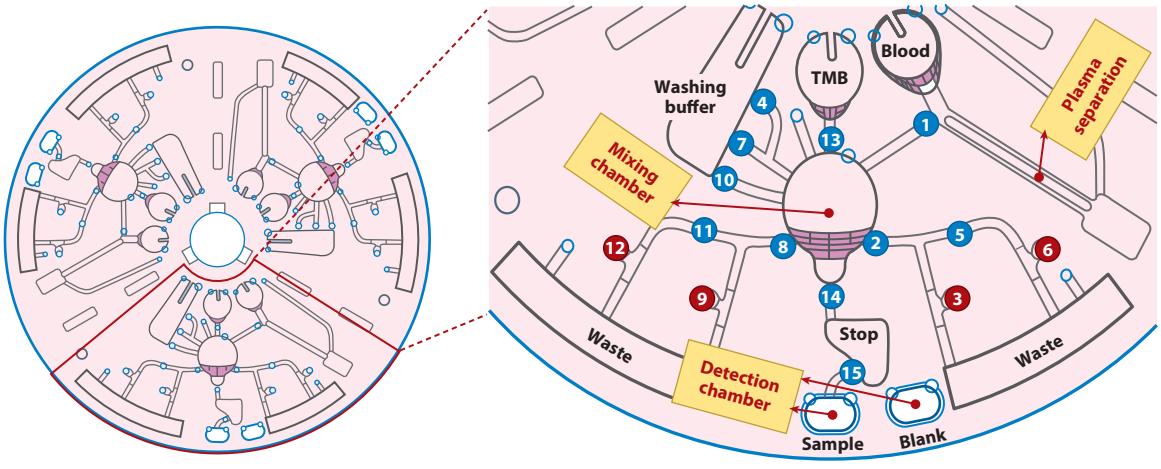

Figure 1

The layout of an integrated centrifugal microfluidic system with three independent platforms for whole blood preparation and enzyme-linked immunosorbent assay (ELISA) operation. The inset shows the function of each part and the order of valve operation for automation (valves are laser-irradiated ferrowax microvalves). Red valves and blue valves are, respectively, normally open and normally closed. The substrate for protein detection is tetramethylbenzidine (TMB). Figure adapted from Reference 13 with permission.

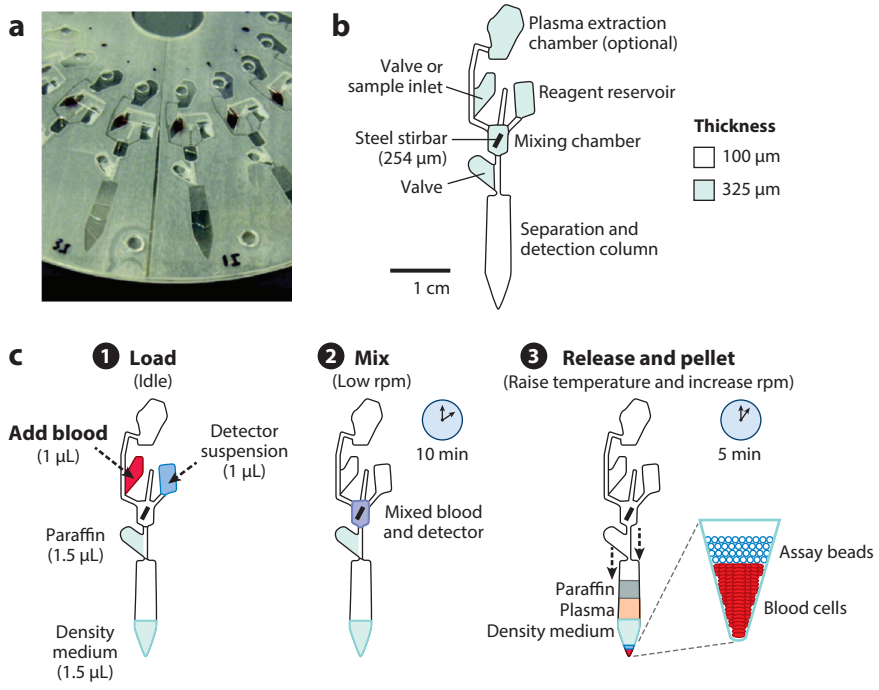

Figure 2

Sample-to-answer blood immunoassay disk using centrifugal sedimentation. (*a*) Bright-field image of part of the disk with multiple devices. (*b*) Microfluidic channel layout. (*c*) Three-step blood-sample assay protocol: (❶) The blood sample and detector suspension (that is, beads and detection antibodies) are loaded. (❷) The disk spins for 10 min to mix the reagents. (❸) The paraffin valve opens after the temperature has been slightly raised, and the reaction products differentially sediment to the separation column. The assay beads and blood cells are pelleted at the periphery of the disk following a 5-min spin at high rpm. Figure adapted from Reference 15 with permission.

2.2. Centrifugation Using Density Media

An innovative modification in centrifugal microfluidics has been the implementation of bead-based immunoassays using density-media centrifugation (15). This allows bodily fluids, such as WB and saliva, to be analyzed directly because cells are separated by centrifugation from the analytes, which are captured on antibody-bearing beads. In this technique, WB is mixed with a suspension of antibody-bearing beads and detection antibodies; this is then layered on top of a medium that is denser than the blood sample mixed with beads but less dense than the bead-antibody-analyte complexes and blood cells. During incubation, the beads functionalized with primary antibodies capture analyte molecules in the sample and further extend to labeled secondary antibodies. The beads are then sedimented by centrifugation and become concentrated at the outermost part of the chamber, where they are optically analyzed; cells, proteins, nucleic acids, and other constituents of WB remain on top of the density medium (**Figure 2**).

2.3. Sedimentation

Sedimentation-aided plasma separation utilizes differences in cell-sedimentation rates caused by differences in the densities of various blood components to separate components into channels.

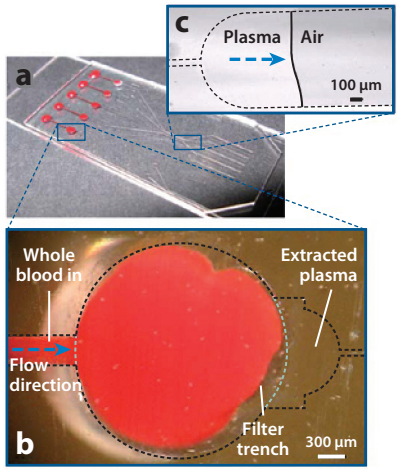

Figure 3

Sedimentation-aided plasma extraction demonstrated with a stand-alone self-powered integrated microfluidic blood-analysis system (known as SIMBAS). (*a*) Whole blood flows are driven by a vacuum in degassed suction chambers. (*b*) The use of a filter trench and gravity-driven blood-cell sedimentation enables blood-cell-free plasma to be extracted. (*c*) Plasma fills the suction chambers. Figure adapted from Reference 7 with permission.

Erythrocytes and leukocytes are denser than the surrounding plasma, and, thus, settle at the bottom of the channel due to gravity. Sedimentation on the macroscale requires hours, yet microfluidic devices greatly reduce sedimentation times to a few minutes or less. Once the sedimentation of cells is complete, the top portion of plasma is drawn into a separate channel. Microfluidic sedimentation for plasma extraction has been successfully demonstrated using various channel configurations (7, 16–20). Channels of varying depths are commonly used to collect cellular sediments in a lower-branch channel, which is deeper, and obtain cell-free plasma in upper-branch channels, which are shallower. Sedimentation may also reduce clogging or hemolysis because the cells do not approach the upper channel or the filter that is dedicated to plasma flow. Blood dilution is not usually required to prevent clogging or aggregation when plasma is separated from WB using sedimentation. Sedimentation techniques can be coupled with other separation schemes, such as filtration in a single unit, to increase throughput (16).

Dimov et al. have developed an integrated blood-analysis system that uses degas-driven flows (7). The device extracts plasma from five distinct 5-µL droplets of WB deposited into identical parallel channels, and subsequently performs simultaneous biomarker detection (**Figure 3**). For plasma separation, an 80-µm-deep trench is used for sedimentation; the upper extraction channel has a depth of 10 µm; 100% separation efficiency was achieved with this device. Nevertheless, sedimentation-aided separation systems have relatively low throughputs because slow flow rates (<50 µL/h) are required to maintain the high purity of plasma. This limited throughput may hinder the use of the sedimentation approach for large volumes of blood.

2.4. Plug-Assisted Separation

Recently, a novel plasma-separation method has been proposed that uses a long two-phase plug flowing through tubing (21). For demonstration, 1 µL of blood was aspirated by capillary action into an oil-filled tube and sandwiched by two plugs of oil. With a low flow rate (0.5 µL/min), cells accumulated at the rear of the moving plug. Plasma was successfully separated from diluted WB

within 120–240 seconds, but higher flow rates led to much weaker separation effects. Interestingly, plug-assisted separation offers a unique opportunity for droplet-based microfluidics. Using the plasma-separation plug as an input to an aqueous phase, the authors were able to produce 86 plasma droplets and 41 blood-cell droplets using conventional T-channel droplet generation. They also demonstrated that droplets containing plasma could be used in simple diagnostics, such as a colorimeter, to measure blood cholesterol levels.

2.5. Hydrodynamic Separation

The contents of WB samples differs greatly in physical properties, such as cellular size and deformability, and density. Thus, specially designed networks of channels or microstructures may produce distinct streamlines for different particles, depending on their physical properties. The flow of blood cells can be directed naturally or forced into the designated channel while the remainder of the WB contents flow elsewhere. This hydrodynamic separation can be used to effectively extract plasma from WB. A wide range of designs have been proposed to extract plasma from blood, including deterministic cell deviation by obstacles (22), Zweifach–Fung bifurcation (23–25), inertial force deviation (1), and centrifugation effects in curved channels or Dean vortices (26–29). Of the various separation methods used, the technique based on the Zweifach–Fung bifurcation law is of particular interest, and has been widely adopted in most of the recent WB-preparation systems.

When the size of cells is comparable to a channel's width, the cells at a bifurcation point tend to migrate into the channel with the highest flow rate, although there is still debate about how to explain this effect (30). Since there are no fluidic obstacles for cell flows, concentrated WB samples with high hematocrit levels can be used without prior dilution, which is a significant advantage over techniques that are prone to clogging. The purity of plasma achieved by separation using Zweifach–Fung bifurcation has been reported in multiple papers to be higher than 99% (23–25). This effect could be further exploited by adding local geometric singularities, such as abrupt channel enlargement (28, 29).

Devices based on hydrodynamic separation are especially suitable for continuous plasma extraction from large-volume samples, since they are based on passive fluidic properties and do not quickly degrade over time. Their designs are simple enough—they do not require any filtering structures or an external force field, which minimizes the complexity of fabrication—making it easy to integrate blood preparation into further diagnostic steps. For example, Heath and coworkers (25) have developed an integrated blood barcode chip consisting of two sequential units: one that separates plasma from WB and one that acts as an in-situ multiparameter protein assay to rapidly measure a panel of protein biomarkers (**Figure 4**). The ability to seamlessly integrate plasma separation with on-chip detection, along with a multitude of other design possibilities made available through easily fabricated microfluidic geometries, leads us to conclude that hydrodynamic separation holds the most promise as a microfluidic blood-fractionation technique.

2.6. Microfiltration

Microfiltration is another example of a miniaturized counterpart of a conventional macroscale blood-fractionation method; microfiltration is simply based on differences in particle size and the selective permeability of WB constituents through pores, slits, or different phases of immiscible media. Size-discriminating sieves, membranes, pores, and packed beads have been widely used to create flow-through filters to separate blood from plasma (31–38). Because unfiltered blood cells get stacked on one side of a filter, there is the possibility that cells may quickly aggregate and obstruct the filter or may lyse when put under high stress from the filter. In addition, some

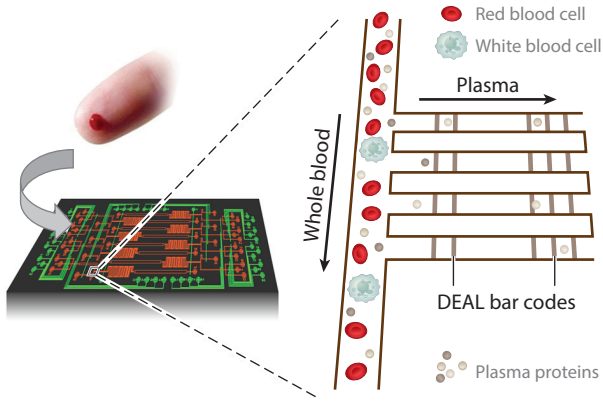

Figure 4

An integrated blood barcode chip that can be used for a protein assay. Plasma is separated from blood collected from a finger-prick by harnessing the Zweifach–Fung effect. Proteins in plasma are skimmed and analyzed in situ within the antibody barcode arrays. Figure adapted from Reference 25 with permission. Abbreviation: DEAL, DNA-encoded antibody library.

erythrocytes may penetrate the filter due to their high deformability. To avoid hemolysis and cell leakage, blood samples should be diluted. The purity of plasma separated by flow-through microfiltration is typically high; yet its yield is significantly lower than that of other methods, mainly because the filters clog quickly. Fluid flows coaxially through the porous membrane in coflow filtration, but crossflow filtration utilizes tangential flows between the fluid and the membrane to wash away aggregated particles. However, the rate of plasma penetration will be significantly decreased due to the reduced pressure between crossflows over the filter resulting in limited plasma separation. Thus, although these techniques are in widespread use, it should be noted that they are efficient only for a certain duration of time and volume as defined by filter obstruction; thus, they require low flow rates, and samples often require dilution. Instead of using hydrodynamic or electrokinetic operation for fluid actuation, Chung et al. (39) used a permanent magnet to pass WB through a filter. Their technique utilized the strong magnetic dipole force occurring between two permanent magnets sandwiching WB drops to pull the fluid through multiple membranes. They successfully extracted 7 mL of plasma from 50 mL of whole undiluted blood in less than 1 minute.

2.7. Microfluidic Paper-Based Separation

Paper is commonly used as a membrane for filtration, but this type of separation fundamentally differs from the microfiltration method because it uses microfluidic paper-based analytical devices (μPAD), in which paper may fulfill dual purposes, acting as both a membrane and a support at the same time. Yang et al. (40) recently used chromatography paper as a substrate to extract plasma from agglutinated red blood cells, and as a support to perform glucose metering (**Figure 5**). In this design, the chromatography paper allowed the plasma to slowly reach diagnostic sites, while preventing red blood cells from moving with the plasma.

Another example of separating blood from plasma was performed without the agglutination of red blood cells; instead, patterned Whatman number 1 filter paper successfully separated plasma from WB (41). The efficiency of plasma separation was confirmed by using a bromocresol green colorimetric assay. For a small volume of analyte, such as from a finger-prick, paper can be

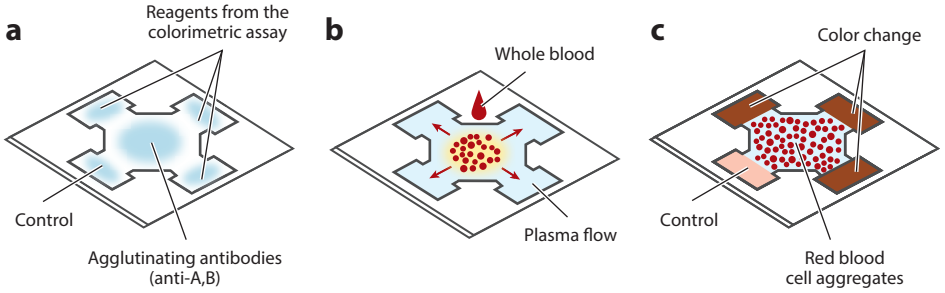

Figure 5

A microfluidic paper-based analytical device (known as μPAD) that provides integrated separation of blood from plasma and consists of a plasma-separation zone in the center and four test readout zones on the periphery. (*a*) The μPAD is spotted with agglutinating antibodies and the reagents from the colorimetric assay, which are left to dry. (*b*) For a diagnostic test, a drop-size sample of whole blood is added to the plasma-separation zone. (*c*) Agglutinated red blood cells remain in the central zone, while separated plasma wicks into the test zones and reacts with the reagents. Figure adapted from Reference 40 with permission.

successfully used to separate plasma and perform a simple diagnostic test with a fast turnaround time. However, paper-based separation is not suitable for large-volume samples or when long separation time is required, since blood rapidly clots on paper. Additionally, most paper-based sample-preparation chips include only simple analysis functions, since extracting prepared samples from the device for further analysis is challenging, which is a major drawback of this technique.

2.8. Acoustic Wave-Driven Separation

Acoustic forces generated by ultrasonic standing waves have been used for plasma separation (42, 43). The pressure wave generates a radiation force on the particles, causing them to rapidly migrate toward the pressure nodes in the standing-wave field, an effect known as acoustophoresis (44). By providing standing waves from embedded interdigitized transducers on the sides of a channel with a wavelength that is twice the width of the channel, a pressure node, where acoustic forces will cancel out one another, can be formed in the center of the channel. Blood cells will then rapidly be focused near the center of the channel while cell-free plasma is continuously drawn from the sides of the channel to separate outlets. Plasma collected using acoustophoresis has been used in microarray detection of prostate-specific antigen (42). An investigation into the effects of acoustophoresis on cell viability or functions has shown that the acoustic waves have no adverse impact on the survival or function of microglia, leukocytes, or tumor cells (45). Because the system can utilize relatively strong forces, higher flow rates can be used for separation, enabling large volumes to be analyzed. However, the requirement for an additional component makes it difficult to build parallelized systems to enhance throughput or integrate with detection units.

3. EXTRACTION FROM CELL LYSATE

Often, as in the case of nucleic acid testing, target biomarkers must first be released from shielding membranes or viral protein capsids. Once large assay-inhibiting particulates have been removed and target analytes have been released into solution via cell or virion lysis, the chemical species of interest must be enriched and extracted from the lysate. This vital step serves to eliminate any inhibitors to downstream processes, to protect the analyte from enzymatic degradation and

ensure stability, as well as to concentrate the analyte to improve the limit of detection and reduce background noise. Many methods of microfluidic cell lysis exist, and these utilize mechanical, thermal, or electrical energy to disrupt cell membranes (46), although the technique most commonly implemented in emerging micrototal analysis systems devices is chemical lysis. This is often carried out using chaotropic agents, such as guanidinium thiocyanate, or, for bacterial lysis, enzymatic degradation by muramidases (47) A more complete review of microfluidic chemical lysis techniques is offered by Nan et al. (48).

Conventional nucleic acid extraction is often achieved through phenol-chloroform extraction, ethanol precipitation, or silica spin-column adsorption. Although these have proven to be effective methods for DNA purification, they require repeated centrifugation at high speeds, repeated manual washing, and, in the case of the first technique, handling of toxic reagents. These multistep extraction protocols remain laborious, nuanced, and dependent on large pieces of external equipment that may not be readily available in areas where resources are limited but the need for clinical diagnoses is high. Thus, extraction from cell lysate could greatly benefit from the streamlined operation and automated capabilities of closed microfluidic systems.

3.1. Micro-Solid-Phase Extraction

Micro-solid-phase extraction (μSPE) uses a microchip for nucleic acid extraction; this represents the miniaturization of common techniques used in commercial spin-column or bead-based products. Like its macroscale counterpart, μSPE utilizes the affinity of nucleic acid for silica surfaces in a chaotropic salt solution (49). PCR inhibitors are then washed away in isopropanol, and the captured nucleic acid is eluted in a polar solvent. Extraction devices have emerged that use packed silica beads (50), silicon micropillars (51), monolithic sol-gel (52), and chitosan-coated silica beads (53), among many other technologies. Recently, Lounsbury et al. (54) developed an integrated sample-to-PCR product microdevice that uses poly(methyl methacrylate) rather than silica, thus circumventing hazardous hydrofluoric acid etching, and making these single-use chips more economically feasible. Processing both dried buccal swabs and liquid whole blood, they were able to achieve a fivefold reduction in processing time.

3.2. Isotachophoresis

Microchip-based isotachophoresis (ITP) has been employed for sample pretreatment and electrophoretic separation (55). ITP uses a heterogeneous buffer system consisting of high-mobility ions in the leading electrolyte and low-mobility ions in the terminating electrolyte; the analytes of interest have intermediate ionic mobility. An applied electric potential separates the ionic species based on their electrophoretic mobility, thus focusing the analytes at the leading and terminating electrolyte interface (**Figure 6**) (56).The efficacy and versatility of this technique has been demonstrated in the purification of genomic DNA from WB (57), the isolation of small RNA from cell lysate (58), the extraction of DNA from malaria-infected erythrocytes (59), as well as the purification of bacterial RNA from WB lysate (60). Additionally, nucleic acid detection has been implemented with ITP using loop-mediated isothermal amplification (a process known as NAIL) on an integrated chip to detect pathogenic *Escherichia coli* O157:H7 cells from whole milk (61). With limited user interaction and a result readout available over a mobile phone, this technique shows that ITP is a promising technology for nucleic acid enrichment and separation in point-of-care applications.

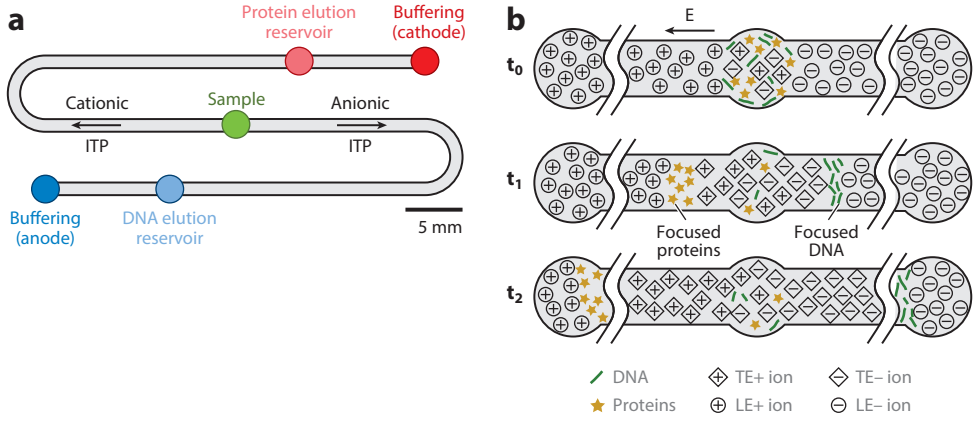

Figure 6

(*a*) Device design for simultaneous purification of nucleic acids and proteins from serum using simultaneous cationic and anionic isotachophoresis (ITP) processes. The channels are 1 mm wide and 100 μm deep, with a volume of 3.8 μL for each branch. Each reservoir holds approximately 8 μL of liquid. (*b*) Simultaneous extraction process in a schematic of the system (buffering reservoirs not shown). The serum sample is mixed with buffer and pipetted directly into the sample reservoir. An electric field is applied, and the DNA and proteins are extracted into each separation channel and focused at anionic and cationic ITP interfaces, respectively. Purified DNA and proteins eventually reach each elution reservoir and are collected for off-chip polymerase chain reaction (PCR) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Figure adapted from Reference 55 with permission. Abbreviations: LE, leading electrolyte; TE, terminating electrolyte.

3.3. Immiscible Phase Filtration and Transport

Another microfluidic method for biomolecular extraction that has been studied in recent years is immiscible phase filtration, which typically utilizes paramagnetic particles (PMPs) as solid-phase transports to shuttle nucleic acids or protein through oil–water or air–water interfaces. These devices do not require pumps or external hardware, except for a permanent magnet, and, therefore, may hold potential for purification technologies geared toward low-resource settings (62, 63). Sur et al. (64) developed a nucleic acid extraction cartridge with an automated magnetic mixer and mover to pull PMPs with adsorbed nucleic acid through liquid wax and into an elution chamber. Berry et al. (65) based their immiscible filtration assisted by surface tension (IFAST) device on a similar principle, but added high-throughput capability to purify multiple messenger RNA samples in parallel. They also demonstrated the versatility of IFAST by passing prostate specific antigen captured on antibody-functionalized PMPs through primary and secondary labeling buffers, thus performing extraction and labeling in one streamlined step (**Figure 7**) (66). Filtration can also be accomplished using air, as den Dulk et al. (67) have proved with their magneto-capillary valve. Their device consists of patterned hydrophilic and hydrophobic surfaces that effectively create air valves through which PMPs loaded with analyte traverse.

Manipulating the droplets using electrowetting (which is called digital microfluidics or DMF) is another technique that utilizes transport through an immiscible phase to process and purify a sample, in addition to many other applications. A DMF device consists of a chip with electrodes attached at the bottom in an array with a common electrode on top. A droplet sandwiched between the top and bottom electrodes is moved between the electrodes on the bottom to carry out steps

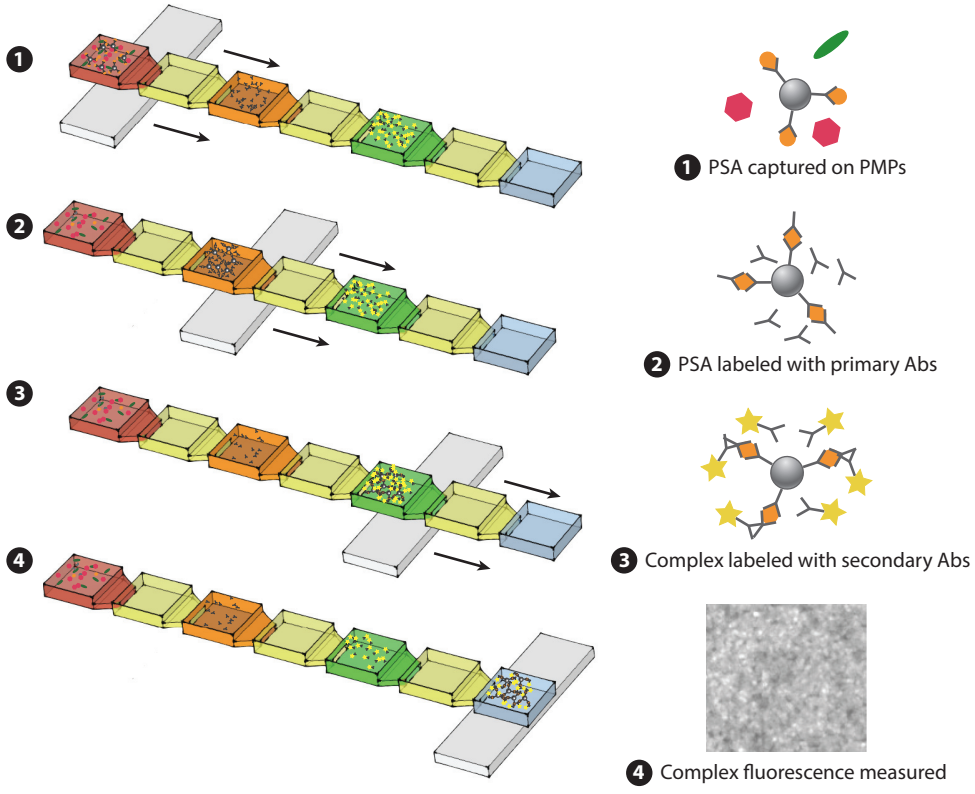

Figure 7

Schematic of operation of the IFAST (immiscible filtration assisted by surface tension) device for immunoassay. (❶) Paramagnetic particles (PMPs) are mixed with prostate-specific antigen (PSA) in the sample well. A permanent magnet then pulls the PMPs through oil into chambers containing primary (❷) and secondary (❸) labeling antibodies before finally pulling the labeled PSA into the readout buffer (❹). Figure adapted from Reference 66 with permission. Abbreviation: Abs, antibodies.

such as mixing, merging, splitting, and incubation. The droplets are surrounded by an immiscible fluid to prevent unwanted merging and to minimize evaporation.

Since Fair and coworkers (68) first reported successfully handling various samples of bodily fluid in DMF devices, the devices have been used for various biochemical assays for diagnostic purposes (69, 70). The extraction of small components from WB, such as nucleic acids (71, 72) and specific hormones (73), using DMF also has been reported. More importantly, many recently developed DMF systems are integrated platforms on a single device that can prepare real physiological samples, including tissue and blood, and then detect or analyze the sample (73–76). An integrated DMF device for an estrogen assay (**Figure 8**) has been developed to process breast tissue homogenate, WB, and serum for the quantitation of steroid hormones (73). The amount of sample required for this method was at least 1,000 times smaller than that required for similar bench-top techniques, and there was a 20- to 30-fold reduction in turnaround time. In addition, Wheeler and coworkers (74) have reported a DMF method that can be used for in-line extraction and analysis of dried blood spots by mass spectrometry. They successfully extracted biomarkers of disorders of amino-acid metabolism from dried blood spots, and correctly identified patients

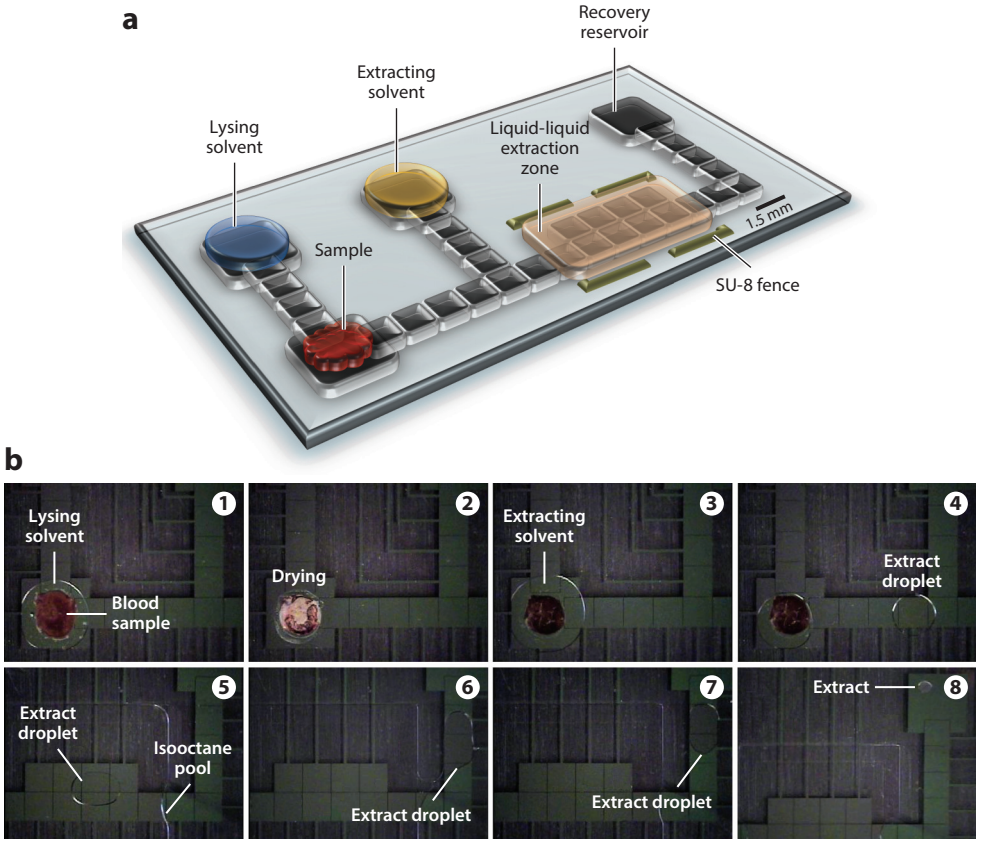

Figure 8

Digital microfluidic device for estrogen assays from blood, serum, and breast tissue. (*a*) Schematic of the device. (*b*) Sequential micrographs show the key steps in extracting estrogen from a 1-μL droplet of human blood. Figure adapted from Reference 73 with permission.

suffering from metabolic disorders using significantly reduced sample volumes and with shorter times for analysis.

4. SAMPLE PREPARATION FOR URINE ANALYSIS

The clinical analysis of urine is one of the most important noninvasive inspections used for medical diagnosis. In addition to its frequent use for tests for drug abuse and toxin exposure, urine can provide crucial diagnostic information about kidney diseases, metabolic disorders, or other pathologies (77). Under normal conditions, urine is acellular, and may have a small amount of proteins. However, when severe pathology is present, urine may contain blood cells, casts of cells, and crystals, as well as proteins such as albumin. Unlike the preparation of blood samples, preparing urine samples for microfluidic assays is straightforward because it does not require pretreatments to prevent severe channel clogging. Nonetheless, since urine is also a complex biofluid that contains diverse entities, it is usually necessary to prepare samples for purification or enrichment, or both, prior to analyzing specific targets urine. Harvesting cells that may have pathological implications

is important, but it requires a large volume of urine and lengthy testing due to the extremely low number of cells. Urine may also contain debris from damaged red or white blood cells, further complicating attempts to isolate specific cells. Moreover, the high urea concentration present in urine degrades cells rapidly (78).

4.1. Small-Molecule Enrichment and Detection

Recently, several attempts have been made to prepare urine samples in a microfluidic format to extract or selectively detect small molecules. For example, the use of a microchip-based liquid–liquid extraction technique to analyze whether amphetamine-type stimulants are present in urine by gas chromatography has been reported (79). Pretreated alkalized urine samples were pumped into a deep microchannel and an organic solvent was pumped into a shallow microchannel. The extracted sample in the shallow microchannel was collected for subsequent analysis by gas chromatography. In this report, the microchannels were partially modified to stabilize the interface between the organic solvent and urine.

SPE is also popular for sample purification and enrichment, and can be easily carried out in a microchip format. For instance, parallel enrichment of ephedrine in urine using an SPE array microchip has been demonstrated (80). Fused-silica capillaries were used as extraction channels; these contained a UV-polymerized porous monolith and were packed with ODS beads for efficient extraction. Fluorescein isothiocyanate-labeled ephedrine was successfully quantified using capillary electrophoresis for separation and laser-induced fluorescence detection. In another report, Craighead and coworkers (81) used an integrated SPE microchip coupled with mass spectrometry. They used an alkyl acrylate-based monolith as the solid phase to demonstrate the effectiveness of preconcentrating and the ability to detect imipramine in human urine samples.

4.2. Protein Enrichment and Detection

Albumin is the primary protein excreted in urine, but other proteins may also be found in low abundance. The overall protein concentration in urine is clinically relevant to several diseases (82), and the level of the most abundant protein, urinary albumin, thus serves as a good diagnostic indicator of such diseases. Recently, the use of paper-based microfluidic devices employing a simple colorimetric assay have been described for analyzing urine samples (82–84). Whitesides and coworkers (83) first used microfluidic paper-based devices to measure the levels of protein as well as glucose directly from urine. They adapted a simple colorimetric protein assay based on the nonspecific binding of tetrabromophenol blue to proteins. In a later study, they suggested using a different detection scheme based on transmittance colorimetry and the thickness of the paper (82). An assay-ready paper was dipped in the urine sample and inserted into a plastic sleeve containing vegetable oil. A handheld detector then showed the results of the urinalysis and quantified the protein in the sample. Similarly, Hashemi et al. (84) have proposed a wax-printed paper device based on the principles of origami; their device could be used to simultaneously conduct multiple colorimetric assays and accommodate one or more samples, including urine, blood, and saliva. As a proof-of-concept demonstration, a urinalysis of protein and glucose was conducted by colorimetric assay. Although qualitative, the assays successfully confirmed the presence of protein and glucose from human urine samples and had diagnostic relevance.

5. SAMPLE PREPARATION FOR SALIVA ANALYSIS

Saliva is a preferred biofluid sample for monitoring patients at home because collecting it is noninvasive. Nonetheless, processing saliva samples for analysis is extremely difficult, mainly because

of the presence of oral particulate matter and adhesive mucins (85), which are responsible for the high viscosity and glycoprotein content. Untreated saliva that contains these interferents often fouls bioanalysis equipment, and makes pipetting inaccurate. To successfully analyze saliva, it is necessary to remove these interferents while maintaining the target concentration. Yager and coworkers (86) have proposed a simple microfluidic method to condition saliva samples before analysis. They used partially membrane-filtered saliva containing a reduced amount of mucins as the input for their microfluidic filtration channel to further eliminate mucins and glycoproteins. This microfluidic filter, called an H-filter, uses laminar flows in narrow microchannels for diffusive extraction of impurities. Using their conditioning protocol, they removed 97% of mucins and 92% of total proteins while retaining a significant amount of target analytes, which corresponds to a threefold enrichment of the analyte compared with direct dilution of the filtrate sample.

5.1. Protein Detection

Singh and coworkers (87) have developed a microfluidic system that integrates the pretreatment of a saliva sample (filtering, mixing, incubation, and enrichment) with subsequent electrophoretic immunoassays (**Figure 9**). This integration enabled pretreatment to be automated, gave a short turnaround time, and prevented sample contamination. Using their integrated chip, they measured the concentration of endogenous matrix metalloproteinase-8 in saliva, which has been identified as a major destructive enzyme in periodontal disease (88).

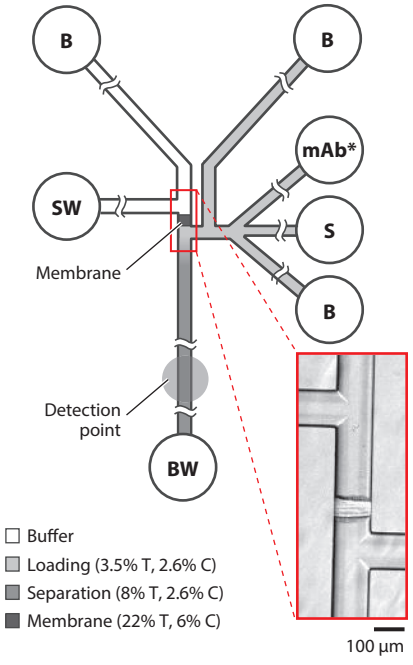

Figure 9

A microchip electrophoretic immunoassay (known as μCEI) device for measuring a putative biomarker of periodontal disease in human saliva. Fluid wells are labeled as follows: S, saliva sample; B, buffer; SW, sample waste; BW, buffer waste; mAb*, fluorescently labeled monoclonal antibody to matrix metalloproteinase-8. The inset shows a bright-field image of the size-exclusion membrane. T refers to total acrylamide crosslinker, and C refers to bisacrylamide crosslinker. Figure adapted from Reference 87 with permission.

Cancer biomarkers have been detected using microfluidic reactors (89). An integrated assay system has been shown to be capable of processing samples, capturing analytes, and detecting biomarkers from samples of serum and whole saliva. Researchers used a microporous array of agarose beads to capture antigen in saliva with quantum-dot-labeled antibodies. Quantum dots were used as detection probes, and this increased the signal by 30 times, which enhanced the detection of multiple cancer biomarkers, leading to a reduction in the detection limit of two orders of magnitude relative to typical ELISAs.

5.2. Small-Molecule Detection

Saliva is the preferred biofluid for assays of drugs taken orally, including methamphetamine. Compared with current laboratory tests for drugs, such as ELISA, gas chromatography, and high-performance liquid chromatography, microfluidic platforms offer a quick cost-effective alternative for detecting in biofluids drugs that may be abused. For example, Meinhart and coworkers (90) have proposed a microfluidic device that could be used to detect methamphetamine in saliva. Their device consists of a flow-focusing channel for transport, salt-induced aggregation of silver nanoparticles, and an interrogation channel where surface-enhanced Raman spectroscopy is performed to detect methamphetamine. They confirmed the presence of methamphetamine in saliva at concentrations as low as 10 nm, which is much less than physiological quantities.

6. CONCLUSIONS

Preparing samples on microfluidic platforms could create low-cost integrated, automated, sensitive, and efficient assays that would be improvements over current diagnostic techniques. Although many advances have been made since the inception of microfluidic diagnostic techniques, with multiple elegant solutions aimed at integration and automation, currently there are a limited number of studies focusing on optimization and mass transport within devices, which could drive better reactor design. Parameters such as sample size, channel geometry, flow rate, and reagent composition are only beginning to be investigated. Additionally, high surface-to-volume ratios in microfluidic reactors modify the adsorption–desorption characteristics of reactants and products, which alter the kinetics. Hence, the challenges of optimizing existing assays to be performed in a microfluidic format are not trivial. Despite the promising successes of microfluidic systems, commercialization of these systems is rare. Their development is a complex and multistep process that requires individual components to be thoroughly investigated and carefully combined to form an integrated platform. In this review we have discussed many promising technologies that advance the field of microfluidic diagnostics toward the ultimate goal of developing publicly available devices. Combining these technologies and using careful parameter optimization could create the next generation of integrated diagnostic assays.

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