Microfluidic systems for drug discovery, pharmaceutical analysis, and diagnostic applications

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Guided reading questions:

1. What is microfluidics? What is the role of microfluidics in drug discovery? How are microfluidics used in pharmaceutical analysis? How can microfluidic devices be used for diagnostic applications?

7.1 Introduction

The ascension of the microfabrication technology at the turn of the century opened several avenues for the biomedical sector. Microscale chips, with micrometre dimension channels, can be used to manipulate fluid flow on micron or submicron scale. The spatial control offered by this technology, known as "microfluidics," has potential applications in handling, processing, and analysis of fluids (Whitesides, 2006). The miniaturized ambit of these devices requires lower sample volumes in nanolitres as opposed to conventional microplate assays which require hundreds of microliters, hence making them economical alternatives. The minute dimensions of the device offer shorter diffusion path lengths, allowing for precise control of fluid flow and faster analysis leading to specificity in the chemical microreactors. Design manipulation, easily achievable by conventional lithographical and novel nanotechnology techniques, provides versatility in mixing of fluids that can be controlled by external

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physical forces such as magnetic and electric fields. These microdevices may either be integrated to the existing devices or be a comprehensive analytical system by itself. The miniaturization provided by these high throughput devices endows a small chip with large number of replicates on, which make massive parallelization possible, thereby increasing efficiency and further cutting down the cost (Lombardi & Dittrich, 2010). The fluid flow properties at microscale are very different from that at macroscale, and this can be exploited using microfluidic devices (Beebe, Mensing, & Walker, 2002). These advantages make them an ideal choice in disciplines spanning across molecular analysis, biodefense programs, and discovery and development of new drugs in pharmaceutical and biotechnological industries.

Initially, the concept of microfluidics was applied to the field of analytical chemistry. Lithographic patterning/etching, used to produce chemical sensors and chemical analytical techniques on glass/silicon substrates, provided proof of concept for their applicability (Harrison et al., 1993; Manz, Graber, & Widmer, 1990). Afterward, chemical and biological sensors that could thwart the threats due to bioterrorism and aid in biodefense sample testing (Liszewski, 2003) were developed. With the surge in the biotechnological methods, proteomics, genomics, and discovery of protein-based therapeutics, microfluidics offers brighter prospects in DNA sequencing and genotyping as well as protein separation and analysis (Chen, Roller, & Huang, 2010; Gomez, 2011). Microfluidic devices have also provided commendable opportunities for drug discovery and development process with their plausible benefits at each stage from target identification (Malmstadt, Nash, Purnell, & Schmidt, 2006) to lead identification/optimization (Jones et al., 2005) and further to preclinical studies (Matsui et al., 2006), clinical trials (Herr et al., 2007), formulation development (Alsenz and Kansy, 2007), and manufacturing stage (Szita et al., 2005). Additionally, these devices have been used for improved confinement of cells in three dimensional scaffolds, cell-based testing, and cell component analysis. The cellular and molecular interactions at a scale proportional to their dimensions (Whitesides, 2003) are much different from that observed at macroscale volumes. An interesting application has been in the field of tissue engineering whereby microfluidic platforms provide three dimensional scaffolds mimicking natural environment for growth and mutual interaction between cells (Li, Valadez, Zuo, & Nie, 2012; Yamada, Sugaya, Naganuma, & Seki, 2012). They have also been investigated for transdermal and pulmonary delivery of drugs (Ashraf, Tayyaba, & Afzulpurkar, 2011; Yeo, Friend, McIntosh, Meeusen, & Morton, 2010) as well as for personalized diagnostic kits (Yager et al., 2006).

In this chapter, we will present an overview of the microfluidic devices that have been researched for drug discovery and drug analysis. First, we discuss the role played by microfluidics in the current paradigm for drug discovery, in identifying druggable targets, progress achieved by high throughput screening, that allowed for thousands of molecules to be screened on a chip, followed by optimizing lead molecules and assessing their pharmacokinetic and pharmacodynamic properties in preclinical systems. Lastly, we discuss the application of microfluidic devices in chemical analysis.

7.2 Microfluidics for drug discovery

Discovering new therapeutics for a pathophysiological condition involves identifying a specific target (Kang, Chung, Langer, & Khademhosseini, 2008). With the help of computational biology and/or experimental methods, such targets can be identified. This is followed by validating the target by a series of complicated cell or animal-based experiments. Once validated, screening of drug libraries, produced by combinatorial chemistry, composed of millions (usually $>10^6$ compounds) of drug molecules to find a few lead molecules for clinical trials is carried out. This is aimed at getting the safest, most reliable, and efficacious pharmaceutical compound that is then filed as a new drug application for approval by regulatory agents like United States Food and Drug Administration (USFDA). The complex and lengthy procedure of discovering a suitable drug candidate is exemplified by the fact that it takes 10−15 years for a drug to reach from bench to bedside and has been estimated to cost approximately one billion USD (Wu, Huang, & Lee, 2010). The attrition rate from thousands of new chemical structures in the drug library to a few lead compounds and a single successful therapeutic agent is a result of the inefficient procedures used in the conventional/current drug discovery and development process.

Progress in use of microscale platforms aims to accelerate the process of drug discovery by efficient and expeditious design of therapeutics and provision of information on biological targets (Lal & Arnsdorf, 2010). High throughput microfluidic devices have shown considerable promise over the conventional methods which required long processing times and expensive equipment, hence delaying the whole drug discovery process. In the following sections, we describe the contribution of microfluidics in various segments of drug discovery.

7.2.1 Identification of druggable targets

The process of drug discovery begins with the identification of the function of a potential drug target and comprehending its role in the disease process. Discovering pharmacological activities was conventionally carried out by testing various substances in living organisms to observe the changes caused in a phenotype. However, toward the end of the 20th century, this process of phenotype-based target identification was largely replaced by a target-based approach. With progressive acquisition of knowledge in the field of molecular biology and improvement in isolation techniques, identification of complex systems that are responsible for a drug's pharmacological response has evolved to be the new approach in identification of drug targets and has reduced the use of living organisms and living tissues (Terstappen, Schlupen, Raggiaschi, & Gaviraghi, 2007).

Drug targets, which may be a cellular receptor, an ion channel, nucleic acids, DNA or RNA, enzymes, polysaccharide, and lipids, are usually chemically well-defined molecular structures capable of interacting with therapeutic drug moieties (Imming, Sinning, & Meyer, 2006). This interaction leads to downstream clinical effects.

The most common drug targets belong to the class of kinases, phosphatases, nuclear receptors, and G protein coupled receptors (Santos et al., 2017). Ion channels proteins represent another attractive target in drug discovery paradigm as they have been implicated in neurological, cardiovascular, and metabolic diseases as well as cancer and immunomodulation (Dunlop, Bowlby, Peri, Vasilyev, & Arias, 2008) (NATURE REVIEWS DRUG DISCOVERY 2019, 18, 339−357). Around 40% of targets in drug discovery belong to the class of ligand-gated ion channels (Yin et al., 2008). They act as the main targets for the currently available pharmaceutical agents as well as majority of those agents in the drug development phase and hence have been the focus of intense research resulting in dedicated conferences and numerous publications (Perrin, Fremaux, & Scheer, 2006; Talwar & Lynch, 2014; Zagnoni, 2012).

As most of these targets are a part of the cell membrane lipid bilayer structure, their functionality depends on the membrane integrity. The proteins may be denatured once dissociated from the membrane and hence are required to be integrated into the membrane throughout the analytical procedure (Suzuki, Tabata, Kato-Yamada, Noji, & Takeuchi, 2004). Target validation employing isolated membrane proteins and ion channels offers many technological challenges as reproducing these nanoscale systems is very complex (Sandison, Zagnoni, & Morgan, 2007). However, incorporating these drug targets in artificially synthesized lipid bilayer membranes and by specifically controlling the membrane architecture and surface characteristics, simulating the natural environment of a drug target, is envisaged as an option for target identification (Zagnoni, 2012).

Microfluidic technology has played a key role in the fabrication of bilayer lipid membranes (BLMs) (Mayer, Kriebel, Tosteson, & Whitesides, 2003). Micron-sized BLMs with integrated membrane proteins and ion channels are advantageous over macrosystems, providing economical and time-saving analysis platforms. These BLMs bear remarkable electric sealing and, hence, are amenable to recording of electrical signals across single membrane protein. On chip planar bilayer structures were first introduced in 2004 by Suzuki et al. (2004). They fabricated a bilayer membrane chip using a silicon wafer having flow channels on both sides that are connected to apertures (Fig. 7.1(a)). Lipid solution and buffer, injected alternatively, resulted in the formation of the lipid bilayer.

The proteins were incorporated in the bilayer using protein laden liposomes. Integrated microelectrodes could be used for determining the membrane potential and, thus, could serve as a tool for ligand binding studies. However, silicon-based devices suffer from many disadvantages including high dielectric loss of silicon leading to high electrical noise. Apart from that, the manufacture of silicon-based devices is time consuming, and the reproducibility of the BLMs is questionable. Other materials or substrates used for fabrication include epoxy photoresist (Cheng et al., 2001), glass (Fertig, Blick, & Behrends, 2002), and Teflon (Mayer et al., 2003), but the resultant BLMs were fragile and unstable.

Polymeric microfluidic devices have the potential to overcome these drawbacks, offering advantages of economy and ease of fabrication. Poly(methyl methacrylate) (PMMA) has been seen as viable alternative due to its good optical and dielectric properties, low glass transition temperature, ease of processing, and ability to bond

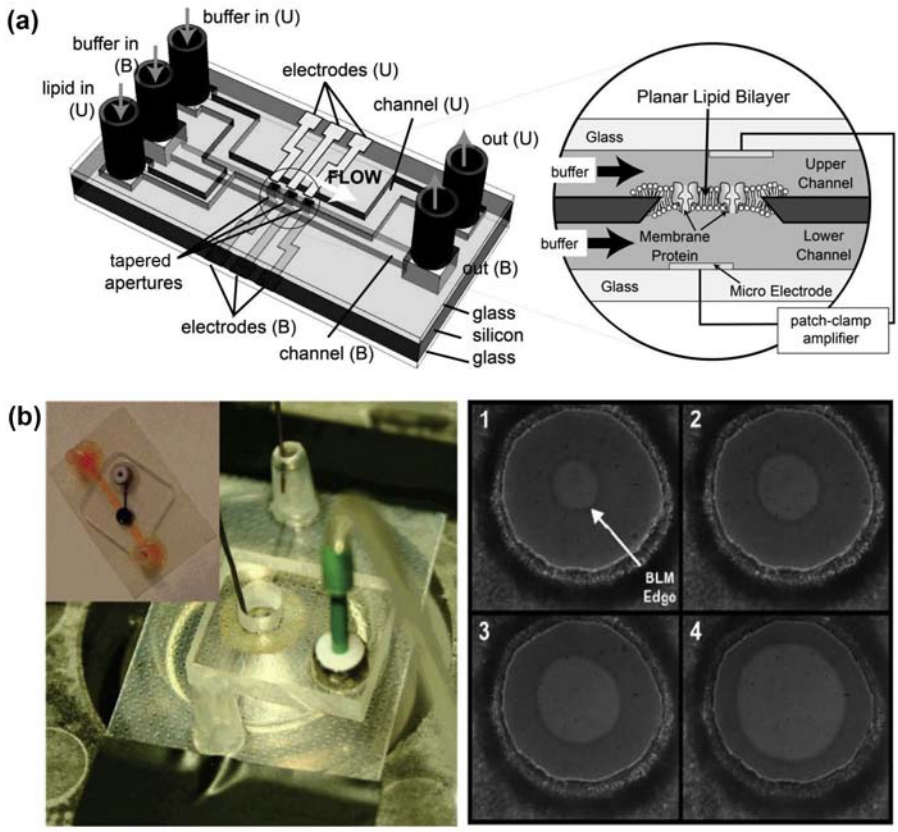

Figure 7.1 Formation of bilayer lipid membranes (BLMs) on microfluidic chips. (a) Conceptual diagram of a membrane fluid chip having fluid channels and apertures. Alternate flow of lipid and buffer solutions lead to formation of BLMs (Suzuki et al., 2004). (b) A microfluidic device with a channel extending out from a trench, where electrodes are inserted in both the upper well (containing lipid) and the lower channel (containing buffer). The bilayer is formed within an aperture upon exposure to air (left), the growth of which is monitored over 20 s (right). The setup was placed over a microscope to observe BLM formation (Sandison et al., 2007).

other materials unlike Teflon (Sandison et al., 2007). Suzuki et al. modified their previous silicon-based design, to make a PMMA-based device providing a tapered aperture for lipid flow and hence achieve a constant amount of lipid solution at the aperture. Further application of a static pressure to control film thickness yielded a more reproducible (90%) bilayer. With further optimization, embedding of four lipid bilayers on a single chip and gramicidin peptide, a monovalent cation channel, incorporated into the bilayer, was achieved (Suzuki, Tabata, Noji, & Takeuchi, 2006). One of the unique advantages of this microfluidic device is that it facilitates easy microscopic observation of the bilayer (Suzuki, Tabata, Noji, & Takeuchi, 2007). Sandison et al. created microfluidic channels on PMMA-coated glass substrates by using hot embossing and laser micromachining (Fig. 7.1(b)). PMMA surface

was chemically treated to render it hydrophobic. Lower channel was filled with buffer, and lipid solution was applied to the upper well, which was later filled with the buffer. Lipid bilayers could be achieved by exposure of the top surface to air (Sandison et al., 2007).

Malmstadt et al. suggested that air required in triphasic PMMA-based BLMs can be problematic, and automation is limited as continuous operator vigilance is needed during device fabrication. Also, an annulus was formed around the membrane due to the solvent, limiting the miniaturization capability (Malmstadt et al., 2006). They developed a novel method based on hydrophobic properties of poly(dimethylsiloxane) (PDMS), used a microfluidic channel. A nonaqueous solution of the lipid was suspended in an aqueous flow stream through a microfluidic channel in PDMS. The hydrophobic solvent, partitions into PDMS, shrinking the lipid membranes together forming a bilayer (Fig. 7.2(a)). Ide and Ichikawa developed a microfluidic device based on successive stacking of a glass slide, plastic sheet, PDMS spacer, and agarose-coated coverslip (Fig. 7.2(b)). Lipid solution is first applied to plastic aperture and sucked by vacuum to form a thin layer over the coverslip. Electrolyte was then added to the well and aperture was moved toward the coverslip, compressing the spacer. Before applying a thin layer of lipid, excess electrolyte was removed. Another layer of electrolyte was applied over this, and excess lipid drained by the means of lateral diffusion, leaving behind central lipid bilayer. The area of the bilayer could be controlled by modulating the aperture movement (Ide & Ichikawa, 2005). This method offers the advantage of specific control of bilayer thickness due to application of vacuum and provision for aperture adjustment.

Kreier et al. developed a solvent free method for creating lipid bilayers, using giant unilamellar vesicles that were made to burst by suction through a micron-sized glass orifice. Ion channel proteins were integrated in the bilayer by incubation of giant unilamellar vesicles to obtain proteoliposomes in a simple and less time-consuming manner as opposed to the previous techniques. Typical gating phenomenon was observed by changes in pH and membrane voltage in the outer membrane protein OmpF obtained from *Escherichia coli* (Kreir, Farre, Beckler, George, & Fertig, 2008). Chip-based bilayers have been used for bacterial toxin binding studies. Using total internal reflection fluorescence microscopy, Cholera toxin B subunit and tetanus toxin C fragment could be detected as low as 100 pM (Moran-Mirabal et al., 2005). It was suggested that this method is adaptable for proteins and nucleic acids as well.

Recently, Schlicht reported a fully integrated microfluidic system to produce artificial lipid bilayers based on the miniaturization of droplet interface bilayer (DIB) techniques (Schlicht & Zagnoni, 2015). The microfluidic platform allowed the controlled positioning and storage of phospholipid-stabilized water-in-oil droplets, giving rise to the scalable and automated formation of DIB arrays which were able to mimic cell membrane processes. Based on the optimization of important parameters such as lipid concentration, immiscible phase flow velocities, and the device geometrical parameters, they were able to quantify diffusive transport of molecules and ions across on-chip DIBs by fluorescence-based assays. To further investigate the effect of inhibitors and promoters of ion channels in drug discovery, it would be beneficial to conduct a solution exchange of droplets to introduce membrane proteins.

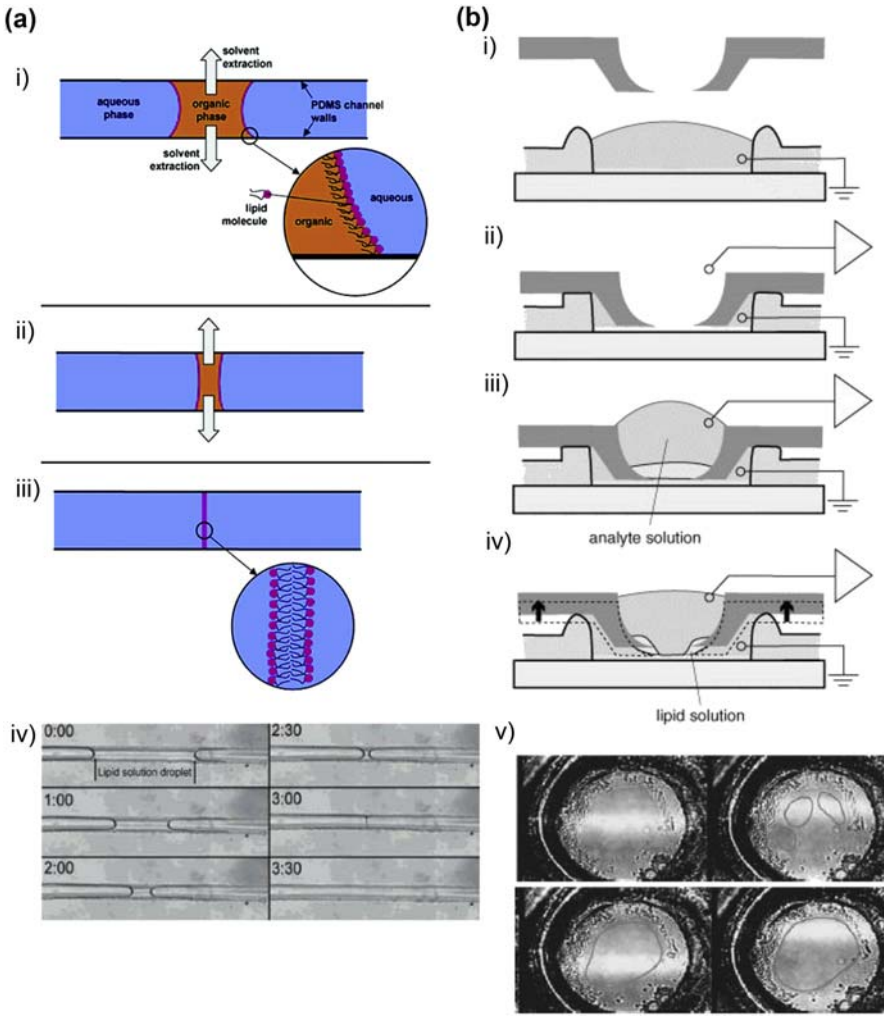

Figure 7.2 Formation of bilayer lipid membranes (BLMs). (a) By microfluidic solvent extraction, (i) droplet of organic solvent with dissolved lipid is formed in an aqueous stream of fluid. Lipids are organized on the hydrophobic−hydrophilic interface (inset). (ii) As solvent enters the poly(dimethyl siloxane) (PDMS), the two interfaces approach one another. (iii) Finally, only the lipid layers are left behind, forming a bilayer membrane. (iv) Images showing solvent extraction from a lipid solution droplet in a microfluidic channel, over a period (minutes: seconds), the BLM, although not visible in the last image, was formed and confirmed by electrical measurements (Malmstadt et al., 2006). (b) By microfluidic bilayer chamber method, (i) A drop of electrolyte was applied to the well of spacer. (ii) A plastic sheet was placed on the spacer and moved downward until the aperture hit the bottom. Then excess electrolyte was removed with a pipette. (iii) Small amount of lipid solution and a sample solution were added sequentially. Alternatively, lipid solution was sprayed through a fine pipette to the edge of the aperture with bubbling without removing the electrolyte in (ii). (iv) After formation of a thick membrane across the aperture, the plastic sheet was moved upwards. The membrane expanded, reached the agarose layer, and thinned to form a bilayer. (v) Successive bright-field images of BLM formation (Ide & Ichikawa, 2005).

To this end, Tsuji and coworkers designed a droplet contact method that allowed the solution exchange of droplets via microfluidic channels (Tsuji et al., 2013). The system allowed the injection of αHL blockers into a droplet and then washing them out. The injection flow rate and the exchange time were adjusted to control the concentration of blockers, which is of great importance since applying differing type of permeating ion, or applying modulators or drugs to the ion channels is often required in ion channel analysis. Moreover, the washing-out experiment demonstrated the binding behavior between ion channels and its ligands. Taken together, the solution exchangeable bilayer system is expected to be a powerful tool for the rapid analyses of ion channels. Similar multiplexed DIBs were prepared by using a mechanically operated linear PMMA chamber array (Barlow et al., 2016). The low cost, linear movable array chip allowed for the simultaneous formation and rapid and high throughput permeation analysis of sub-μL DIBs by the parallelization of DIBs.

These techniques to fabricate BLMs in vitro provide a good platform to identify ion channel proteins as drug targets. Also, once identified, these targets can then be used to screen new therapeutic agents and identify lead compounds for preclinical studies (Kongsuphol, Fang, & Ding, 2013). Syeda and coworkers designed 16-element "DIB-chips" for fast single potassium channel screenings (Syeda, Holden, Hwang, & Bayley, 2008), while Andersson et al. developed arrays of 14 BLMs for the screening of mechanosensitive ion channel (MscL) by forming an ionic reservoir between membrane and the gold electrodes (Andersson et al., 2008) (Fig. 7.3). They can also be used for determination of membrane properties under nonphysiological conditions and gain access to ion channels in intracellular membranes (Kreir et al., 2008).

Cellular receptors and the downstream signal transduction pathways are being increasingly recognized to play a critical role in drug action, and astounding progress has been made in characterizing their behavior. Signal transduction has also been enormously researched with many companies having dedicated programs for signal transduction−based drug discovery (Anonymous, 2000). Enzyme such as tyrosine kinase plays an important role in phosphorylating proteins, forming the essential links in signal transduction pathways (Wang et al., 2008). Wang et al. recently developed a novel microfluidic device combining the function of electroporation and flow cytometry to measure the translocation of fluorescently tagged tyrosine kinase to the cell membrane, at a single cell level. It was demonstrated that cells stimulated through antigen receptor retained more kinase than their nonstimulated counterparts. These results could have a marked impact in target-based drug discovery as kinases are frequently involved in common diseases such as cancer (Wang et al., 2008).

Analysis of protein molecules from a single cell has recently been envisaged as a potential tool to identify specific targets. Recently, single cell analysis has gained considerable attention in microfluidics-based drug discovery as these devices are able to perform manipulation, lysis, labeling, separation, and quantification of the protein contents in a single cell (Huang et al., 2007; Liu & Singh, 2013). Although, this technique is not amenable to live cell monitoring, it provides for simultaneous detection of multiple targets, endowing higher sensitivity in a high throughput capacity. Using single cell analysis chip, the number of β_2 adrenergic receptors was determined. The integrated microfluidic chip facilitated cell and chemical handling,

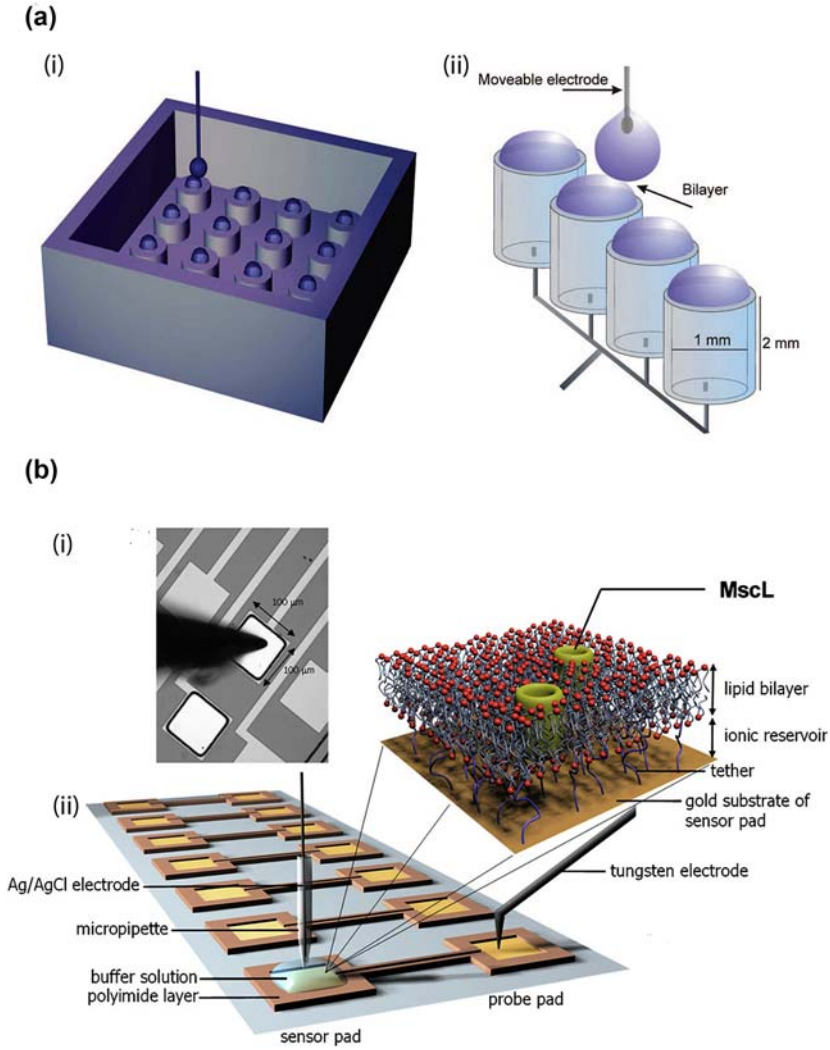

Figure 7.3 (a) Ion-channel screening chip. (i) Simplified schematic of the chip. Each well holds 1.5 µL of solution and presents a convex surface under the oil. A droplet containing the desired IVTT mixture is suspended from the moveable Ag/AgCl electrode, which is grounded. Ion channel blockers and a control (buffer only) are distributed in the wells, each of which contains a Ag/AgCl electrode connected to the working end of the patch clamp amplifier. A DIB is formed between the IVTT droplet and the control well to verify normal channel function. Subsequently, the DIB is separated and the droplet is moved to the next well, and so on. (ii) Detail showing the wiring of 4 of the 16 wells. (Syeda et al., 2008). (b) The engineered tethered bilayer membrane array. (i) Optical microscope image of the probe pad and the tungsten electrode. (ii) Graphical representation of the engineered tethered bilayer membrane array. The lower left corner shows the gold sensor pad covered by a tethered bilayer lipid membrane (tBLM) incorporating MscL ion channels. (Andersson et al., 2008).

cell lysis, electrophoretic separation, and detection of lysate using laser-induced fluorescence (LIF) (Gao, Yin, & Fang, 2004; Wu, Wheeler, & Zare, 2004). Separation of proteins and peptides has also been achieved on miniaturized electrophoretic cells (Schulze & Belder, 2012; Sikanen, Aura, Franssila, Kotiaho, & Kostiainen, 2012). Some of these techniques have been dealt with in greater detail in the subsequent section on analysis.

Understanding of interactions between receptors and their ligands provide insightful information on disease progression and exploration of such drug-receptor pairs provides us an opportunity to discover drugs selectively targeting a particular receptor (Goldberg, Lo, Abele, Macka, & Gomez, 2009). Modulation of physiological events such as cell differentiation and death, release of neurotransmitters and hormones is a result of activation/suppression of signal transduction pathways, which are often coupled to cellular receptors. This activation/suppression is in turn due to binding of specific ligands to these receptors. Much of the research work in discovering new receptor ligands has been focused on binding studies of low molecular weight molecules to macromolecular receptors, followed by screening of biochemical changes. However, it has been reported that lack of a biochemical event does not necessarily translate into lack of receptor activation. Other cellular components and events like second messengers, downstream processes, gene transcription, and change in receptor configuration shall be investigated. This, however, is not possible with the conventional assay procedures (Gurwitz & Haring, 2003). High throughput ligand binding assays provide a suitable alternative to perform multiple tasks on a small chip. Moreover, the discovery of many new "orphan" receptors, for which no ligands are currently known, offers a promising avenue for drug discovery.

Microfluidic devices are beneficial for ligand binding studies as they are able to reduce interaction times, enhance sensitivity and throughput (Kang et al., 2008), and aid in separation of complexed and uncomplexed molecules (Bange, Halsall, & Heineman, 2005). For these binding studies, receptor or ligand molecule can be immobilized on a PDMS substrate by adsorption (Makamba, Hsieh, Sung, & Chen, 2005) or covalent bonding (Sui et al., 2006) or by microcontact printing as achieved for solution hybridized oligonucleotides (Razumovitch, Meier, & Vebert, 2009). These binding interactions are usually quantified by the measurement of equilibrium dissociation constant (K_d) of the ligand−receptor complex.

Goldberg et al. demonstrated the interaction of glycopeptide antibiotics, teicoplanin, and vancomycin, immobilized on a PDMS microchannel with 5-carboxyfluorescein-d-Ala-d-Ala-d-Ala (5-FAM-$(DA)_3$). The K_d was reported to be similar to previously reported values as measured by commercial systems, even though it utilized a smaller amount of reagents (Goldberg et al., 2009). Centrifugal microfluidic platforms, which are disc-shaped microfluidic devices, have also been developed whereby the fluid flows by simple rotation of the disc. Interaction between phenothiazine antidepressants and calmodulin, attached to a green fluorescent protein, was studied. Drug binding affected the fluorescence properties and hence concentration of the drug bound to the protein receptor could be determined (Puckett et al., 2004). BLMs described earlier have been used extensively for ligand binding studies in the past two decades. Recently, phospholipid bilayers were patterned with bovine serum albumin by lithography. Following repeated cycles of patterning, ganglioside GM1 was coated along the

microfluidic channels in different concentrations, and its interaction with varying concentrations of cholera toxin B was studied (Shi, Yang, & Cremer, 2008). Javanmard et al. demonstrated a novel method of coupling microfluidic device with shear force spectroscopy to study the interaction between protein molecules and DNA base pairs. The method could be used to measure the affinity of bond between the interacting molecules by measuring the drag force required to detach the ligand bound to the microfluidic channel when receptor attached on surface of microbeads is pressure driven through these channels (Javanmard, Babrzadeh, & Davis, 2010). Cheow and coworkers reported a protein−ligand binding assay based on a modified commercially available microfluidic platform, the Fluidigm Dynamic Array integrated fluidic circuit (IFC) which was originally designed for multiplexed nucleic acids analysis (Cheow et al., 2014). The analysis was streamlined and automated, where only a loading of 48 protein samples and 48 ligands by pipetting was needed before the benchtop instrument automatically combined the proteins and ligands, for instance chromatin binding proteins and various histone peptides in a pairwise way in 2304 independent chambers of 9 nL for fluorescence anisotropy imaging. The binding affinities turned out to be close to those achieved by a conventional microtiter plate platform which requires two orders of magnitude more reagents and more time. More recently, Glick et al. pioneered an in vitro tool for host−pathogen screening with protein arrays in order to understand important pathogenic processes (Glick et al., 2016) (Fig. 7.4). Around 2700 synthetic genes were arrayed and expressed as insoluble transmembrane proteins within a microfluidic platform using a cell-free protein expression system, and then screened against two important pathogenic proteins, the simian virus 40 (SV40) and hepatitis delta virus (HDV) to find new interactions. The effectiveness of using this microfluidic platform was demonstrated by performing a high-throughput screen of pathogen−membrane protein interactions, since specific interactions of interest were further validated by coimmunoprecipitation or protein−fragment complementation assay of luciferase activity.

7.2.2 Hit identification and lead optimization

After the identification of a druggable target, the next step in the drug discovery process is to identify a "hit" which involves phases of hit identification (HI), lead identification (LI), and leading to lead optimization (LO). A "hit" is a chemical or biological moiety that binds to a specific target which has been implicated in an ailment. Screening and optimization of millions of "hits" results in several "lead" compounds. This whole multiphase process, in which "leads" are optimized by an initial screening involving multiple "hits," is ascribed as a "hit-to-lead" process (Goodnow, 2006). Synthesizing and screening the right drug which can potentially be used and carried forward through a drug development program and enter a clinic starts from right identification of hits and leads. These steps are imperative and crucial since drug discovery is an expensive process (Katsuno et al., 2015). An error at this stage may lead to an expensive failure at a later stage.

Drug candidates may either be derived from combinatorial libraries or be of natural origin, and drug libraries have been estimated to be in the order of 10^{63}

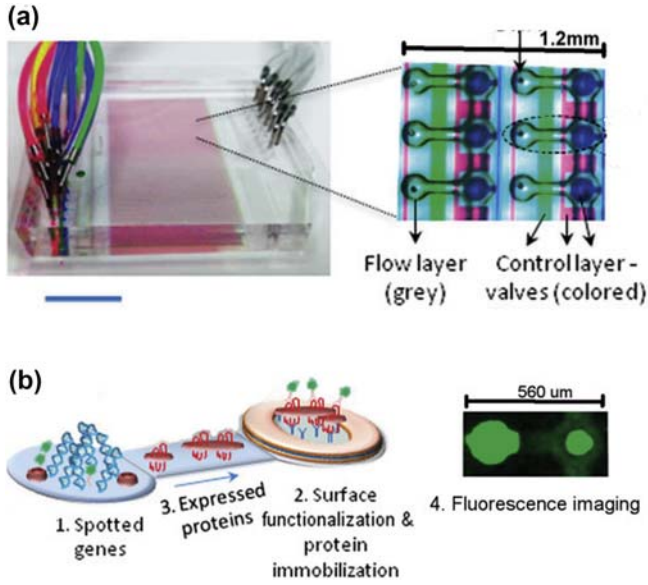

Figure 7.4 Membrane protein array generated by integrated microfluidic platform. (a) An integrated microfluidics platform (Left) was used for on-chip expression of membrane proteins, to serve as "baits" for protein interactions or modifications (29). The device consists of two polydimethylsiloxane (PDMS) layers, a flow layer with 64 × 64 unit cells array (gray), and a control layer with micromechanical valves (colored) that manipulate the flow of fluids in the experiment (Center). The sandwich valves (pink) separate neighboring unit cells; the neck valves (green) divide each unit cell into a DNA compartment and a reaction compartment. The button valves (blue) enable surface patterning to promote binding of proteins to an antibody surface. The button valves serve as mechanical traps of molecular interactions (MITOMI) and allow measurement at equilibrium concentration. MITOMI increases the sensitivity of the system, facilitating detection of weak and transient interactions (SI Appendix, Fig. S6). (b) Combining the microfluidic platform with microarray technology enables programming of the device with up to several thousand spotted genes (Right). Using assembly polymerase cycling assembly (PCR) (SI Appendix, Fig. S1), we added c-Myc (N-terminal) and His6 (C-terminal) tags to the open reading frame (ORFs), creating synthetic genes. On-chip in vitro protein expression, following the synthetic gene programming, combined with the corresponding antibody surface patterning, facilitates the self-assembly of an membrane protein array (MPA) using cell-free transcription and translation (TNT) (rabbit reticulocyte). The immobilized bait proteins are labeled with fluorescent antibodies and quantified by using a microarray scanner. Expressed proteins form a green circle below the button valve (Right) (Glick et al., 2016).

(Bohacek, McMartin, & Guida, 1996). Microfluidic chip−based combinatorial chemistry and high throughput screening together aim to result in a paradigm shift, leading to development of methods of sequential synthesis and testing of thousands of compounds in parallel (Knight, 2000; Li et al., 2018; MacConnell, Price, & Paegel, 2017).

7.2.2.1 Synthesis of drug libraries

Recognition of drug targets has kept pace with the fast progress in genomic and proteomic tools. Pharmaceutical companies on the other hand are facing challenges in generating drug compounds at fastest possible rate, in an inexpensive manner. Synthesis of drug libraries has been described as the biggest impediment in the drug discovery process (Jones et al., 2005). Improved methods in combinatorial chemistry have resulted in rapid synthesis of large number of chemical compounds and produced enormous drug libraries. This has been further accelerated by the improvement in the design of the microfluidic reactors. These microfluidic reactors can be classified into three types, based on the flow pattern, namely (i) flow thorough type, (ii) droplet or slug type, and (iii) batch type (Keng et al., 2012). The most common flow through type enables multiple reagents to be maintained at a temperature, which can be pressure driven through the channels. These reactors have been used greatly in extraction procedures as well as multiple chemical syntheses (Keng et al., 2012). Such application was firstly performed by Warrington and colleagues from GlaxoSmithKline (GSK) to synthesize a small set of pyrazoles using Knorr chemistry (Garcia-Egido, Spikmans, Wong, & Warrington, 2003). The semiautomated synthesis using this technique achieved a residence time in the microreactor of 210 s and ensured near quantitative conversion rates. In another example, an Automated Lead Optimization Equipment Platform, which could significantly reduce time gaps between the synthesis-assay-design cycles, was developed. Its software was equipped with an algorithm which could build predictive bioactivity models and prioritizing the selection of starting materials for subsequent compound generations (Pickett, Green, Hunt, Pardoe, & Hughes, 2011). More recently, Reutlinger et al. presented the optimization of the on-chip reaction and assembly of a combinatorial library of imidazopyridines through the Ugi chemistry (Reutlinger, Rodrigues, Schneider, & Schneider, 2014). The synthesis was conducted at a throughput of 0.3 s per compound with a total reaction volume of only 5 μL.

Parallel combinatorial synthesis in multiple microfluidic reactors has also been demonstrated utilizing continuous flow of reagents in microfluidic channels. A multiple microfluidic reactor assembly was fabricated to synthesize carbamates in a multistep procedure (Sahoo, Kralj, & Jensen, 2007). However, this method sacrifices the advantages of an integrated system for several reactions to be carried out on a single chip. Researchers then looked to fabricate a consolidated device with multiple layers of parallel chips. A multilayer glass chip was developed for a 2×2 series synthesis in parallel (Kikutani et al., 2002). Complexity and expense of fabrication of this multilayered device was a concern. Recently, Dexter and Parker exhibited parallel combinatorial synthesis of compounds on a single layered microfluidic chip (Fig. 7.5(a)). They fabricated a single layer PDMS chip for synthesizing a 2×2 series of amide formation products (Dexter & Parker, 2009).

However, the continuous flow reactors are not suitable for multistep reactions, especially involving sequential synthesis. A modified technique (batch microfluidics) in which specific microvalves control the delivery of reagents in batches has

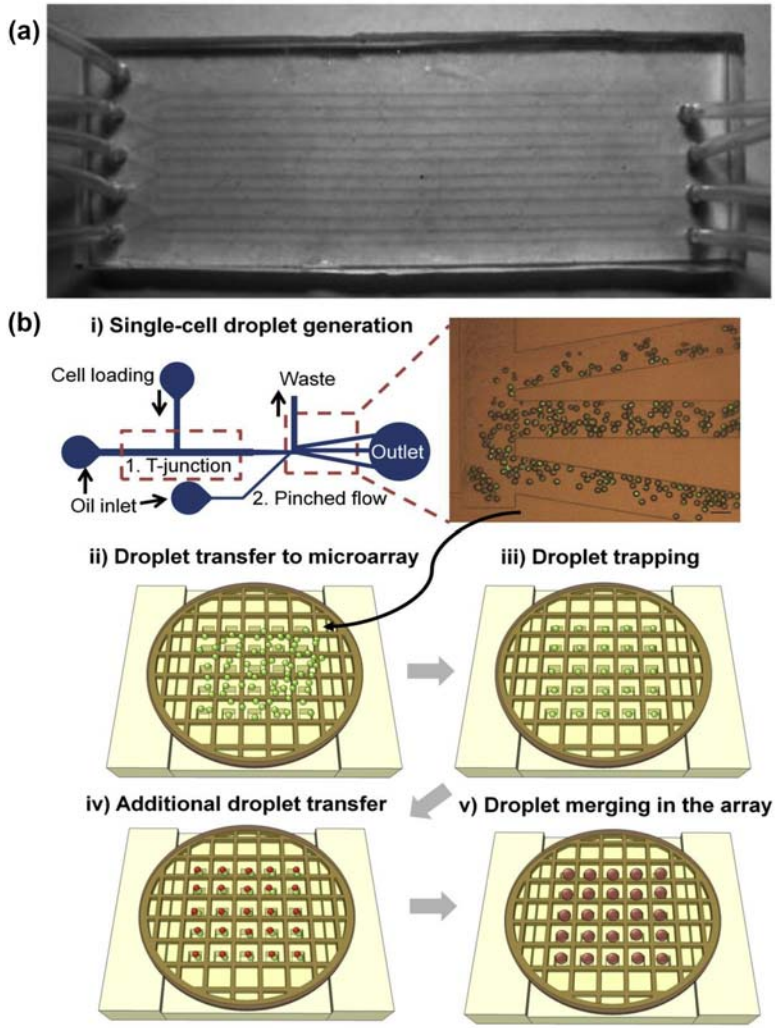

Figure 7.5 Different types of microfluidic reactors. (a) A continuous poly(dimethylsiloxane) (PDMS)-based microfluidic flow reactor for 2 × 2 parallel combinatorial synthesis. The tubing has been inserted at each inlet and outlet port (Dexter & Parker, 2009). (b) Schematic of a microdroplet manipulator, including functions for (i) droplet generation, (ii) transfer of droplets to a microwell array, (iii) migration of droplets into the wells, (iv) trapping of second droplets, and (v) oil change to induce droplet merging (Um, Rha, Choi, Lee, & Park, 2012).

been developed. These isolated batches can be delivered to the microfluidic reactor chamber at specific time points in a reaction cycle, exercising greater control over the reaction (Lee et al., 2005). A fluoride radiolabeled imaging probe, in nano/microgram scale, was synthesized in five sequential processes involving fluoride concentration, water evaporation, radiofluorination, solvent exchange, and hydrolytic deprotection.

A newer technology known as droplet microfluidics has recently come to the fore due to its merits such as consuming very little materials and reagents for large-scale studies, and a high degree of automation which facilitates high throughput screens (Shembekar, Chaipan, Utharala, & Merten, 2016). It is based on compartmentalization of each assay in a small droplet, usually in the range of 1 pL−10 nL which is 10^3−10^9 times smaller than the volume required by conventional systems, surrounded by an immiscible oil and can be manipulated and processed in a high throughput manner (Brouzes, 2012). Each of these droplets can act as a tiny microfluidic reactor, notably reducing the reagent volumes required. A mesh-grid design microwell array was fabricated by Um et al., which allows for continuous addition and trapping of picolitre single cell droplets in the microwells (Fig. 7.5(b)). Due to miniaturization, the device provides high throughput screening (HTS) of the droplets (Um et al., 2012), but multistep reactions using these devices are still a big challenge.

Besides the small molecular chemicals, the microchannel reactor was also employed in the synthesis of macromolecular therapeutics, such as DNA and proteins. Short synthetic oligonucleotides were joined under thermal cycling in a microfluidic PicoArray device to form DNA constructs up to 10 kb in an instant. The fabricated DNA construct was shown to express relevant proteins and may be used for cell free protein expression on a large scale (Zhou et al., 2004). Mei et al. developed a microfluidic array device for synthesis of chloramphenicol acetyltransferase and luciferase and reported the yield to be 13−22 times higher than that achieved in micro-centrifuge tube, with a 5−10 times longer lasting protein expression. The device composed of an array of units that allowed for fabrication of different proteins, protein expression, and nutrient supply. The device is also capable of synthesis and analysis of proteins on a single chip, potentially eliminating the need to harvest proteins thereby reducing wastage and increasing process efficiency (Mei, Fredrickson, Simon, Khnouf, & Fan, 2007). A droplet-based microfluidic method was recently developed for on-chip protein synthesis. Production of a water-in-oil-in-water (W/O/W) emulsion was accomplished by formation of a water-in-oil emulsion on a PMMA chip, up first, followed by complete emulsion formation on a PDMS/glass microchip. Synthesis and expression of a green fluorescent protein from a DNA template was successfully demonstrated using a microfluidic platform (Wu et al., 2011). Recently, Timm reported a microfluidic-based cell-free system as a bioreactor for the production of a single dose of a therapeutic protein (Timm, Shankles, Foster, Doktycz, & Retterer, 2016). This new design integrated a long, serpentine channel bioreactor channel, and a nanofabricated membrane to allow exchange of materials between parallel "reactor" and "feeder" channels. The membrane was designed to facilitate the exchange of metabolites, energy, and inhibitory species between the two channels, and its surface could be modified to tune the exchange rate of small molecules, which enabled extended reaction times and higher yields than conventional tube−based batch synthesis.

Most of the devices developed use PDMS as the substrate materials due to its excellent optical properties as well as its mouldability. However, PDMS is incompatible with many organic solvents and adsorbs many hydrophobic compounds due to its surface properties. Keng et al. fabricated a microfluidic platform that is operated by

electrowetting-on-dielectric (EWOD). The device was made from inorganic materials coated with perfluoro polymer and offers flexibility in use with organic and hydrophobic reagents (Keng et al., 2012). The device was shown to be suitable for diverse chemical reactions with minimal consumption of reagents, with suitability for multistep procedures requiring several solvent exchange rounds.

7.2.2.2 High throughput screening

Microfluidics-based devices have been put to efficient use to generate drug libraries which provide a powerful source that need to be screened to explore new drugs. To screen these large combinatorial libraries of compounds, pharmaceutical industry has looked at HTS methodologies in the past two decades. The conventional screening methods were able to screen 5000−20,000 compounds over a few years, resulting in inefficient screening of only 2%−20% of the compounds on the whole library. However, HTS or newly termed, ultrahigh throughput screening (uHTS) methodologies aim to screen 10,000−100,000 compounds over a period of 24 h, resulting in generation of 2−18 million screening results per year (Beggs, 2001). This logarithmic increase in screening capability has given a boost to the hit-to-lead discovery process.

Traditionally, high density microplates including 96, 384, 1536 and those with >1536 wells have been used extensively for HTS (Battersby & Trau, 2002; Brandish et al., 2006). However, liquid handling on a microliter scale in these microplates was found to be difficult due to their inability to be integrated with robotic liquid handling technologies as well as suitable detection platforms. Microfluidic platforms can further miniaturize the HTS platforms, lowering the assay volume required (Ding et al., 2015). Also, these platforms can be easily modeled for convenient liquid handling and integrated with analytical devices. Microfluidic HTS platforms for confining reagents have been studied in both serial and parallel configurations. Using serial method, compounds are screened in a successive manner with only one detector unit. However, in this approach, the throughput is largely dependent on flow rate and concentration of the sample as well as acquisition speed of the detector. In contrast, parallel screening offers faster analysis, segregating multiple samples into miniaturized compartments of a high-density microplate, and analyzed by a single detector (Ding et al., 2015). But parallel analysis is limited by the miniaturization capacity and hence the extent of parallelization (Thorsen, 2004). Nevertheless, both methods have been extensively used in microfluidic HTS. Generally, cell-based microfluidic HTS platforms include cell culture (El-Ali, Sorger, & Jensen, 2006; Wu, Huang, & Lee, 2010), introduction and transportation of samples (Melin & Quake, 2007; Stone, Stroock, & Ajdari, 2004), and characterization of cell viability (Barbulovic-Nad, Yang, Park, & Wheeler, 2008), with an effort to demonstrate the integration of these different components into a single microfluidic device. Among current microfluidic platforms for cell-based HTS, there are three major modes to manipulate microfluids: perfusion flow mode, droplet-based mode, and microarray mode (Du, Fang, & den Toonder, 2016), which will be elaborated in the following.

Microfluidic microwell arrays are a versatile tool for cell culture and high throughput experimentation through cell-based assays. They enable assays with

many biological samples on a 2D solid substrate and are particularly important in drug screening. Nearly 50% of all drug discovery processes rely on cell-based assays (Fox et al., 2006). Seeding many cell types on a single chip offers the advantages of testing the effect on drugs on different cells types. It also offers the potential of testing many compounds on a single cell type in high throughput. A multiwall microelectrode array was fabricated using PDMS by conventional soft lithographic process. The array was then coated with a cell adhesive layer of poly-D-lysine followed by patterning a nonconducting agarose gel layer to isolate the individual neuronal microcircuits and record individual action potentials of drugs like bicuculline and N-methyl-D-aspartic acid (Kang, Lee, Lee, & Nam, 2009) (Fig. 7.6). Chen et al. developed a complementary microwell and microcolumn system for screening of drugs. They used microelectromechanical systems (MEMS) to first fabricate a microwell array on a glass substrate, to culture the cells. Employing a similar process, they fabricated complementary microcolumns that will carry the drugs to be topically applied onto the cells. The system was found to be suitable to deliver high throughput identification of epidermal growth factor receptor inhibitors (Chen, Huang, & Juang, 2011). An integrated multilayer microdevice incorporating a drug/medium concentration gradient generator, flow controlling microvalves, and microchambers for cell culture was recently fabricated by Liu et al. for testing the apoptosis behavior in a cisplatin resistant cancer cell line (Liu et al., 2012). A vertical perfusion mode was adopted in this device, as shear stress due to horizontal fluid flow can adversely impact the cells. Using the setup, sequential loading of cells, medium, drugs, and air was achieved in successive layers of the device.

The combination of two or more clinically available drugs, administered either simultaneously or sequentially, may enhance the therapeutic efficacy, as well as reduce the drug toxicity and resistance as a result of its multitarget treatment mechanisms. More importantly, drug combination is also considered as an effective way to increase the efficiency of drug discovery since most drug combinations are conducted using existing drugs which have passed the strict clinical and safety studies (Ashburn & Thor, 2004). Therefore, Ding et al. developed a low-cost, high-efficiency microfluidic print-to-screen (P2S) platform for high-throughput screening of anticancer drug combinations (Ding et al., 2015). The P2S platform utilized a microfluidic impact printer to generate large-scale combinatorial droplets containing multiple anticancer drugs, so-called combinatorial library in microarrays. Then, the hydrogel-based cell culture matrix was treated with the droplet arrays before stained and imaged for HI (Fig. 7.7). Compared with conventional techniques, the P2S platform completely automated the combinatorial library generation and significantly accelerated the screening process by performing thousands of cell-drug interaction analyses in parallel, which could facilitate the discovery cycle of potent drug combinations. Furthermore, taking advantage of the sequential operation droplet array (SODA) technique, Du and coworkers developed a drug combination screening platform with multisteps involving cell culture, medium changing, drug dosage and stimulation, and cell viability assay in an oil-covered nanolitre-scale droplet array system (Du et al., 2013). The drug consumption for each screening testing was substantially reduced to 5 ng−5 mg, considered as a significant reduction compared with conventional

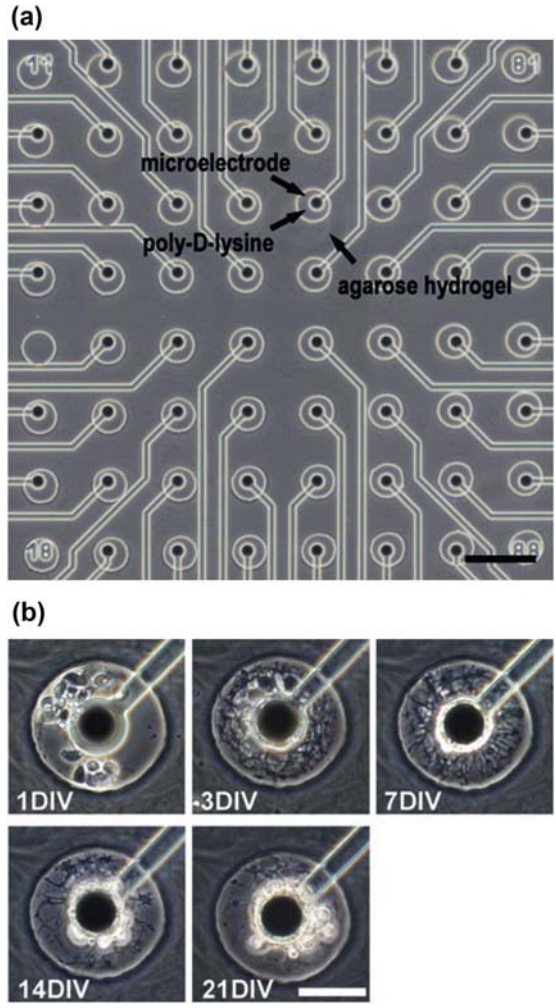

Figure 7.6 Microfluidic microarrays for cell-based high throughput screening. A multiwell microelectrode array, (a) phase contrast image of agarose microwells on a microelectrode array. Each microwell is composed of a microelectrode, poly-D-lysine coated surface, and agarose hydrogel wall, scale—200 μm. (b) The growth of neuronal conduits in microwells over a period of 3 weeks, scale—50 μm (DIV—days in vitro) (Kang et al., 2009).

drug screening systems. Despite a lot of progress in developing microscale arrays for cell culture, cell seeding in these arrays is a challenge. Kang et al. addressed this issue by developing a simple wiping method to seed cells in microwells. A coverslip was used to slowly wipe the cells suspended in the growth medium across the surface of the microwell array. Cell concentration, microwell geometry, and wiping speed controlled the cell seeding density (Kang, Hancock, Brigham, & Khademhosseini, 2010). They also developed an algorithm and software for automatic counting of cells

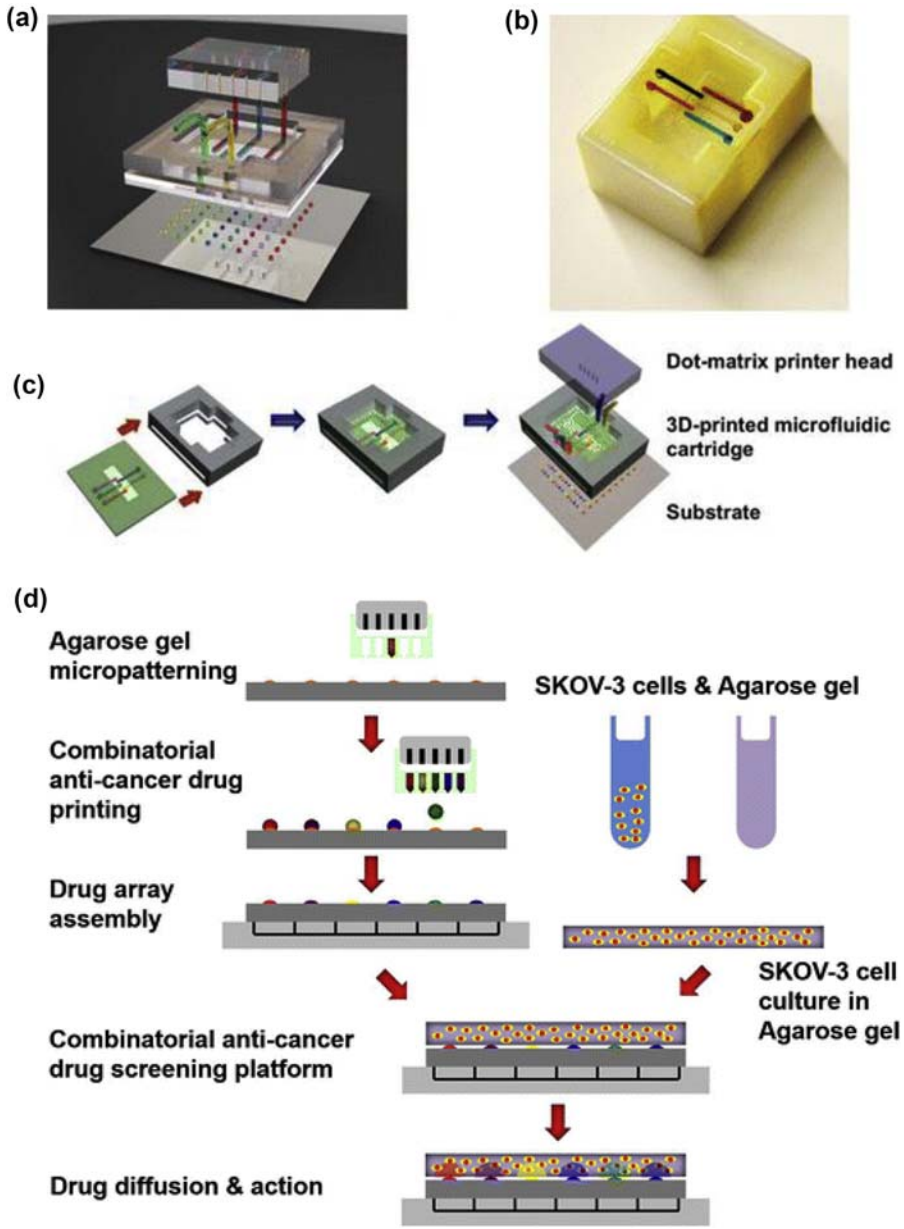

Figure 7.7 Microfluidic impact printing. (a) Illustration of the microfluidic impact printer, consisting of a dot matrix printer head, a traveling stage, and a microfluidic cartridge with a 3D printed adapter. (b) Microfluidic impact printer prototype. (c) Plug-and-play assembly of the microfluidic impact printer head (Ding et al., 2015).

in a microwell array. The software, named as Arraycount, detects the cell count from the fluorescent cell images in high throughput. The results were in close correlation between cell counts from the manual methods (Kachouie, Kang, & Khademhosseini, 2009).

Studying single cell characteristics offers the advantage over observing the behavior of a group of cells as single cell characteristics might be hugely different from the entire population of cells. Microwell arrays have been developed to confine single cells for observation of these cells and their progeny over a period. One of the first studies pertaining to single cell confinement in microwell arrays for drug screening was reported by Rettig and Folch (Rettig & Folch, 2005). PDMS microwells were fabricated by conventional soft lithography, and controlled seeding of single cells into microwells could be achieved by optimizing the geometry of the microwells. It was observed that microwells with an aspect ratio (diameter: depth) close to one had more than 85% wells with single cell occupancy for both adherent and non-adherent cells (Rettig & Folch, 2005). An interesting round bottom microwell array was recently developed by Liu et al. by creating PDMS microwell arrays by reverse molding using polystyrene microspheres melted on a glass substrate (Liu, Liu, Gao, Ding, & Lin, 2010) (Fig. 7.8). The size of these microwells could be tuned to 10−20 μm, which is difficult to achieve by conventional soft lithography. The PDMS microwells were then used to confine single cells by pouring excess of cell suspension over the microwells, which allowed the cells to settle in. The enzymatic activity of cells was studied by carrying out the carboxylesterase assay using calcein AM. Fluorescence intensities from single cells could be captured to reveal different kinetic behavior of entrapped cells, which was related to cell viability. Another novel way of constraining single cells in microwells was demonstrated by Wang, Shah, Phillips, Sims, and Allbritton (2012). The flexibility of PDMS was exploited by stretching the patterned PDMS array using a tube that delivered the cells onto the array. After loading, the tube was withdrawn and cells settled in the microwells, which were then amenable to further analytical treatments. They also demonstrated that cells within the microwells could be isolated by deforming the PDMS substrate using a microneedle (Wang et al., 2012). A further example was illustrated by Lew and coworkers who devised a plastic microwell array by using economical materials like shrink wrap film and tape. A carbon dioxide laser was used to cut holes in the tape which acts as a mask to etch wells in the shrink wrap by oxygen plasma (Lew, Nguyen, & Khine, 2011).

In a perfusion flow mode of drug screening, the microfluidic devices need a series of generic components for introducing reagents and samples, transferring fluids within a microchannel network, as well as combining and mixing reactants. By using the technique of reversible sealing of elastomeric PDMS, Ali et al. developed a double-layer device making use of a PDMS substrate layer with microwells and a PDMS cover layer with arrays of microchannels (Khademhosseini et al., 2005). Multiple cell types were seeded into microwells in the substrate layer through the microchannels of the first PDMS cover layer which was replaced with an orthogonally aligned second cover layer to deliver different fluids to the patterned cells for screening. Another method of on-chip drug screening relies on the controlled diffusive mixing of solutions in

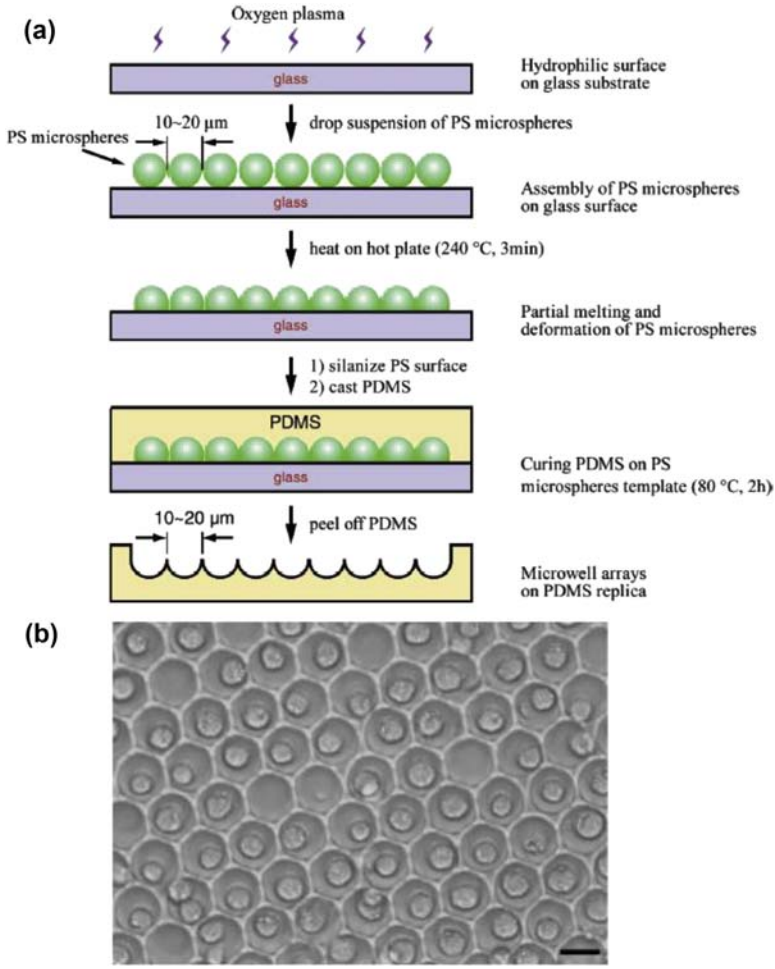

Figure 7.8 Fabrication of microwell assays. (a) Schematic illustration for the fabrication of PDMS microwell arrays. (b) Cell arrays on the microwells and single cell enzyme activity analysis. Micrograph of dense trapping of Ramos cells (Liu et al., 2010).

continuous laminar flow inside a network of microfluidic channels where drug concentration gradients were generated. Chung and coworkers reported a microfluidic device for high throughput capture and imaging of single cells by shear force in a continuous flow channel (Chung, Rivet, Kemp, & Lu, 2011). Coupled to gradient generators, this device was able to study heterogeneity in calcium oscillatory behavior in genetically identical cells and investigate kinetic cellular response to various chemicals. Due to the development of individual components, microfluidics also has potential in developing integrated automatic systems to perform cell culture, drug release, and cell activity detection together for drug discovery. Weltin et al. developed a microfluidic system for drug screening by real-time on-line monitoring of human

cancer cell metabolism process (Weltin et al., 2014). The optically transparent multifunction microsystem comprised cell culture chambers and four chemo- and biosensors modules for the detection of pH, oxygen, lactate, and glucose, respectively. The drug screening application was demonstrated by monitoring the change and recovery effects of cellular metabolism induced by the addition of substances to the medium.

The droplet mode is the last type of microfluids manipulation in cell-based HTS which can perform a broad spectrum of experimental chemistry and biology screening. The typical droplet systems use water-in-oil emulsion droplets, where the continuous oil phase can prevent cross-contamination between reagents in neighboring droplets and minimize the nonspecific binding between the channel surface and reagents of dispersed phase. Another advantage of droplet system is that droplets can be split or merged to start or stop reactions or to conduct washing steps. Clausell-Tormos and coworkers first designed a droplet-based microfluidic platform where cells or multicellular organism could survive and proliferate for more than several days (Clausell-Tormos et al., 2008). This system offered a screening with a 1000-fold smaller volume and a 500 times higher throughput. Brouzes et al. reported a microfluidic droplet-based system for high throughput cytotoxicity screening of single cells (Brouzes et al., 2009). The cells were encapsulated in individual microdroplets and coalesced with optically coded droplets from a chemical library to identify drug composition and drug concentration in each droplet, before the coalesced droplets were merged with fluorescent dye droplets to stain the cells for the on-line fluorescence assay. Recently, Cao and coworkers developed a microfluidic droplet device for an assay of toxic effects on *Escherichia coli* via the generation of multidimensional concentrations of antibiotics (Cao et al., 2012). This device allowed more than 5000 distinct experiments with different combinations of antibiotic concentrations in a single experiment, making it suitable for the drug screening with the advantages of reduced experimental complexity and higher information density.

Multiplexed screening platforms have also been developed to screen multiple samples in one run. The ability to analyze multiple proteins, nucleic acids as well as small molecules reduces assay time, reagent volume, and cost. Multiplexed measurements provide the ability to increase the throughput without a simultaneous increase in the density of the microfluidic array. Multiplexing technology has been applied to two different types of microfluidic platforms: planar arrays and suspension (particle-based) arrays. For protein and DNA analysis, planar arrays have been used, whereby protein molecules have been patterned as microarrays onto substrates using lithography (MacBeath & Schreiber, 2000). Such systems offer application-specific advantages ranging from study of protein−protein interactions to establishing proteins as targets for small molecules and specific functions of enzymes. Suspension arrays on the other hand offer the advantages of studying the properties of compounds in solution, thereby providing ease of sample modification, higher throughput, and increased batch to batch uniformity (Nolan & Sklar, 2002).

A multiplexed system could be used to screen a compound against multiple kinases or study protein−protein interaction and detect changes in enzyme conformation (Xue, Wainright, Gangakhedkar, & Gibbons, 2001). In this report, four kinases

were screened against a substrate. The reaction products/substrates could be separated by electrophoretic separation on a chip and analyzed. Multiplexed screening of picolitre-sized droplets that could be manipulated using an array of electrodes has also been reported. For example, caspase-3 activity, a marker of apoptosis which is an important tool in cancer drug discovery, was measured after human cervical adenocarcinoma HeLa cells were treated with different concentrations of staurosporine. The technique termed as digital microfluidics (DMF) was compared against conventional techniques involving 96-well plate. It resulted in a 33-fold reduction in sample volume together with a lower detection limit for caspase-3 analysis compared with conventional techniques. This can be attributed to the lack of delamination in apoptotic cells in the DMF platform that uses droplet manipulation system instead of pipetting or aspiration of liquids with conventional techniques (Bogojevic, Chamberlain, Barbulovic-Nad, & Wheeler, 2012).

Analyzing multiple samples by multiplexing, however, poses a challenge in sample recognition. Hence, it is necessary to have an encoding scheme integrated into the system to allow for rapid and precise analyte identification. Encoding schemes based on spectrometric (Han, Gao, Su, & Nie, 2001), graphical (Evans, Sewter, & Hill, 2003), electronic (Service, 1995), and physical techniques (Vaino & Janda, 2000) have been developed. An exhaustive review on various encoding techniques has been published by Braeckmans, De Smedt, Leblans, Pauwels, and Demeester (2002). Spectrometric techniques utilize specific wavelengths to analyze a compound. In contrast, graphical methods use certain optical elements that are chemically patterned onto the microarray. These techniques require much sophistication and are expensive and may require considerable amount of time for fabrication and integration.

Pregibon et al. recently developed a novel encoding scheme for multiplexed platforms (Pregibon, Toner, & Doyle, 2007). In this system, two poly(ethylene glycol) based monomer solutions, one being a fluorescent dye and another being an acrylate probe, were made to flow through microfluidic channels. The solutions during flow were exposed to ultraviolet light using conventional techniques of continuous flow lithography to develop a patterned particle (Pregibon et al., 2007). Morphological properties of the particles were determined by a photomask, inserted into a fluorescence microscope (Pregibon et al., 2007). A simple dot coding scheme was used on the photomask that could generate over two million particles, with each having a unique code. Although the particle size achieved in this method was larger than previous methods, the authors demonstrated that the sample volume required will be manageable, together with providing higher sensitivity and reproducibility. The system was able to detect DNA at concentrations as low as 10^{-18} mol, without signal amplification, proving it to be a completely integrated encoding device, with advantages of low cost, high efficiency with virtually unlimited number of codes possible, and all this achievable with the services of a simple fluorescence microscope.

Inkjet printing technology has been purported as a highly efficient screening alternative, providing efficiencies greater than 200,000 compounds per day, currently achievable with the microfluidic platforms described earlier. The technology offers capabilities to simultaneously deposit cells and drugs to be tested in a small picolitre volume. Postprocessing, the cell characteristics can be studied to evaluate the

drug effects. Such a novel platform was developed by Rodríguez-Dévora, Zhang, Reyna, Shi and Xu (2012). They developed an inkjet printer—based method to pattern green fluorescent protein expressing *Escherichia coli* cells grown on a soy agar medium, on a coverslip. Live/Dead assay, used to assess bacterial cell viability, demonstrated high number of cell survival after imprinting. Fast screening utilizing low volumes to assess effect of three antibiotics patterned together with the bacterial cells could be carried out. This bioprinting approach was compared to the standard micropipetting approach and was found to yield similar results at much lower volumes (Rodríguez-Dévora et al., 2012).

These microfluidic platforms have significantly enhanced the profile of high throughput screening, leading to optimization of hits and leads, before the leads are put through preclinical testing for evaluation of their preliminary pharmacokinetic and toxicological properties.

7.2.3 Preclinical evaluation

Interaction with the molecular targets begins the journey of the drug in the human body. When a drug is administered, it must get absorbed across mucous membranes, followed by its distribution to its target site and metabolism to an inactive metabolite to get eliminated from the body. It should also be devoid of any toxic effects. These characteristics, known as Absorption, Distribution, Metabolism, Elimination, and Toxicology (ADMET) are essential factors in determining the path of the drug in the later stages of the drug discovery process. A fine balance between these pharmacokinetic characteristics is needed for the development of a drug from a chemical entity (Muster et al., 2008). Unsatisfactory ADMET profile accounts for attrition of 50%—60% drug candidates at the preclinical development stage (Smith, 2007), with lack of efficacy and undesirable toxicity being the major causes (Kramer, Sagartz, & Morris, 2007). It has been reported that the lack of efficacy accounts for 30% of failures of new drug entities and toxicity further accounts for another 30%. If these are detected at later stages in the drug development process, the overall cost of the program will be increased, as cost escalates with each stage (Kola & Landis, 2004). Therefore, pharmaceutical companies are nowadays adopting the *fail early; fail cheap* approach to identify the toxicological properties of drug compounds. This is done in lieu of savings if toxicological properties are identified at a much later stage or even after the launch of the product, necessitating an inevitable and highly expensive market recall. It has been reported that number of market recalls as a percentage of number of approvals in United States has reduced from 27.2% in 1980s to 5.2% in 2000s (Qureshi, Seoane-Vazquez, Rodriguez-Monguio, Stevenson, & Szeinbach, 2011). This has, in part, been the contribution of more novel and efficient toxicity screening platforms that have been developed in the past two decades. It also underlines the importance of proficient preclinical programs and the role played by them in drug development.

In vitro toxicological testing in cell models provides useful information about the drug candidates, much before the expensive animal experiments and first-in-human clinical trials are conducted. In vitro experiments have been long touted to replace

animal testing, especially due to the ethical concerns surrounding animal experimentation (Wen, Zhang, & Yang, 2012). Moreover, in vitro toxicity in excised animal organs may not be extrapolated to correctly reflect human toxicities. On the other hand, in vivo preclinical testing in live animals requires large amount of compound under investigation, which is usually available in limited quantities and may be prohibitively expensive (Muster et al., 2008).

7.2.3.1 *In vitro evaluation*

Three dimensional (3D) cell culture mimics the natural environment of the cells, including cell−cell and cell−extracellular matrix interactions as opposed to planar two dimensional (2D) cultures that are used to maintain cells (Pampaloni, Reynaud, & Stelzer, 2007). An excellent collation of advantages of 3D cell culture over 2D format has been provided by Zhang and van Noort (2011). Also, these 3D cultures offer an ex vivo alternative to live animal testing and potentially reduce the cost of toxicity screening during drug development. Nonetheless, 3D cell cultures present a few shortcomings, especially with sample handling and imaging. Since these cultures are thicker than conventional "petri-dish" cultures, they are difficult to adapt to conventional microscopic techniques. Liquid handling in patterned microstructures requires sophisticated micro/nanolitre scale devices. However, the advantages of studying the cells in an environment outweigh the technological shortcomings, which, too, are being addressed simultaneously.

As hepatotoxicity has been the leading cause of failure at the clinical trial stages and postlaunch market withdrawals, many researchers have looked at developing in vitro cell-based hepatotoxicity assays. It is important to notice here that most of these agents went through preclinical animal testing and were assumed to be safe (Kaplowitz, 2005). Microfluidic 3D cell culture platforms aim to address this problem and have been designed to provide deeper insights into cell behavior, when exposed to cytotoxic agents. A multiwell 3D cell culture platform was designed using soft lithography to coculture primary hepatocytes with mouse 3T3-J2 fibroblasts. A PDMS stencil containing through holes in a 24-well format was first applied to a polystyrene plate, followed by application of collagen-I through the holes. After removal of the PDMS stencil and application of a 24 well PDMS blank, hepatocytes were cultured on the 24 wells, which attached to the collagen, surrounded by fibroblasts. The hepatocyte morphology was maintained in the wells for 4−6 weeks. Albumin and urea synthesis, measured as markers of protein synthesis and nitrogen metabolism and typically considered as a measure of liver function, was reported to be normal. On the other hand, pure cultures were reported to be morphologically unstable, and there was a loss of albumin and urea synthesis (Khetani & Bhatia, 2008).

Kane et al. designed a microfluidic 8×8 array, composed of PDMS. Each well in the array had two chambers, a primary chamber whose bottom was made of glass coated with collagen, for coculturing rat hepatocytes and 3T3-J2 fibroblasts. The collagen aided selective adhesion of hepatocytes. Continuous perfusion of medium and removal of waste products was achieved by microfluidic tubing connected to the chamber. The secondary chamber, which was separated from the primary chamber

by a thin PDMS membrane, was linked to microfluidic channels supplying humidified air with 10% carbon dioxide at 37°C. They also reported similar results of increased albumin and urea production (Kane, Zinner, Yarmush, & Toner, 2006). Such microfluidic platforms have also been used to assess cardiotoxicity, neurotoxicity, embryotoxicity, and cytolysis, a summary of which has been provided in a review by Wen et al. (2012). These microfluidic devices, which can emulate an organ in vitro, are referred to as organ-on-a-chip devices.

Although the above listed cell-based assays provide information about a compound's therapeutic and toxic properties on the tissue under consideration, they do not tell anything about the effect on the whole body or interactions with other organs and related dose dynamics. As a drug in the body goes through the complex process of ADME, collectively called as pharmacokinetics (PK), with contribution from different organs, cell culture using cells-on-a-chip or organ-on-a-chip technology fails to capture these responses. Of late, scientists have developed miniaturized multicompartment cell culture platform better known as body-on-a-chip devices. These can promote tissue−tissue interactions by creation of environment and flow conditions scaled down to in vivo tissue sizes. They can also aid in studying interactions between organs in a high throughput manner, enabling the study of multiorgan metabolic and toxicity profiles of a compound. Microscale systems designed for physiologically based pharmacokinetic modeling (PBPK) having different compartments for different tissues can help to understand parameters such as tissue-to-blood perfusion, enzyme kinetics, liquid-to-cell ratio, and physiological stress on a particular tissue/organ (Esch, King, & Shuler, 2011).

Novel microfluidic systems called as microscale cell culture analog (μCCA) have been developed for multiorgan toxicity analysis. A multiorgan culture system termed as "Integrated discrete multiple organ culture" or "wells-within-a-well" system was designed by Li et al. Cells from different organs were cultured in small wells in their respective medium in a bigger well. They cultured primary cells from liver, kidney, lungs, central nervous system, blood vessels as well as human breast adenocarcinoma cancer cell line, MCF-7. For testing the toxicity of a model drug, the bigger wells were flushed with a medium containing the drug, tamoxifen. The effect of tamoxifen was evaluated, and its comparative toxicity toward various organs was also examined. Apart from this, the system offers another advantage in enabling the analysis of anticancer activity of a drug with respect to its effect on normal tissues. The authors did not delve upon multiorgan interactions; this, in principle, can be adapted for this purpose and its capabilities should be further investigated (Li, Bode, & Sakai, 2004).

In a model based on PBPK to emulate dynamics of human body, different compartments hosting different cell types were connected through microfluidic channels to mimic blood circulation. Four different cells were cultured on a μCCA, including hepatocytes (HepG2/C3A), bone marrow cells (MEG-01), uterine cancer cells (MES-SA), and a multidrug resistant (MDR) uterine cancer cell line (MES-SA/DX-5). In a combination drug therapy of chemotherapeutic doxorubicin, with MDR modulators cyclosporine and nicardipine, treated for 24 or 72 h, a selective toxicity toward MES-SA/DX-5 was observed, a synergy not observed in conventional 96-well plate assays. This device could, thus, be used in drug screening and selection of potential MDR modulators, as well as gather dose required and dose response

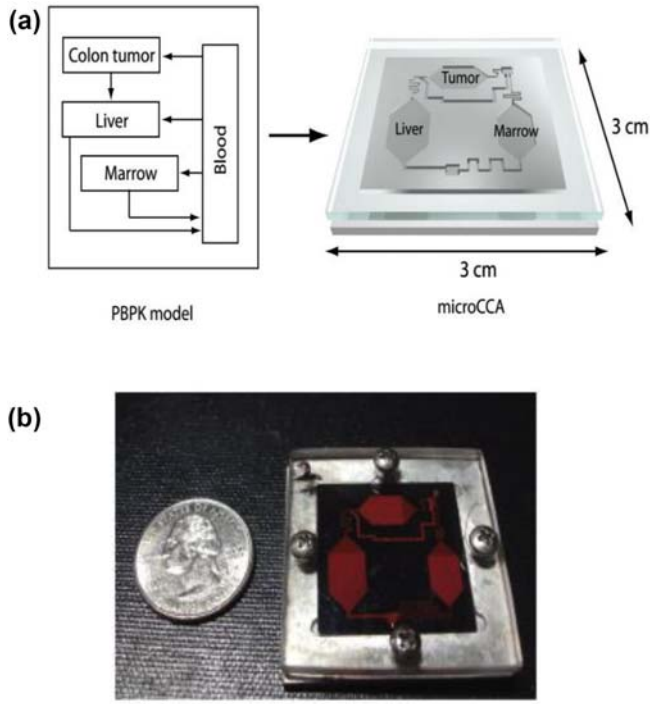

Figure 7.9 A mathematical PBPK model and a corresponding physical mCCA based on the human body. (a) A μCCA consists of liver, tumor, and marrow chambers, interconnected with channels mimicking the blood flow pattern in the human body. (b) An assembled μCCA with red dye for visualization of chambers and channels (Sung & Shuler, 2009).

curves for subsequent in vivo animal experiments or clinical trials (Tatosian & Shuler, 2009). 3D hydrogel cultures in μCCA format were developed by Sung and Shuler (Fig. 7.9). Three types of cells, hepatocytes (HepG3/C3A), myeloblasts (Kasumi-1), and colon cancer cells (HCT-116), were embedded in different chambers in 3D hydrogels, representing different organs. The cytotoxic effect of tegafur, a prodrug of active anticancer drug, 5-fluorouracil, commonly used in colon cancer was tested using this device. An interesting revelation as compared to conventional 96-well plate assay was that, although, the liver cells in μCCA showed metabolism of tegafur similar to 96 well plate, the metabolism lead to death of hepatocytes, an effect which was unnoticeable in well plate assays (Sung & Shuler, 2009). The literature is replete with tegafur toxicity data, particularly its hepatotoxicity (Maruyama, Hirayama, Abe, Tanaka, & Matsui, 1995). In such a scenario, development of microfluidic systems providing critical toxicity information in in vitro models bodes well for preclinical drug testing.

7.2.3.2 Ex vivo evaluation

Apart from in vitro microfluidic cell culture platforms, some researchers have also looked at ex vivo microfluidic platforms by isolating animal tissues, particularly liver,

and culturing excised explants to analyze the toxicity of various compounds. It has been reported that precision cut liver slices fare better than hepatocytes alone with respect to metabolic activity (Graaf, Groothuis, & Olinga, 2007). Continuous perfusion of nutrient medium can further reduce the loss of metabolic activity and prolong protein expression in these slices. Microfluidic devices have been designed to continuously replenish the spent medium and remove waste material from these slices. van Midwoud et al. designed a PDMS-based "perifusion" device, with liver slices supported on polycarbonate membranes. The term "perifusion" was used instead of perfusion as the medium flowed around the slices. PDMS membranes were purposely kept thin to allow for efficient gaseous exchange. Comparable metabolic activity of 7-ethoxycoumarin to well plate based method was observed in this device (van Midwoud, Groothuis, Merema, & Verpoorte, 2010). Another PDMS based device was developed to analyze ethanol toxicity in liver explants. Using this device, concentrations as low as 20 mM produced a decrease in mitochondrial metabolic activity as well increased lactate dehydrogenase activity, a marker of cell death. These effects were observed in a concentration dependent manner, together with a decrease in albumin and urea synthesis (Hattersley, Greenman, & Haswell 2011). Such devices utilizing excised tissues represent clinically more relevant models to replace animal experimentation.

7.2.3.3 *In vivo evaluation*

Microfluidic platforms have also been used to assist in vivo animal experiments, for blood sampling, sample preparation, and analysis (Kang et al., 2008). An automatic blood collection microfluidic chip based on PDMS was developed by Wu et al. for withdrawal of blood from mice without the need of trained personnel. The device consisted of two layers, holding channels for blood inlet, outlet, heparin block, blood reservoir, and sample wells. A microfluidic device was used for processing blood samples from mice for determining hematotoxicity. In this device, a microcavity array was created by master molding PDMS structures to form a sieve-like structure that separates leukocytes from other blood cells (Fig. 7.10). Benzene toxicity was assessed by staining the leukocytes and counting them over a period of 2 weeks (Hosokawa et al., 2012). Microfluidic platforms have been designed to be integrated with novel analytical techniques such as matrix-assisted laser/desorption ionization−mass spectrometry (MALDI-MS), which can facilitate fast sample analysis with high precision and resolution of many metabolites in biological samples (Lee, Lee, Kim, & Kim, 2008; Xu, Little, & Murray, 2006).

These microfluidic systems have, thus, played a critical role in various stages of the drug development process. Beginning with the identification of targets, to synthesis of compounds for generation big compound libraries, to HTS and preclinical development, microfluidics has been effectively adapted to reduce the consumption of reagents and make the drug discovery process more efficient and cost effective. In the subsequent section, we will discuss about the application of microfluidics in analysis of chemical and biological drugs. We also discuss the role played by these devices in detection of diseases and routine diagnostic purposes, which may reduce the healthcare costs.

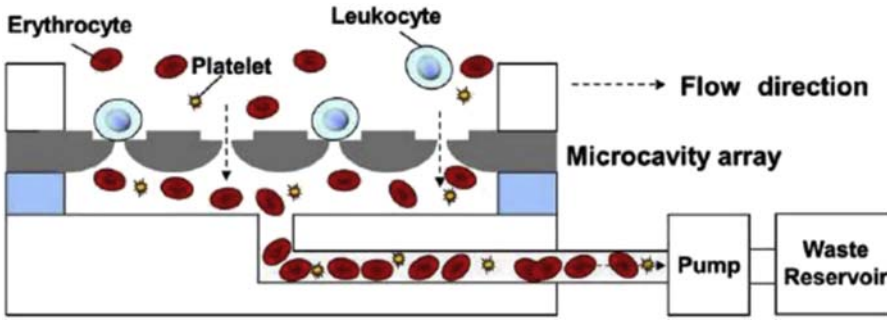

Figure 7.10 Microfluidic platforms for ex vivo experiments. For leukocyte counting and assessment of hematotoxicity. A microcavity array was created by PDMS to form a sieve-like structure that retained the leukocytes, while allowing other blood cells to pass through. The cells can then be separately analyzed for toxicity due to various drugs (Hosokawa et al., 2012).

7.3 Microfluidics for pharmaceutical analysis and diagnostic applications

Miniaturization of analytical tools has been propelled by the recent surge in the development and maturation of microfabrication techniques. The better control of physical processes and parameters at the micron scale has further fueled the interest in microscale analytical systems as new paradigms for pharmaceutical analysis. These systems are aimed at both reducing the sample volume and time of analysis, besides being amenable to integration with the other platforms and potential for high throughput. High parallelization made analysis of multiple compounds fast and easy (Lee et al., 2009). Moreover, design modification can provide integrated facilities for handling fluids, thermal and spatial control for targeting specific detection components to enhance selectivity (DeMello, 2006). Given that the mass fabrication of micron-sized platforms now is possible via sophisticated instruments, the cost of production of these devices has come down, providing an opportunity to develop single use analytical device, and thereby reducing the possibility of cross-contamination (Lion, Reymond, Girault, & Rossier, 2004). In the following sections, we will discuss the application of microfluidic device in chemical/drug analysis followed by a brief description on microfluidic-based diagnostic applications.

7.3.1 Microfluidics for pharmaceutical analysis

Although there have been many mechanistic and experimental advancements in analysis of drugs, the basic analytical equipment and components have not changed much over the past few decades. Recently, with the application of microscale techniques adopted from the semiconductor industry, scientists are now poised for choices to carry out analytical assessments at an order 5−9 times lower than conventional counterparts (DeMello, 2006). Microscale analytical devices, also termed as micro total analytical systems (μTAS), comprise microchannel networks, which aim to replicate the analysis

procedures on physically shrunk platforms, without compromising the analytical efficiency or sensitivity. Apart from this, μTAS can be designed to attain a high level of automation, thereby making multiplexed assays possible and providing a system that reduces manual errors and helps to increase the assay accuracy. In particular, these devices have been more popular in analysis of biological molecules like proteins and nucleic acids (Guo, Rotem, Heyman, & Weitz, 2012; Meagher & Thaitrong, 2012) and have been the subject of other chapters in this book. Here, we would limit our focus to discuss about the application of microfluidics in the analysis of drug entities in both pharmaceutical and biological samples.

Analysis of pharmaceutical compounds has been carried out using high performance liquid chromatography (HPLC) equipped with various detection modules including ultraviolet and fluorescence spectrophotometers as well as mass spectrometry, electrophoresis, potentiometry, colorimetry, radioisotopic assay, microbiological methods, enzymatic methods, surface plasmon resonance based biosensor assays, surface-enhanced Raman spectroscopy (SERS), conductivity, and chemiluminescence (CL) among many others. Microfluidic platforms, due to the flexibility in their design, are docile to integration with most of the above listed analytical methods. With the increasing demand for highly sensitive and miniscule working volume platforms, it is imperative that the chosen methods detect low amounts of the analyte. CL-based methods have been used for microfluidic detection of vitamin B_{12} and L-phenylalanine. It was reported to be a highly sensitive technique which was capable of detecting vitamin B_{12} as low as 5 pg/mL (Kumar, Chouhan, & Thakur, 2009). This testing stands on luminol oxidation by hydrogen peroxide in the presence of externals catalyst ions such as cobalt (II) and copper (II) and amino acid like L-phenylalanine under alkaline conditions. The resultant product is a blue compound (3-aminopthalate ion) that can be detected at a wavelength of 425 nm (Chen, Gao, He, & Cui, 2007; Wang et al., 2007).

Lok et al. developed a microfluidic chip to detect the concentration of vitamin B_{12} using a continuous flow microfluidic chip (Fig. 7.11). The device consisted of three layers which contained two passive mixing reaction chambers and a double spiral microchannel network as an optical detection unit. The first layer was located on top of the second one, where there were a mixing chamber and a clockwise spiral detection unit. The third layer had the other mixing chamber and an anticlockwise spiral detection module. The mixing chambers were designed in layers to counter the problem of mixing in the laminar flow. The spirally designed detection channels present a better CL signal to the optical detector as compared to a single loop unit. The microchip also had a chamber for acidification of vitamin B_{12}, as cobalt present in vitamin B_{12} complex is not released passively to catalyze the reaction. Using the device, up to 0.3 pg/mL of vitamin B_{12} could be detected (Lok, Abdul Muttalib, Lee, Kwok, & Nguyen, 2012). In another CL-based microfluidic chip based on the same principle of oxidation of luminol catalyzed by copper sulfate was used to detect L-phenylalanine, as the CL signal increased in the presence of L-phenylalanine in alkaline medium. PDMS was used to fabricate the device by soft lithography. The device was provided with four sample inlets and one outlet and was able to detect

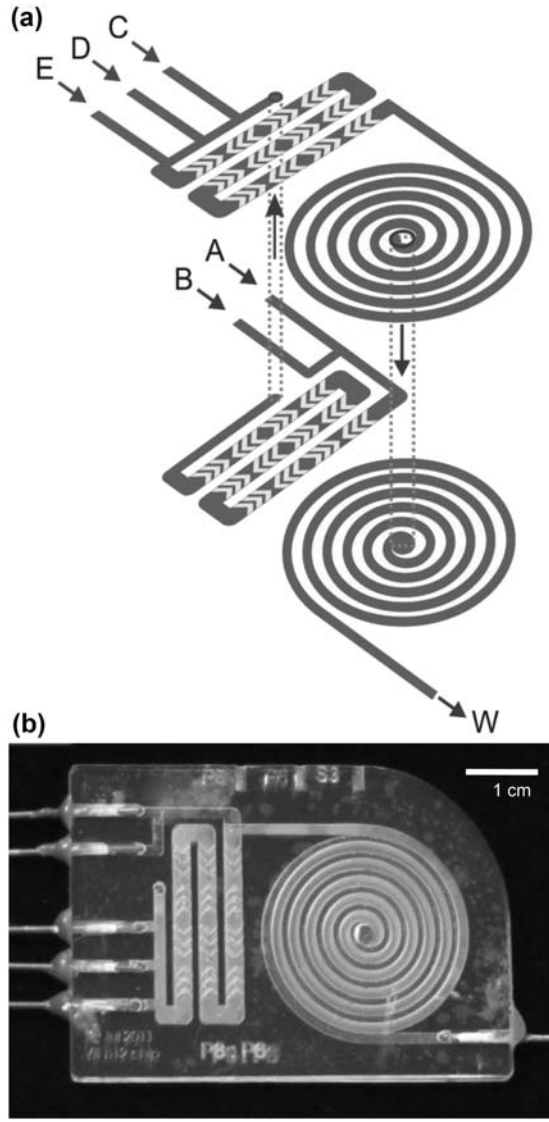

Figure 7.11 Lab-on-a-chip for determination of vitamin B12 concentration: (a) microchannel network (A to E are inlets; W is outlet to waste; arrows indicate the fluid flow); (b) Fabricated microchip. The chip measures 45 6 30 6 3 mm (Lok et al., 2012).

around 39 pg/mL of L-phenylalanine in commercial soft drinks as well as pharmaceutical injections (Kamruzzaman et al. 2012).

In contrast to CL, a larger number of microfluidic devices rely on the electrochemical detection of analytes for drug analysis. Won et al. developed a microfluidic device on glass slides for simultaneous detection of five sulphonamide drugs.

The device was provided with modules for preconcentration and electrokinetic (EK) separation of drugs using the field-amplified sample stacking (FASS) and field-amplified sample injection (FASI) techniques in two parallel channels (Shiddiky & Shim, 2007). Subsequent electrochemical detection of sulphonamides was carried out at the end of separation channel which consisted of a silver/silver chloride, platinum wire, and aluminum-gold nanoparticles modified carbon paste electrode. The device was able to detect femto-molar level concentration of sulphonamide drugs and provides an opportunity to simultaneously detect these drugs in clinical samples. Later, they further developed a highly sensitive and robust microfluidic device integrating the preconcentration, separation, and electrochemical detection for simultaneous analysis of multiple sulphonamide drugs (Won, Chandra, Hee, & Shim, 2013). The microfluidic device comprised both FASS and FASI channels for the preconcentration process and the EK separation for sulphonamides detection by chronoamperometry. In this study, they pioneered the simultaneous detection of preconcentrated sulphonamides in a microfluidic device. Thanks to the optimization of various experimental parameters affecting the analytical performances of the method, the detection limits of the analytes were brought down to approximately 1−2 fM. In addition, the reliability of the proposed method was confirmed by detecting the spiked concentrations of drugs in various meat samples. Wu et al. developed a micellar electrokinetic chromatography (MEKC) technique with the help of microchips for the detection of antibiotics (Wu et al., 2015). An on-line multiple-preconcentration device coupled FASS and reversed-field stacking (RFS) for the simultaneous analysis of three antibiotics including kanamycin, vancomycin, and gentamycin by microchip MEKC with LIF detection. This strategy allows the detection of antibiotics in river water samples, which could be successfully focused and well separated with high efficiency and sensitivity. In addition, Chong et al. also developed a portable microchip electrophoresis (MCE) coupled with on-chip contactless conductivity detection system for the detection of vancomycin in human plasma (Chong, Thang, Quirino, & See, 2017). To increase the sensitivity, a new online multistacking preconcentration technique based on field-enhanced sample injection (FESI) and micelle-to-solvent stacking (MSS) was designed and implemented in MCE-C^4D system combined with a commercially available double T-junction glass chip. The cationic analytes from the two sample reservoirs were injected under FESI conditions and subsequently focused by MSS within the sample-loading channel. They achieved a detection limit of vancomycin at 1.2 μg/mL, recoveries in spiked human plasma around 99.0%−99.2%, as well as intraday and interday repeatability relative standard deviation (RSDs) of 2.6% and 4.3%, respectively. Similarly, Rudasova et al. developed a novel MCE method for the rapid detection of N-acetylcysteine, a pharmaceutically active ingredient with isotachophoresis separations and conductivity detection (Rudasova & Masar, 2016). The repeatability and accuracy of N-acetylcysteine determination in all samples were more than satisfactory with the RSD and relative error values $< 0.7\%$ and $< 1.9\%$, respectively, while a recovery range was achieved at 99%−101%. This work showed the analytical potential of the microchip isotachophoresis for the quantification of pharmaceutical samples that contain analyte(s) at relatively high abundances.

Taking the advantage of electrochemical detection, recent researchers also designed nontraditional microfluidic devices for drug analysis. For instance, Shiroma et al. developed a simple, cost-effective, and sensitive paper-based microfluidic device with electrochemical detection for the analysis of paracetamol and 4-aminophenol (Shiroma, Santhiago, Gobbi, & Kubota, 2012) (Fig. 7.12(a) and (b)). The separation channels of a width of 2.0 mm were created on paper via a wax printing process to define the shape of the device, while the electrochemical detection system was located at the end of the channels through sputtering, where the preseparated drugs were detected by applying a potential of 400 mV on the Au working electrode. A baseline separation of paracetamol and 4-aminophenol was obtained by injecting the sample at 12.0 mm from the working electrode which was already long enough to minimize the effect of interfering substances in the sample. Using this paper-based device, they achieved detection limits of 25.0 and 10.0 μmol/L for paracetamol and 4-aminophenol, respectively. This study provided a promising tool for lab-on-a-paper technology combining paper-based separation channels with electrochemical detection.

Besides CL and electrochemical detection, a microfluidic chip integrated to laser-induced fluorescence scanner was developed for the detection of β_2 agonist drugs like clenbuterol (Fig. 7.12(c−e)). These drugs increase muscle mass and have been often misused in farm animals (Martinez-Navarro, 1990) as well as in power sports by athletes (Delbeke, Desmet, & Debackere, 1995; Hesketh et al., 1992), making it vital to analyze them in a rapid and accurate manner. The three layers of the device consisted of a fluidic channel, a PDMS membrane, and a pneumatic control layer interspersed with many pneumatic microvalves and micropumps to enable the delivery of reagents. Glass was used to fabricate the fluidic channel and pneumatic control layer by standard lithography and etching to create microchannels. The PDMS membrane was then sandwiched between the two layers and generated pneumatic valve and pump effect due to deflection by compressed air. The drugs could be detected within 30 min and at a concentration as low as 0.088 ng/mL (Kong et al. 2009). Ho et al. developed a cost-effective and robust microfluidic system to quantify the amount of active pharmaceutical ingredients (APIs), especially artemisinin and its derivatives (Ho, Desai, & Zaman, 2015). The detection relied on an indirect way. Under alkaline condition in the presence of catalyst hematin in blood, luminol reacts with hydrogen peroxide which comes from the cleavage of artemisinin and derivatives, giving rise to a chemical compound that emits chemiluminescent signals at 425 nm. The system was able to quantify the artesunate tablets with results comparable to the conventional 96-well plate in the spectrophotometer and comparable to the conventional HPLC. More importantly, each chip could be used for 3 times given proper cleaning between the usages, thus reducing the cost per test to about $0.5. More recently, Zeid et al. developed a facile, rapid, and highly sensitive PMMA microchip-based EK chromatographic method for the simultaneous detection of two gabapentinoid drugs, gabapentin (GPN) and pregabalin (PGN) (Zeid et al., 2017). β-Cyclodextrin (β-CD) was used as the additives to optimize the separation, which enabled the analyses of both fluorescently labeled compounds. The sensitivity of the technique was enhanced by 14- and 17-fold for PGN and GPN, so that it could detect both analytes with a detection

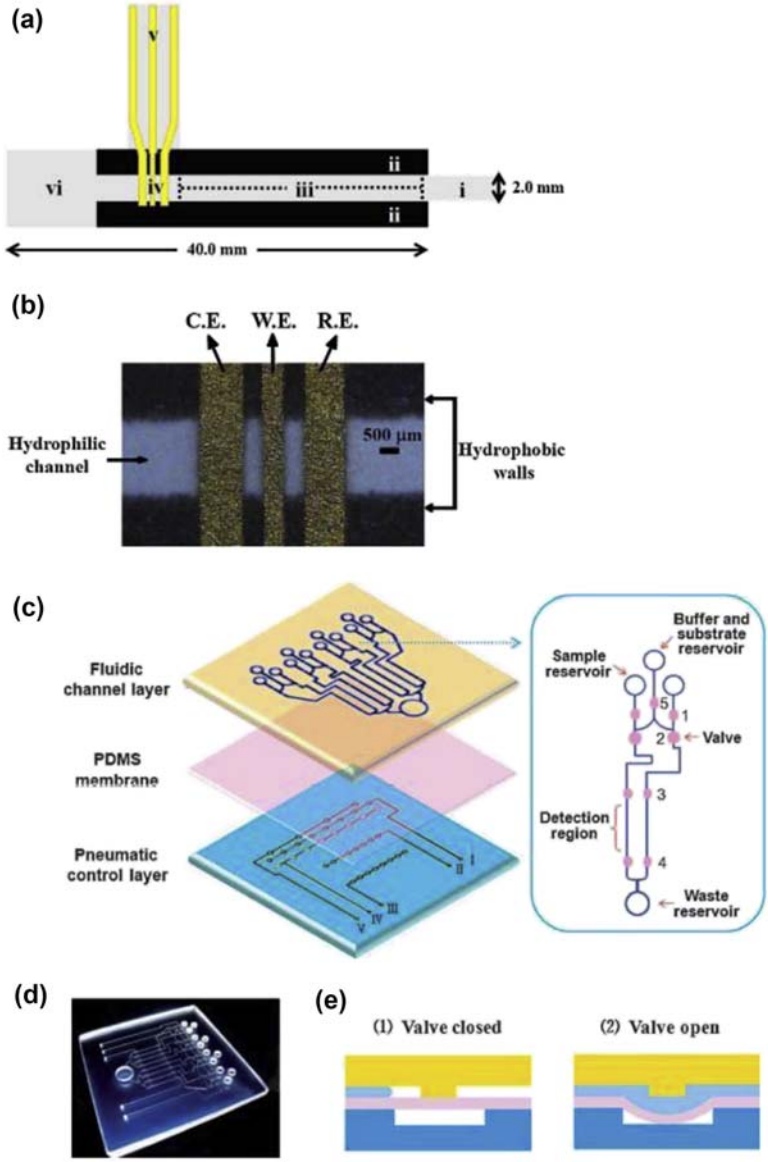

Figure 7.12 (a) Main parts of the paper-based microfluidic device with electrochemical detection: (i) eluent entrance, (ii) hydrophobic walls, (iii) region of sample addition, (iv) electrochemical detection system with three gold electrodes, (v) contact pads, and (vi) absorbent pads. (b) Photograph of the electrochemical detection system (Shiroma et al., 2012). (c) Schematic of a multilayered microfluidic device for analysis of drugs. The device comprised three layers, the top and bottom layer made of glass and a poly(dimethylsiloxane) (PDMS) membrane was sandwiched between two layers. (d) Photograph of the device. (e) Cross-sectional view of the microvalves showing closed and open position (Kong et al., 2009).

limit lower than 3 ng/mL. More importantly, the device was used for the analysis of PGN and GPN in biological fluids, with an extraction recovery rate greater than 89%.

Besides the integration of luminance/fluorescence and electrochemical based detection in microfluidic devices, other analytical techniques, including HPLC, mass spectroscopy, and SERS have also been investigated for microfluidics-based drug analysis. For example, Andreou reported a microfluidic device that detected trace concentrations of drugs of abuse in saliva samples within minutes using SERS (Andreou, Hoonejani, Barmi, Moskovits, & Meinhart, 2013) (Fig. 7.13). They utilized a flow-focusing microfluidic device to tailor the spatial arrangement and flow rate of the various streams for optimal SERS signals. The analyte, methamphetamine, was introduced into the central stream that was focused by two side streams containing Ag-NPs and salt, diffused laterally into the side streams, especially into the side

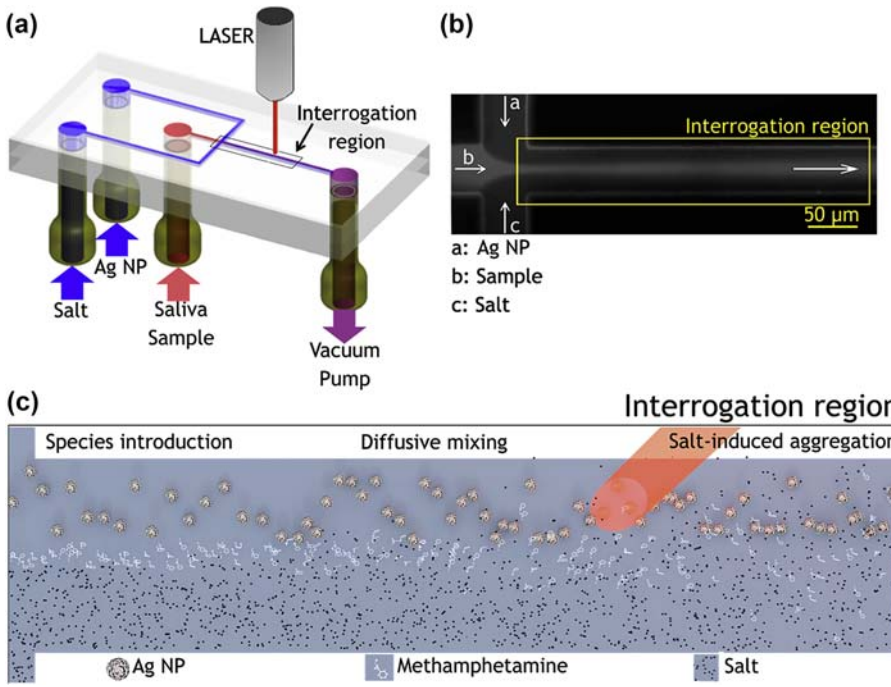

Figure 7.13 Flow-focusing microfluidic device used for controlled Ag-NP aggregation. (a) Ag-NP suspension, a saliva sample, and salt solution are loaded in the device and driven through it by a vacuum pump. (b) At the flow-focusing junction, the sample stream is enveloped by the side streams, and diffusion drives lateral mass transport between the laminar flows, here visualized with a fluorescent dye. (c) Schematic of the reaction: Ag NP, analyte, and salt solution are introduced to the channel from the left and flow toward the right. Analyte molecules resident in the focused stream diffuse laterally into the side flows. Salt ions also diffuse into the colloid stream inducing controlled nanoparticle aggregation, creating SERS (surface-enhanced Raman spectroscopy)-active clusters that convect downstream. Interrogating the region rich in colloid dimers, which provide intense plasmonic enhancement, we are able to achieve optimal SERS-based detection (Andreou et al., 2013).

containing the Ag-NPs where it may adsorb. The Ag-NPs were much bigger than any of the other chemicals involved in the process. As a result, they diffused at a slower rate than the salt ions, an aggregating agent of Ag-NPs. However, salt ions were required to travel a greater distance since they must pass through the central stream before reaching the side stream where Ag-NPs were located. This gave the analyte enough time to be adsorbed by nanoparticles before significant Ag-NP aggregation was induced. Therefore, the strongest SERS signal was only readable at a downstream location where significant aggregates (predominantly dimers and other small order aggregates) appeared. This device exhibited several advantages over conventional SERS-based techniques. It promoted the interaction between NPs and analytes in the solution along the laminar flows, while the controlled aggregation induced by the salt gave rise to a reproducible and reliable region where SERS signal was maximized. Baharfar et al. reported a microfluidic device for on-chip electromembrane extraction of trace amounts of ephedrine (EPH) and clonidine (CLO) in human urine and plasma samples, which was coupled with HPLC-UV analysis (Baharfar, Yamini, Seidi, & Karami, 2017). The device was composed of polymethylmethacrylate plates with a polypropylene sheet in between. Under the electric field, the analytes were converted to ionized form, crossed the supported liquid membrane, and then extracted into the acceptor phase. The effectiveness of the technique was determined by the analysis of real biological samples from urine and plasma. In terms of relative recoveries, a high accuracy of 94.6%−105.2% and RSD of repeatability of less than 5.1% were obtained. In addition, Zhu et al. developed a microfluidic chip based nano-HPLC integrated to tandem mass spectrometry (nano-HPLC-Chip-MS/MS) for simultaneous detection of 14 types of abused drugs and metabolites (e.g., cocaine, benzoylecgonine, cocaethylene, norcocaine, morphine, codeine, amphetamine, methamphetamine, and methadone) in the hair of drug abusers (Zhu et al., 2012). The microfluidic chip was prepared by laminating polyimide films and was coupled to an enrichment column, an analytical column and a nanospray tip. The microfluidic chip was inserted into the HPLC-Chip cube interface, which was mounted directly on the MS source for data acquisition. Similarly, Kirby et al. reported a device which incorporated microfluidics and a miniature mass spectrometer for the quantitation of drugs abuse in urine (Kirby et al., 2014) (Fig. 7.14). Apart from other microfluidic devices, this system was designed to deliver droplets of solvent to dried urine samples before conveying extracted analytes to an array of nanoelectrospray emitters for MS/MS analysis. This design increased the efficiency of drug analysis, where cocaine, benzoylecgonine, and codeine could be quantified from four samples in less than 15 min. In addition, they achieved a limit of quantitation (LOQ) for cocaine at 40 ng/mL, deeming it compatible with the performance criteria for laboratory analyses established by the United Nations Office on Drugs and Crime. Collectively, these results indicated that the technique was suitable for on-site screening, and the study became a proof of concept for integration of microfluidics with miniature mass spectrometry.

Besides the analysis of drugs in the pharmaceutical and biological samples, researchers also expanded the applications of microfluidic-based devices to the analysis of cells and artificial organs exposed to drugs in vitro. Snouber et al. reported

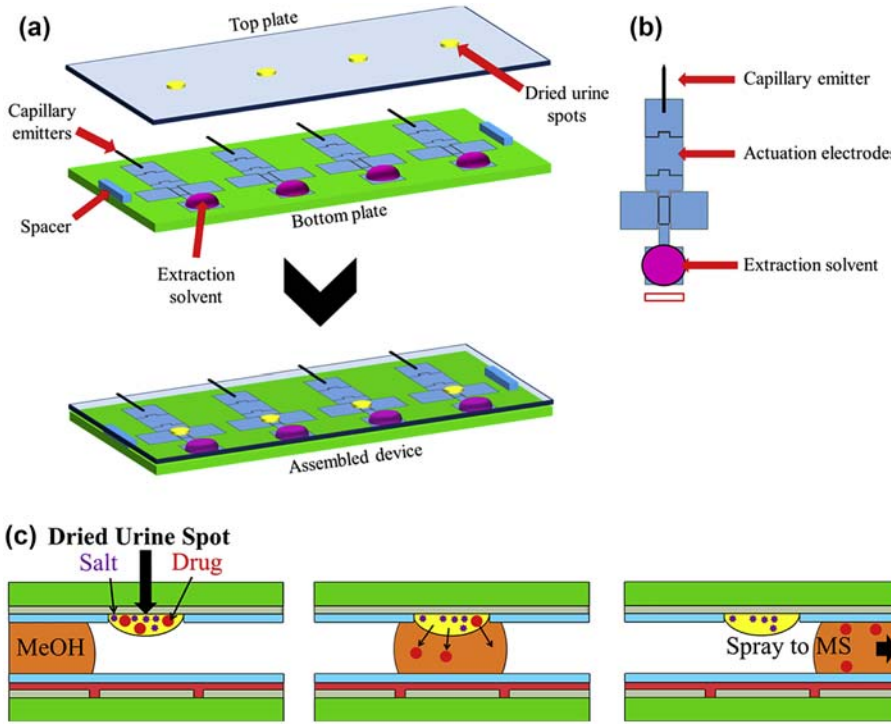

Figure 7.14 Digital microfluidic device used for extraction of drugs from dried urine. (a) Three-quarter view schematic of device, which features four independent DMF modules mated to pulled-glass capillary nanoelectrospray ionization emitters for direct analysis by tandem mass spectrometry. Urine is affixed and dried onto hydrophilic anchors located on the top plate. When assembled, the top and bottom plates are separated by a 360 μm thick spacer. (b) Top-down schematic of a single module, which features five 7 mm × 7 mm, two 2 mm × 5 mm, and one 7 mm × 5 mm actuation electrodes. The red outlined scale bar is 7 mm. (c) Side-view scheme (left-to-right) of sample cleanup illustrating the selective extraction of drug from dried urine (Kirby et al., 2014).

a "metabolomics-on-a-chip" approach to test secondary drug toxicity in bioartificial organs (Choucha Snouber et al., 2013). A microfluidic biochip was utilized to investigate the metabolic response of HepG2/C3a cells subjected to an anticancer prodrug flutamide and its active metabolite hydroxyflutamide (HF) by nuclear magnetic resonance spectroscopy to determine cell-specific molecule-response markers. The metabolic response of flutamide led to a disruption in glucose balance and mitochondrial dysfunction, illustrated by a decrease in the extracellular glucose and fructose consumptions and tricarboxylic acid cycle activity. Their findings illustrated the potential of metabolomics-on-a-chip to be used as an in vitro alternative method to predict the toxicology or function of drugs. In addition, Gao et al. reported an integrated cell-culture based microfluidic device for high-throughput drug screening with an online electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-Q-TOF MS) (Gao, Li, Wang, & Lin, 2012). The multiple gradient

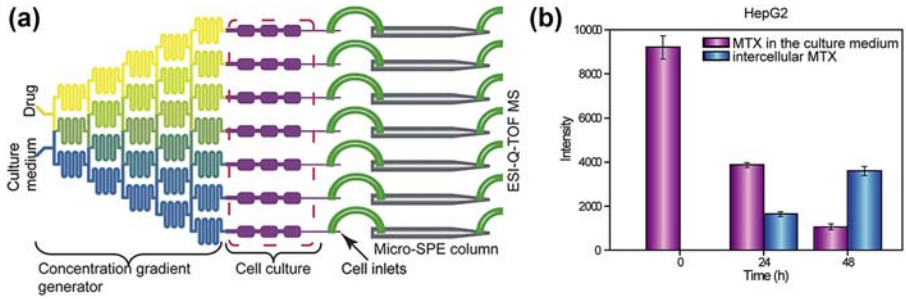

Figure 7.15 Integrated microfluidic device for drug absorption and cytotoxicity assays. (a) A schematic drawing showing the upstream concentration gradient generator, the downstream cell cultivation modules, and sample pretreatment module prior to ESI-Q-TOF MS detection. (b) Time-dependent accumulation of intracellular MTX in HepG2 cells on the microfluidic device (Gao et al., 2012).

generator was then coupled to an array of microscale cell culture chambers and on-chip solid-phase extraction (SPE) columns for sample pretreatment prior to mass analysis (Fig. 7.15). Drug absorption and cytotoxicity could be simultaneously determined using this integrated system. The device was composed of two functional parts: one part with upstream drug gradient generators and downstream cell culture chambers where liquid diffusion and mixing, cell cultivation, cell stimulation, drug absorption, and drug-induced cytotoxicity assay could be achieved, whereas the other had an integrated on-chip SPE column for sample clean-up and concentration process before the MS examination. To be a proof of concept, the absorption of methotrexate and its effects on HepG2 and Caco-2 cells were investigated, where the percentage of apoptotic cells appeared to be drug dose dependent. Comparing the results from ESI-Q-TOF MS analysis with the cytotoxicity assay, it was found that high intracellular drug concentrations increased cell cytotoxicity. Overall, this integrated system provided an easy online tool to screen drugs rapidly with low drug consumption, high throughput, and high sensitivity, thus accelerating the development of new effective and safer drugs.

In summary, microfluidic devices can play a crucial role in detecting drugs and pharmaceuticals and can be routinely used in chemical, pharmaceutical, and clinical settings with high precision and economical effectiveness.

7.3.2 Microfluidics for diagnostic purposes

The conventional diagnostic techniques based on sophisticated macroscopic equipment such as gas chromatography−mass spectrometry are only feasible in large air-conditioned laboratories, which are equipped with trained workforce and devices for sample handling, together with ample ancillary resources needed for efficient diagnosis. But, this is not attainable outside the realm of these laboratories, especially in the developing world, particularly in rural areas (Lee, Kim, Chung, Demirci, & Khademhosseini, 2010; Yager et al., 2006). Miniaturized versions of analytical platforms have been recently conceptualized, primarily based on microfluidic

technology, to perform diagnostic analysis of metabolites and biomarkers associated with diseases, consuming minimal amounts of reagents, with high efficiency and speed, making the device a portable point of care, self-usable system. This not only reduces the logistic issues with sample handling and transfer but also provides patients with the luxury of testing for various markers, such as blood glucose, in the comfort of their homes, which is particularly suitable for geriatric patients. These microfluidic diagnostic devices may reduce the healthcare costs associated with diagnosis.

Microfluidic biosensors, as they are commonly known, have been fabricated for a variety of purposes. Particularly important among these are on-chip enzymatic assays. As enzymes have the potential of converting a large amount of substrate molecules into product in a fraction of a second, in a highly selective manner, they offer an exciting avenue for chemical analysis. Enzyme assay on a chip may be either homogenous or heterogeneous. In the former, all the reactants are in solution phase (Hadd, Raymond, Halliwell, Jacobson, & Ramsey, 1997) while in the latter, either of the enzyme/substrate/inhibitor needs to be immobilized on a solid surface (Krenkova & Foret, 2004; Mao, Yang, & Cremer, 2002). In some cases, enzyme immobilization is carried out on microchannel walls or onto some support inside the channels. This provides the advantages of enzyme recycling, placement of enzyme at specific locations on microchannels, and analysis in a continuous flow environment, make immobilized assays a preferable choice (Kim, Lee, & Koh, 2009).

Enzyme immobilization has been more carried out on microspheres, also known as microbeads, due to their similar size properties to microchannels, as well as large surface area for enzyme attachment (Peterson, 2005). They offer the advantage of being contained at appropriate locations by using mechanical barriers or magnetic devices. Kim et al. developed a microfluidic device for glucose detection. The device consisted of two separate chambers, for reaction and detection. In the reaction chamber, microbeads were covalently bound to enzyme, glucose oxidase and were supported by microfilters. A poly(ethylene glycol) based microarray (fabricated by photolithography) encapsulating a horseradish peroxidase formed the detection chamber. The bienzymatic reaction was used to detect the conversion of non-fluorescent substrate (Amplex Red fluorescence indicator) to a fluorescent resorufin, with glucose concentrations in the range of 1−10 mM detected successfully by fluorescence microscopy and quantified by a software (Kim et al., 2009). In a modified version, Sheng et al. used glucose oxidase modified magnetic nanoparticles, constrained in the microchannel with the aid of external magnetic field, for the amperometric analysis of glucose. The device offers a simple alternative to other such devices, as no mixing is needed, achieving higher sensitivity. Detection was linear with a range between 25 μM and 15 mM. The device also possessed a separation channel that avoided the entry of macromolecules, thereby eliminating the need to preprocess the sample. This allowed for the serum samples to be directly used for glucose analysis (Sheng, Zhang, Lei, & Ju, 2012). Other devices integrating enzyme and immunoassays were fabricated for simultaneous detection of glucose and insulin (Wang, Ibanez, & Chatrathi, 2003).

Microfluidic-based devices have been commonly used in the detection of disease biomarkers including metabolites (e.g., glutathione (GSH)), enzymes, pathogens

(e.g., bacteria and DNA), and even cells. Before the widespread use of diagnostics for assessment of complex diseases, it was necessary to develop sophisticated bioassays capable of quantitatively analyzing disease biomarkers (Herr et al., 2007). GSH is an intracellular thiol-containing tripeptide which is a biomarker of oxidative stress (OS) and an important antioxidant in the cells of organisms. The rapid and accurate detection of GSH content is important for the early diagnosis and prevention of diseases. Hao et al. established an effective and rapid method based on microfluidics and LIF detection to analyze the intracellular constituents and GSH in single cells (Hao, Liu, Zhang, Li, & Jing, 2014). A hydrostatic pressure approach was utilized to inject the hepatocytes, and a low electrical potential was used to drive the single hepatocyte to the detection point. By the modification of the microfluidic device surface and optimization of injection voltage and separation, the analytes can be measured with high efficiency (e.g., 10 s for single cell analysis) smaller reagent volumes and less waste production.

Besides such small molecules, nonimmunoassays have also been employed in the diagnosis of macromolecules. Electrochemical methods of detection are recognized as one of the most sensitive as they do not involve any label tagging for studying the fate of biological compounds. Electrodes serving as sensors have found their niche in medical diagnostics due to relative ease of fabrication and integration with analytical devices. With several markers being pliable for electrochemical analysis, many methods of electrode fabrication have been pursued (Quinton et al., 2011; Wartelle, Schuhmann, Blochl, & Bedioui, 2005). Screen-printed electrodes (SPEs) based on carbon are another form of electrochemical analytical device that has been studied for the detection of chemicals like nitric oxide (Miserere et al., 2006) and biomarkers for cancer (Wan et al., 2011). They are useful for electrochemical immunosensor assays as they offer advantages such as low background current, ease of chemical modification on carbon surface, and relative inertness of carbon-based materials. A variety of materials such as nylon, glass, alumina, organic films have been used for electrode fabrication and present an interesting alternative for point-of-care testing (Miserere et al., 2006; Schuler, Asmus, Fritzsche & Moller, 2009).

Recently, Yan et al. fabricated SPEs on vegetable parchment as a substrate for disposable immunosensor fabrication in detection of prostate-specific antigen (PSA) (Fig. 7.16). Electrodes were printed from carbon and silver/silver chloride ink. The device was then integrated to a paper-based microfluidic device, to absorb detection solution and immersing the electrodes in electrolyte. The immunosensor was fabricated by coating the SPEs with a sheet of functionalized graphene, containing the enzyme-linked antibody on gold nanoparticles. The assay could detect PSA as low as 2 pg/mL and presents itself as a suitable method for detecting potential disease specific biomarkers, allowing for early diagnosis of a disease (Yan, Zang, Ge, Ge, & Yu, 2012). Shin et al. designed an aptamer-based electrochemical biosensor integrated with a microfluidic platform for online detection of secreted protein biomarkers from an organ-on-a-chip device (Shin et al., 2016). The sensor was modified with aptamers specific to a cardiac injury biomarker in extremely low abundance, the creatine kinase (CK)-MB, by electrochemical impedance spectroscopy (EIS) rather than immunoassay. Interestingly, the application of aptamers as the antigen receptors significantly

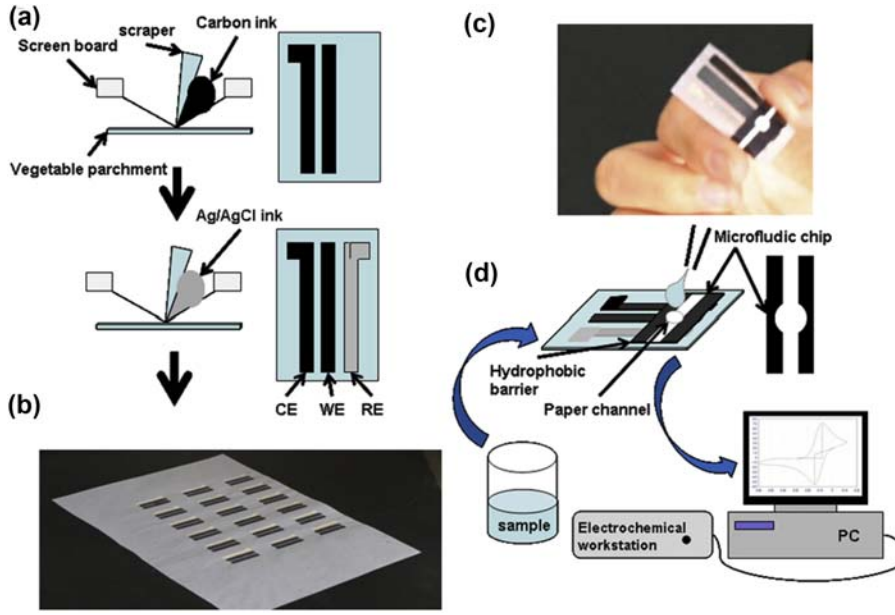

Figure 7.16 Screen printed electrodes as microfluidic platforms for immunosensor applications. (a) Carbon and silver/silver chloride electrodes were printed on a sheet of vegetable parchment, with WE, working electrode; RE, reference electrode; CE, counter electrode. (b) A sheet of vegetable parchment with 18 electrodes. (c) A hydrodynamic paper-based electrochemical sensing device for the measurement of prostate-specific antigen (PSA). (d) The detection processes of PSA (Yan et al., 2012).

increased the sensitivity and shelf life of the biosensor compared to antibody-based biosensors. They also believe that the unique microfluidic electrochemical biosensor based on the aptamer-capturing mechanism paves the way for measuring a wide variety of other biomarkers of interest.

Dried blood spot (DBS) samples on filter paper have emerged in popularity as a sampling and storage vehicle for a wide range of clinical and pharmaceutical applications. Shih et al. reported a DMF coupled to nanoelectrospray ionization mass spectrometry (nESI-MS) for the quantification of succinylacetone, a marker of hepatorenal tyrosinemia in DBS samples (Shih et al., 2012) (Fig. 7.17). The new system was fabricated by sandwiching a pulled glass capillary emitter between two DMF substrates. Droplets are driven in the DMF device by applying AC fields between electrodes on the top and bottom plates. When a droplet touches the inlet of the capillary, it spontaneously fills by capillary action in seconds. Then, a DC potential is applied between the top-plate DMF electrode and the mass spectrometer to generate a spray for MS analysis. The system was validated by application to on-chip extraction, derivatization, and analysis of succinylacetone with comparable performance to gold-standard methods.

Many bacteria-induced infectious diseases exhibit similar symptoms, such as common digestive and respiratory-borne ailments. However, it is difficult to identify

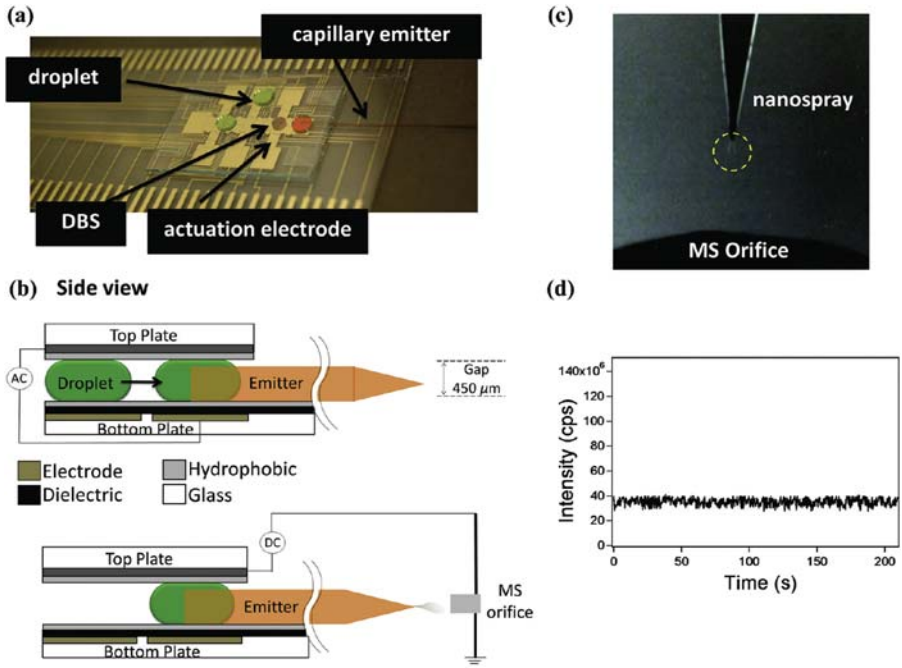

Figure 7.17 Digital Microfluidics−nanoelectrospray Ionization-Mass Spectrometry (DMF−nESI-MS) interface. (a) Image of a device (bearing colored droplets and a punched dried blood spot sample) mated to a capillary emitter. The contact pads on the sides of the device mate with a 40-pin connector for automated droplet control. (b) Side-view schematics. (top) AC electric potentials are applied between the top and bottom substrates to actuate the droplets. (bottom) DC electric potentials are applied between the top plate and the MS orifice to generate a nanoelectrospray. (c) Image of spray generated at the tip of the capillary emitter. (d) Total ion count as a function of time from a 15 μL droplet of tyrosine (5 μM). The spray was stable for >200 s, with an RSD of 7.3% (Shih et al., 2012).

the pathogenic bacteria solely based on these clinical presentations, resulting in delayed treatment and symptom deterioration. Therefore, a fast and accurate diagnostic method is critical for effectively identifying bacterial pathogens to facilitate the selection of appropriate treatment. Xia et al. has reported a rotate & react SlipChip (RnR-SlipChip) for simultaneous visual detection of multiple bacterial pathogens by LAMP (Xia et al., 2016). The device was composed of two round PDMS-glass hybrid chips that were coaxially aligned by a plastic screw-nut suite. One-step rotation after the sample loading allowed immediate mixing and reaction of multiple bacteria samples with LAMP reagents on the chip. After the optimization of LAMP conditions, a fluorescent signal-to-noise ratio of about 5-fold and a detection limit of 7.2 copies/μL genomic DNA were achieved, while five common digestive bacterial pathogens including *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica*, *Vibrio fluvialis*, and *Vibrio parahaemolyticus* were visually identified in 60 min with a relatively high success rate. In addition, Hsieh et al. reported an integrated microfluidic platform

for the fast, sensitive, and quantitative detection of pathogenic DNA (Hsieh, Patterson, Ferguson, Plaxco, & Soh, 2012). The platform relied on electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP), a powerful alternative to polymerase cycling assembly (PCR) with greater sensitivity, accuracy, reaction speed, and amplicon yield. The real-time, quantitative electrochemical detection of LAMP amplification was achieved by monitoring the intercalation of DNA-binding methylene blue (MB) redox reporter molecules into newly formed DNA molecules with a set of integrated electrodes including two platinum counter and reference electrodes and a gold working electrode. Highlighting the advantages of real-time electrochemical detection and LAMP within a microfluidic device, the MEQ-LAMP method omits the usage of bulky and sophisticated optical detectors and temperature controls while ensuring robust microfluidic DNA amplification, thus making it a promising method for the diagnosis of other nucleic acid−based biomarkers, such as viral RNAs.

In contrast to antibody-free assay mentioned above, immunoassay is largely employed in the detection of macromolecular biomarkers for diagnosis purposes. Herr et al. designed a microchip-based device for the detection of disease biomarkers in human saliva. The device called microchip electrophoretic immunoassay (μCEI) was provided with molecular sieves fabricated using hydrogel, to enrich the sample, followed by electrophoretic separation to resolve a fluorescent antibody bound to an enzyme, responsible for tissue decay. Using 20 μL of saliva, they demonstrated rapid (<10 min) measurement of the collagen-cleaving enzyme matrix metalloproteinase-8 (MMP-8) in saliva from healthy and periodontally diseased samples. Using this method, they could dispense the need for using matched antibody pairs as well as to immobilize the antibody (Herr et al., 2007).

Enzyme-linked immunosorbent assay (ELISA) has been the mainstay of clinical diagnostics for detection of disease-related macromolecular biomarkers. However, the conventional macroscale ELISA protocols are laborious, sluggish, requiring multiple reagent addition and washing steps, often resulting in inconsistent results due to manual glitches. Furthermore, commercially available ELISA kits as well as instruments used are costly (Lai et al., 2004). Chip-based ELISA methods offer the advantage of faster antigen−antibody reaction with the consumption of significantly less reagents (Cesaro-Tadic et al., 2004; Murakami et al., 2004). Microfluidic ELISA platforms have been researched in great detail in the past decade (Herrmann, Veres, & Tabrizian, 2006; Holmes, She, Roach, & Morgan, 2007). Lee et al. developed a fully automatic ELISA platform for detecting antigen and antibody for hepatitis B virus, on a disposable plastic disc, made of poly(methyl methacrylate), having arrangements for conducting immunoassays from whole blood. The device had facilities for plasma separation and chambers for storage of buffers, reagents, substrates, collection of waste, mixing the reagents, and detection of the product (Fig. 7.18). Using just 150 μL of blood, the assay could be performed as opposed to double this volume in conventional methods, whereas the whole assay could be carried out in 30 min, whereas conventional well-plate based ELISA yields result in a minimum of 2 h (Lee et al., 2009). Miniaturization did not compromise the sensitivity of the device, and similar detection limit could be achieved. Recently, they developed an advanced chip to carry

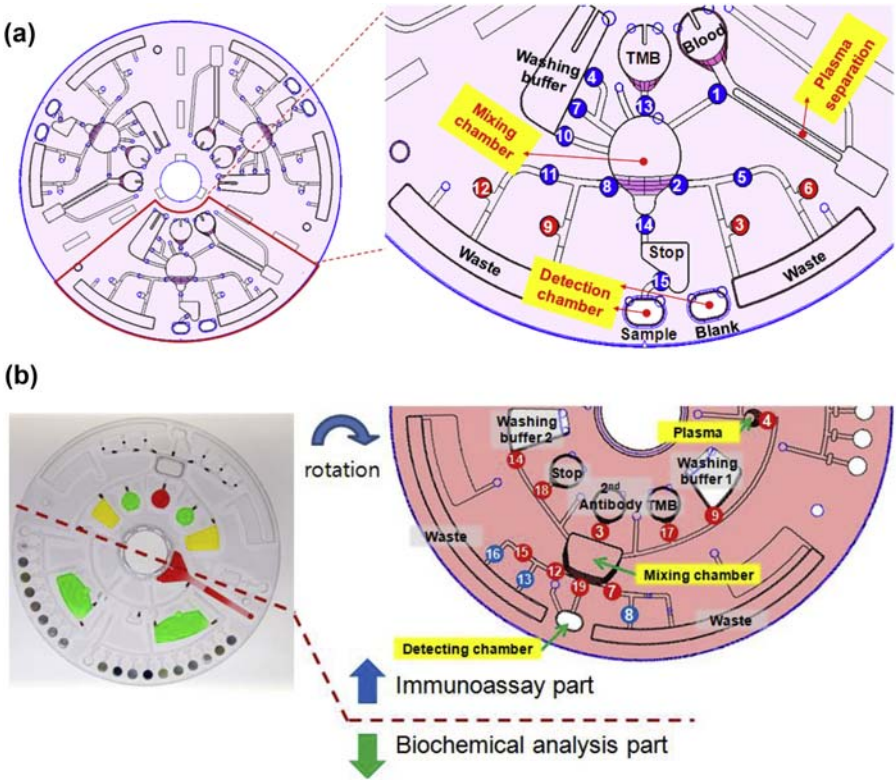

Figure 7.18 Disc-based immunoassays. (a) Disc design showing the detailed microfluidic layout and functions. The number indicates the order of the laser irradiated ferrowax microvalve (LIFM) operation (Lee et al., 2009). (b) Photograph of a disc. Detection wells on the clinical chemistry side are preloaded with lyophilized reagents. Other chambers for liquid type reagent are loaded with food dye solution for demonstration. In the right-hand side, the disc design shows the detailed microfluidic layout. The number indicates the order of the LIFM operation. The top half of the disc for the immunoassay part is rotated for easier demonstration. The blue circles with numbers are (NO)-LIFM. The other half of the disc for the clinical chemistry analysis part is shown in the bottom (Lee et al., 2011).

out the immunoassay as well as biochemical assessment of whole blood. The chip had automated arrangement for plasma separation, mixing, incubation, and detection. The freeze dried reagents for both assays were stored in dedicated compartments, and the detection was carried out by optical measurement at 10 different wavelengths to accommodate various reactions (Lee et al., 2011). Their group has also developed a multiplexed immunoassay, based on three different biomarkers to improve detection efficiency (Park, Sunkara, Kim, Hwang, & Cho, 2012).

As a basic criterion, the point-of-care diagnostic devices meant for the developing world must be inexpensive and integrated, dispensing the services of ancillary equipment (Mabey, Peeling, Ustianowski, & Perkins, 2004; Martinez et al., 2008).

In the wake of this cost consideration, paper-based microfluidic devices offer a potential alternative to glass/polymer-based open channel microsystems. These devices can also enable a multitude of sample outlets from a single inlet, ensuring simultaneous analytical assays, without the need for an external pumping device. Being light in weight and easy to stack makes their shipment logistically much easier than glass/polymer-based devices. Paper-based microfluidic devices have been researched in detail by the laboratory of George Whitesides at Harvard, termed as 3D microfluidic paper analytical devices (μPAD) (Bruzewicz, Reches, & Whitesides, 2008; Martinez, Phillips, Butte, & Whitesides, 2007; Martinez et al., 2008; Martinez, Phillips, & Whitesides, 2008). In addition, Dou et al. reported a versatile and low cost PDMS/paper-hybrid microfluidic device coupled to LAMP for the fast, sensitive, and instrument-free detection of the main meningitis-causing bacteria, *Neisseria meningitidis* (*N. meningitidis*) (Dou, Dominguez, Li, Sanchez, & Scott, 2014). The chip was composed of one top PDMS layer, one middle PDMS layer, and one glass slide for reagent delivery, LAMP reaction, and structure support, respectively. Chromatography paper was used in between as a 3D substrate for the prestorage of DNA primers for LAMP reactions to improve the detection sensitivity. This versatile hybrid system provided not only on-site qualitative diagnostic analysis but also confirmatory testing and quantitative analysis in laboratory settings with a detection limit of three copies per LAMP zone, which was relatively close to single-bacterium detection sensitivity. Furthermore, simple pathogenic microorganism detection was achieved without a laborious sample preparation process or the use of centrifuges, making it a promising point-of-care (POC) diagnosis for a broad spectrum of infectious diseases, especially for developing countries.

Noncommunicable diseases (NCDs) are now leading cause of global mortality, thus there is a growing need for cost-effective and noninvasive methods to diagnose and treat this class of disease. To this end, Warren and coworkers developed a paper-based diagnostic device with tailored synthetic biomarkers specific to colorectal cancer and thrombosis, a representative solid tumor and cardiovascular disorder, respectively (Warren, Kwong, Wood, Lin, & Bhatia, 2014). These synthetic biomarkers were composed of nanoparticles chemically modified by ligand-encoded reporters via protease-cleavable peptides. The nanoparticles passively target diseased sites in vivo, such as solid tumors or blood clots, where upregulated proteases cleaved the peptides and released reporters that were detectable by paper lateral flow assay (LFA) in the urine. LFAs utilize the sandwich complex to capture antibodies that are adsorbed onto a highly porous test strip which wicks fluids and conveys the analytes to the capture regions. The immobilized analytes are then visualized by a detection probe coupled to NPs (typically gold or latex nanospheres) that generate a colored line visible to the eye without enzymatic amplification. With over 500 proteases encoded by the human genome, this technique could be further tailored for the diagnosis of additional NCDs such as fibrosis and inflammation, as well as infectious diseases including malaria and hepatitis B to provide facile and cost-effective diagnostics for global health. Microfluidic devices have also been developed for diagnosis of lysozyme (Giuffrida, Cigliana, & Spoto, 2018), lysosomal storage

disorders (Shen et al., 2012), cancer (Chen, Bai, & Chang, 2011; Das et al., 2015; Hindson et al., 2011; Lien et al., 2010; Piraino, Volpetti, Watson, & Maerkl, 2016; Tang, Vaze, & Rusling, 2012), H1N1 influenza (Lee et al., 2012), herpes simplex virus (Zubair et al., 2011), and Johne's disease in cattle (Wadhwa, Foote, Shaw, & Eda, 2012) at the molecular level. In principle, all microfluidic diagnostic devices consist of a molecular sensing unit coupled to a signal converter (transducer) that aptly reads out the results quantitatively. An elaborate review on different mechanistic approaches of biosensors is provided by Mohanty and Kougianos (2006). Since the biosensors vary greatly in their design and hence their sensitivity and efficiency, the readers are referred to a few state-of-the-art reviews for more detailed information (Choi, Goryll, Sin, Wong, & Chae, 2011; Mohammed & Desmulliez, 2011).

Beyond the molecular level, microfluidics-based systems have been widely used for the detection of cells and subcellular level vesicles (e.g., exosomes) in disease diagnosis, especially for tumors (Garcia-Cordero & Maerkl, 2020). Tumor cells can dissociate from primary and metastatic tumor sites and travel through the bloodstream in single or clusters of tumor cells, also known as circulating tumor cells (CTCs). Isolation of CTCs from the peripheral blood of patients has emerged as a valid alternative source of tumor tissue that can be utilized for molecular characterization of diseases (Kirby et al., 2012). The aim of CTC diagnostic is to retrieve single or clusters of CTCs in reasonable numbers with high purity from large volumes of whole blood (>5 mL) and at low shear stress to minimize cell damage (Garcia-Cordero & Maerkl, 2020). Initially, magnetic-based cell separation systems were widely developed and took advantage of antibody−antigen interactions to bond an antibody-decorated magnetic particle to a cell via its surface antigens. The FDA approved a macroscale immunomagnetic isolation system called CellSearch for CTCs of metastatic cancers such as breast, colorectal, and prostate cancers (Li, Stratton, Dao, Ritz, & Huang, 2013). The first microfluidic technology to capture CTCs from whole blood was called the "CTC-chip," which was reported by Nagrath and coworkers who made use of microposts coated with antibodies against EpCAM (Nagrath et al., 2007). In addition, magnetic-based microfluidic systems are more involved in the isolation and detection of CTCs. For example, Issadore and coworkers used the Hall effect to detect and count magnetically labeled CTCs (Issadore et al., 2012). A micro-Hall detector array was prepared on a substrate, while a microfluidic channel was bonded on top of the Hall detectors. A blood sample was first focused by the flow-focusing configuration of the microfluidic channel to establish a single stream of cells. As the cells moved over a Hall detector, each magnetically labeled CTC induced a Hall voltage and was, thus, counted. This device exhibited a higher CTC detection sensitivity than the CellSearch system and a high throughput of 10^7 cells per minute. A microfluidic magnetic disc has also been used to separate rare, circulating endothelial cells (CECs) from peripheral blood mononuclear cells (Chen et al., 2011). These CECs have been associated with many diseases, and the low CEC concentration in blood impedes their detection. A magnetic disc was used to trap the cells attached to immunomagnetic beads. Human umbilical vein endothelial cells (HUVECs) were used as a model for CECs and stained with anti-CD146-phycoerythrin antibody, which was tagged to antiphycoerythrin magnetic beads that

attracted these cells to the magnetic disc. This magnetic disc had an inlet channel, connecting channels, and a waste reservoir. When the disc was rotated, the centrifugal force propelled away the nonmagnetic cells through the connecting channels to the waste reservoir, effectively retaining just the target cells (HUVECs) in the inlet reservoir.

Besides magnetic separation, affinity chromatography also makes use of antibody–antigen interactions to directly capture the CTCs, without the need of magnetic labeling. Generally, antibodies are conjugated to the surfaces of solid structures, which are then immersed in a fluid of biological sample where the antigens of targeted cells bond with the antibodies. To enhance the binding efficiency, the interactions between CTCs and antibody-modified surfaces have been featured by microstructures or nanostructures to one of the channel walls (Park et al., 2016; Sheng et al., 2014; Stott et al., 2010; Yoon et al., 2013).

Cells are often isolated from an ambient sample by means depending on their physical properties including stiffness, size, and density. Such physical property–based separation systems are advantageous in terms of label-free sorting, high system throughput, and low cost (Mao & Huang, 2012). Hur et al. designed a high-throughput and label-free platform for cell isolation and enrichment from heterogeneous solution using cell size as a criterion (Hur, Mach, & Di Carlo, 2011). This technique utilized a cell isolation mechanism in parallel expansion–contraction trapping reservoirs through the irreversible migration of particles into microscale vortices.

After the cell capture, CTCs were subjected to either immunostaining on-chip, analysis of surface, and intracellular signaling proteins by western blots, or release for off-chip analysis (Garcia-Cordero & Maerkl, 2020). In particular, Kirby et al. developed a geometrically enhanced differential immunocapture (GEDI) microfluidic device that coupled an antiprostate specific membrane antigen (PSMA) antibody to a 3D geometry of channel that specifically arrested CTCs with low nonspecific binding of leukocyte (Kirby et al., 2012). The GEDI device achieved higher sensitivity compared to the commercially available CellSearch system. The patient-derived CTCs were also subjected to on-chip drug treatment with docetaxel and paclitaxel, where CTCs of docetaxel-resistant patients did not show any evidence of drug activity. These measurements stood as the first functional assays of drug-target engagement in living CTCs. Chen et al. reported a novel microfluidic platform integrated with cell recognizable aptamer-encoded microwells for isolating single tumor cells with satisfied single-cell occupancy and unique bioselectivity (Chen, Wu, Zhang, Lin, & Lin, 2012). After the optimization of microwells, the single-cell occupancy was significantly increased from 0.5% to 88.2% due to the aptamer, which enabled the analysis of single-cell enzyme kinetics for the target cells in short time periods (5.0 min) and small volumes (4.5 mL). Microfluidics-based cell isolation has also been coupled to other detection techniques for facile detection. Pallaoro and coworkers reported a detection platform that combined microfluidics with SERS for the continuous identification of individual cells in a microfluidics channel (Pallaoro, Hoonejani, Braun, Meinhart, & Moskovits, 2015). They specifically designed SERS biotags (SBTs), which were based on a silver nanoparticle dimer modified by a Raman active reporter molecule and an affinity biomolecule, providing a unique label where SERS

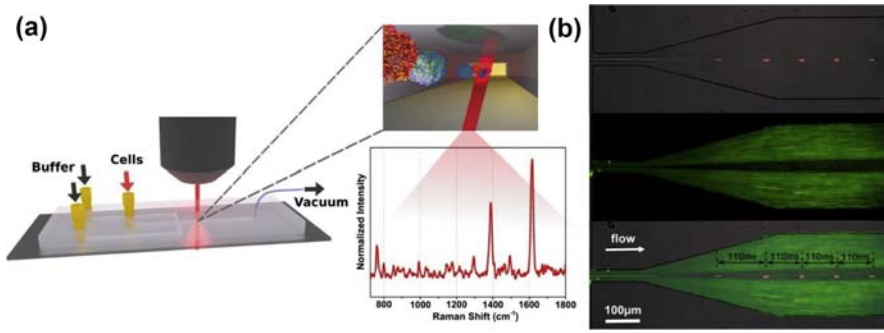

Figure 7.19 Graphical depiction of device layout and flow dynamics. (a) Schematic of setup and concept. Cells, prelabeled with a cocktail of cancer-specific (NRP) and control (UC) SBTs (the latter binding both cell types), are injected into the device, where they are flow-focused before passing through the Raman laser. (b) Simultaneous bright-field and epifluorescence (Cy3 channel, colorized orange) image of a single cell in the channel as a function of time illustrating the efficacy of flow focusing (top). Epifluorescence image (FITC channel, colorized green) of 200 nm polymer beads separately injected into the buffer channels to highlight the sheath flow (center). Montage merging the two former images (bottom), showing the overall flow dynamics in the device (Pallaoro et al., 2015).

spectrum could be deconvoluted (Fig. 7.19). SBTs were incubated with a mixture of cancerous and noncancerous prostate cells before being injected into a flow-focused microfluidic channel and forced into a single file. Cancer cells passing through the focused laser beam in the downstream were successfully identified among a large proportion of noncancerous cells by their Raman signatures. This technique achieved reliable results from all the cell mixture ratios tested, the lowest being one in 100 cells.

Exosomes are a subset of extracellular vesicles produced by tumor cells and nonmalignant cells which contain mitochondrial DNA, RNA, proteins, lipids, and metabolites (Poudineh, Sargent, Pantel, & Kelley, 2018; Simpson, Lim, Moritz, & Mathivanan, 2009). Exosomes are ubiquitous in most body fluids and are present in the circulation at early stages of cancers, making them potential cancer biomarker candidates (Dong et al., 2019). The typical exosome analysis workflow requires the isolation and quantitation of exosomes followed by the characterization of intravesicular and extravesicular contents, size, and morphology (Im et al., 2014; Zhang et al., 2018). The investigation of exosome contents at the molecular level employs a broad spectrum of techniques including western blotting, immunoassays, qRT-PCR, sequencing, flow cytometry, mass spectrometry, and many more (Garcia-Cordero & Maerkl, 2020). But current techniques for isolating exosomes (e.g., ultracentrifugation, filtration, and precipitation) are laborious due to incorporating multiple steps and are limited by low purities, especially when dealing with raw biological fluids that inherently contain a high proportion of proteins, other EVs, and cells with similar physical and/or biomolecular characteristics as exosomes (Contreras-Naranjo, Wu, & Ugaz, 2017). To this end, many studies have investigated microfluidic methods for exosome capture and analysis which are mostly based on immune-affinity (He, Crow, Roth, Zeng, & Godwin, 2014; Liu et al., 2018; Zhang et al., 2018; Zhao,

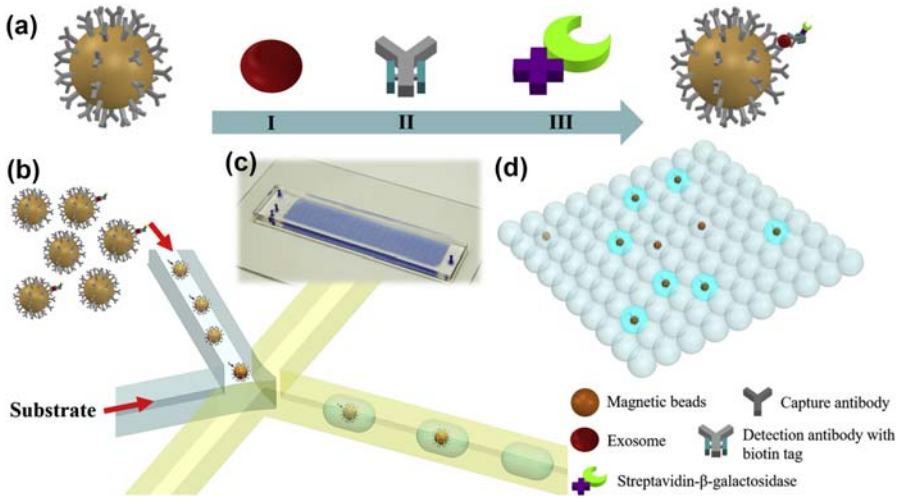

Figure 7.20 Schematic showing the droplet digital ExoELISA for exosome quantification. (a) Single exosome immunocomplex constructed on a magnetic bead. (b) Substrate and beads are coencapsulated into microdroplets. (c) Droplet digital ExoELISA chip. (d) Fluorescent readout for counting the positive droplets with the target exosomes (Liu et al., 2018).

Yang, Zeng, & He, 2016). In particular, Liu et al. reported an immunosorbent assay for digital qualification of target exosomes with the help of droplet microfluidics (Liu et al., 2018) (Fig. 7.20). The exosomes were immobilized on magnetic microbeads by forming sandwich ELISA complexes that were labeled with an enzymatic reporter producing a fluorescent signal. The microbeads were then isolated and encapsulated into enough droplets to ensure only a single bead was present in each droplet. This droplet-based single-exosome-counting enzyme-linked immunoassay (droplet digital ExoELISA) approach allowed the absolute counting of cancer-specific exosomes with an unprecedented accuracy and a minute limit of detection (LOD) of 10 exosomes per microliter ($\sim 10^{-17}$ M).

In addition to immune-affinity assay, size-based chromatography method has also been utilized in microfluidic systems for exosome isolation (Davies et al., 2012). Particularly, the herringbone chip (EVHB-Chip) is an exemplar model of microfluidic exosome isolation. This chip is capable of processing several milliliters of serum and capturing extracellular vesicles by its nanostructured surface with better performance than ultracentrifugation and magnetic beads, demonstrated by 94% tumor-extracellular vesicle (EV) specificity, a LOD of 100 EVs per μL sample, and a 10-fold increase in tumor RNA enrichment (Reategui et al., 2018). Other label-free microfluidics-based techniques include exosome trapping by nanowires (Wang et al., 2013), nanopillar-based sorting (Wunsch et al., 2016; Zeming, Thakor, Zhang, & Chen, 2016), and viscoelastic flow sorting (Liu et al., 2017), as reviewed elsewhere (Contreras-Naranjo et al., 2017). In recent years, researchers have also developed integrated microfluidic platforms for both isolation and analysis of exosomes, including overall exosome levels (Liang et al., 2017; Woo et al., 2017; Zhang, He, & Zeng, 2016) and

the detection of disease specific subpopulations of exosomes (Im et al., 2014; Vaidyanathan et al., 2014), and the internal component analysis of proteins (He et al., 2014) and RNAs (Shao et al., 2015). The exosomes derived from serum, plasma, whole blood, or other biological fluids such as urine, were isolated and analyzed by surface biomarkers including CD9, CD63, CD24, CD81, and EpCAM with enhanced sensitivity as low as ~50 exosomes per μL in overall exosome level or 0.3 pg per mL for IGF-1R analysis.

In summary, microfluidic technologies have been widely applied in the diagnosis of various disease biomarkers at the molecular, subcellular, and cellular levels with great success. It is expected that microfluidic technology will play a crucial role in medical diagnostics in the coming years, essentially with the development of disposable, sample-to-result devices, making routine diagnosis a more personalized approach (Eicher & Merten, 2011; Foudeh, Fatanat Didar, Veres, & Tabrizian, 2012).

7.4 Examples of commercial microfluidic devices

The application of microfluidic devices has increased over the years as these devices now have a wide range of roles, such as examining cell behavior, studying signaling pathways and immune responses, and determining clinical efficacy of new treatments (Sinha, Subedi, & Tel, 2018). The initial designs of microfluidic chips were extremely complex, but now, numerous companies have manufactured their own version of simplified yet efficient microfluidic platforms.

Dolomite Microfluidics developed several different microfluidic platforms for drug assays. One platform is a direct write microfluidic platform with internal channels and a cloudy exterior (Shankles, Millet, Aufrecht, & Retterer, 2018). This platform requires minimal consumables to produce small-scale high-throughput screening (Gencturk, Mutlu, & Ulgen, 2017). Another platform is called the Micromixer Chip, a static mixer in a serpentine-design which generates advection. This platform exploits lamination of three input fluid flow streams which significantly decreases the mixing time required to complete diffusion of triblock copolymer and siRNA (Feldmann et al., 2017). Dolomite Microfluidics have successfully designed their products to improve reproducibility and particle size control for a wide range of users (Dolomite, 2020).

POC lab-on-a-chip devices have also improved and simplified POC diagnostics. Abbott Laboratories designed an i-STAT system device which analyzes blood chemistry by combining microfluidics and electrochemical detection (Volpatti & Yetisen, 2014). The handheld device is capable of quantifying analytes and performing immunoassays; thus the combined device increases patient satisfaction due to minimal wait times (Abbott, 2020). Claros Diagnostics Inc. have also designed a benchtop microfluidic device, consisting of three main components: a blood-collector device, disposable cartridge, and a reader. This device was created to be a time-efficient diagnostic device that can detect the elevated PSA levels in prostate cancer (Maj-Hes, Sevcenco, Szarvas, & Kramer, 2019). Only a low sample volume is required, and utilizing this device can significantly decrease the healthcare system cost and number of hospital

visits required by the patient. It has now been approved to be used as a prostate cancer diagnostic test in Europe and is seeking approval from the USFDA (MIT Technology Review, 2020).

Commercial automated microfluidic products for genomic analysis are also available. Fluidigm C1 rapidly isolates, processes, and profiles individual cells, thus only requiring one tool to both extract and analyze cell activity and responses (See, Lum, Chen, & Ginhoux, 2018). This integrated microfluidic system is an automated tool with high precision and accuracy (Sinha et al., 2018). Bio-Rad has a droplet microfluidic device called ddSEQ which successfully isolates and monitors thousands of genes per cell. This device can be coupled with Illumina, a sequencing system for single cell transcriptomic experiments to produce valid reads (Romagnoli et al., 2018).

7.5 Future trends

The future of microfluidic devices for applications in drug discovery appears bright with a lot of research activity being focused on the development of miniaturized chips. However, the concern remains about the integration of these devices with ancillary equipment including electrical accessories, pressure pumps, and platforms for analysis of samples. Due to their increased acceptance and potential benefits as economical alternatives to conventional benchtop macroscale equipment, it is important to develop integrated "everything-on-a-chip" systems that are widely accepted in all stages of drug discovery and development. This may entail an interdisciplinary effort from engineers and researchers working on fluid dynamics to design micropumps, analytical equipment manufacturers to scale down analysis systems to commensurate the chip size, and finally researchers working on microfabrication to further miniaturize the platforms and making them adaptable to these ancillary systems.

Industry has played a crucial role in this respect so far. Fluidigm Corporation, a venture by Dr. Stephen Quake from Caltech, has developed various platforms for microfluidic device integration. Based on the technology known as multilayer soft lithography, three dimensional structures can be created from elastomers to form integrated valves (NanoFlex), pumps, and channels. Besides this, Caliper Life Sciences also developed several automated/semiautomated robotics controlled liquid handling systems (Zephyr) that can be potentially integrated with microfluidic devices. These microfluidic integration tools are expected to aid microfluidic-based drug discovery by improving efficiency and scalability.

With the rate of approval of new drugs declining in the past few years and the pharmaceutical industry still lacking effective tools in discovering new drugs, microfluidic platforms come with a ray of hope, chaperoning routine assays in a more efficient manner and hopefully allowing more highly efficacious and safe drugs to be discovered.

On the other hand, miniaturized devices as diagnostic kits have made inroads into the households of many diabetic patients as blood glucose monitors. Miniaturized devices for other applications not only face fabrication and technical issues of being an integrated and comprehensive system, but they also face the challenge of patient

acceptance, having a direct interface with the end user. Although their acceptance is propelled by the convenience of use, the future of such devices for routine practice in other pathological conditions would rely on how the users perceive them. A sizable population, particularly in remote villages and tribal areas in developing world, lack good education, and training to use such devices might prove to be a daunting task. Moreover, it would require sincere effort on the part of clinicians and marketing professionals to persuade the patients in developed world, who have been so used to visiting a clinic to get their routine biochemical checkups, to adopt such self-usable devices. In addition, it must be ensured that such devices are safe to use and dispose, without causing any serious environmental hazards.

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