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Microfluidic devices for drug discovery and analysis

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Abstract: Microdevices, since their inception in the last decade of the twentieth century, have changed our view of science, due to their potential applications in fields ranging from optics, semiconductors and the microelectronics industry to drug discovery and development, point-of-care clinical diagnostics, sensitive bioanalytical systems and other areas of the biological sphere. In this chapter we review the potential applications of microfluidic platforms for drug discovery applications comprising high-throughput screening in target selection, lead identification/optimization and preclinical testing. Application of microfluidics in chemical analysis, as well as analysis of metabolites in blood for studying pathology, is also discussed.

Key words: microfluidics, drug discovery, high-throughput screening, drug analysis, point-of-care diagnostics.

7.1 Introduction

The ascent of microfabrication research and development at the turn of the century has opened several avenues for the biomedical sector. Micron-scale chips, with micrometre dimension channels, can be used to manipulate fluid flow at the micron/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 scale of these devices requires lower sample volumes (in nanolitres) than conventional microplate assays (requiring hundreds of microlitres), hence making them economical alternatives. The minute dimensions of the devices offer shorter diffusion path lengths, allowing faster analysis and precise control of fluid flow, and leading to specificity in chemical microreactors. Design manipulation, easily achievable by conventional lithographical and novel

nanotechnology techniques, provides versatility in mixing fluids that can be controlled by external physical forces such as magnetic and electric fields. These microdevices may either be integrated into existing macrodevices, or constitute comprehensive analytical systems by themselves. The miniaturization provided by these high-throughput devices allows for a large number of replicates on a small chip, enabling massive parallelization and thereby increasing efficiency and lowering costs (Lombardi and Dittrich, 2010). The fluid flow properties at microscale are very different from those at macroscale, and this can be exploited using microfluidic devices (Beebe *et al.*, 2002). These advantages make them an ideal choice in disciplines spanning across molecular analysis, biodefence programmes, and the discovery and development of new drugs in the 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 analytical techniques on glass/silicon substrates, provided proof of concept for their applicability (Harrison *et al.*, 1993; Manz *et al.*, 1990). Afterwards, chemical and biological sensors that could thwart the threats of bioterrorism, and aid in biodefence sample testing (Liszewski, 2003), were developed. With the surge in biotechnological methods, proteomics, genomics, and the discovery of protein-based therapeutics, microfluidics offers brighter prospects in DNA sequencing and genotyping as well as in protein separation and analysis (Chen *et al.*, 2010; Gomez, 2011). Microfluidic devices have also provided valuable opportunities for drug discovery and development processes, with their benefits at each stage from target identification (Malmstadt *et al.*, 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 the manufacturing stage (Szita *et al.*, 2005). Additionally, these devices have been used for improved confinement of cells in three-dimensional (3D) scaffolds, cell-based testing and cell component analysis. The cellular and molecular interactions at a scale proportional to their dimensions (Whitesides, 2003) are very different from those observed at macroscale volumes. An interesting application has been in the field of tissue engineering, whereby microfluidic platforms provide 3D scaffolds mimicking the natural environment for growth and mutual interaction between cells (Li *et al.*, 2012; Yamada *et al.*, 2012). They have also been investigated for transdermal and pulmonary delivery of drugs (Ashraf *et al.*, 2011; Yeo *et al.*, 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, and in the progress achieved by high throughput screening (which has allowed thousands of molecules to be

screened on a chip), followed by optimizing few lead molecules and assessing their pharmacokinetic and pharmacodynamic properties in preclinical systems. Later, 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 *et al.*, 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-based or animal 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, which is then filed as a new drug application for approval by regulatory agents such as the United States Food and Drug Administration (FDA). 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 go from bench to bedside, and it has been estimated to cost approximately 1 billion USD (Wu *et al.*, 2010). The attrition, from thousands of new chemical structures in the drug library, to a few lead compounds, to a single successful therapeutic agent, is a result of the inefficient procedures used in the conventional/current drug discovery and development process.

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

7.2.1 Identification of druggable targets

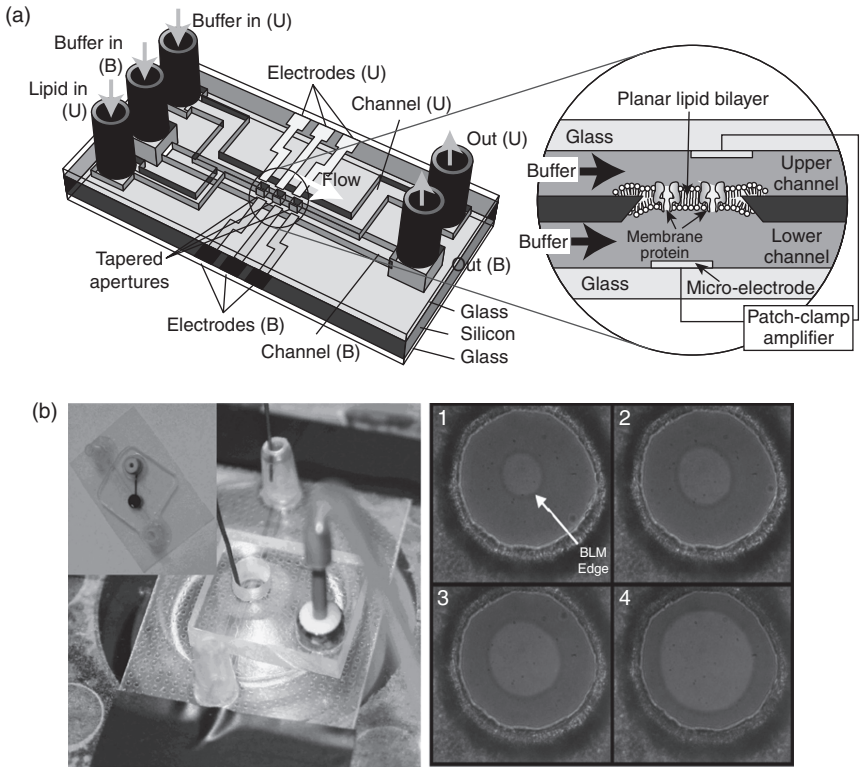
The process of drug discovery begins with the identification of the function of a potential drug target and comprehending its role in the disease process. Discovering pharmacological activities was conventionally carried out by testing various substances (usually plant extracts) in living organisms to observe the changes caused in a particular phenotype. However, towards the end of the twentieth 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 it has reduced the use of living organisms and living tissues (Terstappen *et al*., 2007).

Drug targets, which may be a cellular receptor, an ion channel, nucleic acids, DNA or RNA, enzymes, polysaccharides and lipids, are chemically well-defined molecular structures capable of interacting with therapeutic drug moieties (Imming *et al*., 2006). This interaction leads to downstream clinical effects. Common drug targets belong to the classes of kinases, proteases, phosphatases and G protein coupled receptors. Ion channel proteins represent another attractive target in the drug discovery paradigm, as they have been implicated in several cardiovascular and neuronal disorders (Dunlop *et al*., 2008). 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 *et al*., 2006; 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 *et al*., 2004). Target validation employing isolated membrane proteins and ion channels offers many technological challenges, as reproducing these nano-scale systems is very complex (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 (BLM) (Mayer *et al*., 2003). Micron-sized BLMs with integrated membrane proteins and ion channels are advantageous over macro-systems, providing economical and time saving analysis platforms. These BLMs bear remarkable electric sealing, and hence are amenable to recording 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. 7.1a). 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



7.1 Formation of bilayer lipid membranes (BLM) on microfluidic chips. (a) Conceptual diagram of a membrane fluid chip having fluid channels and apertures. Alternate flow of lipid and buffer solutions leads to formation of BLMs. (Suzuki *et al.*, 2004). (b) A microfluidic device with a channel extending 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 that appeared as a bright region in the centre (Sandison *et al.*, 2007).

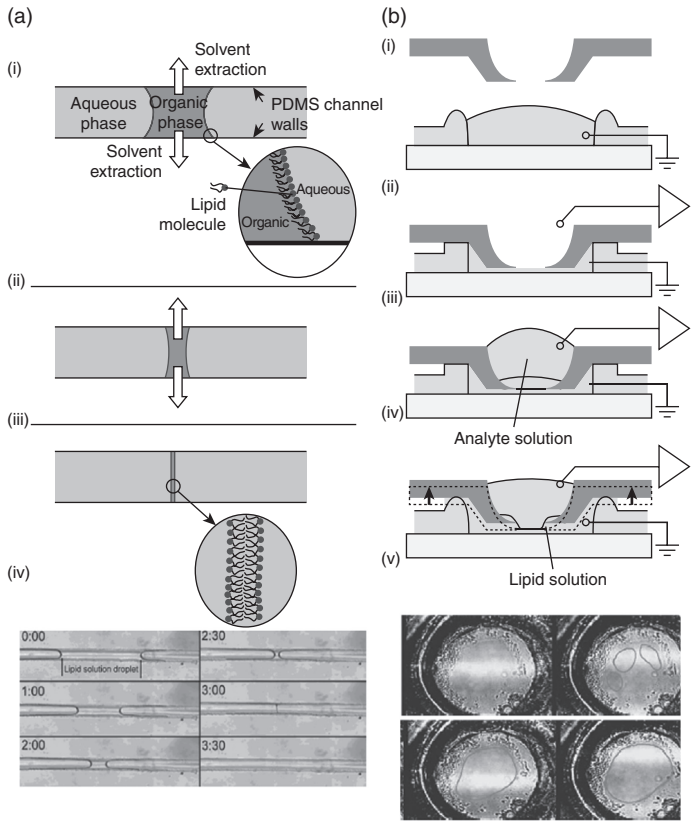
studies. However, silicon-based devices suffer from many disadvantages, including high dielectric loss of silicon leading to high electrical noise. Apart from that, the manufacture of silicon-based devices is time consuming, and the reproducibility of the BLMs is questionable. Other materials used for fabrication include epoxy photoresist (Cheng *et al.*, 2001), glass (Fertig *et al.*, 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 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 *et al*., 2006). One of the unique advantages of this microfluidic device is that it facilitates easy microscopic observation of the bilayer (Suzuki *et al*., 2007). Sandison *et al*. created microfluidic channels on PMMA-coated glass substrates by using hot embossing and laser micromachining (Fig. 7.1b). 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 the 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). A non-aqueous solution of the lipid was suspended in an aqueous flow stream through a microfluidic channel in PDMS. Solvent, being hydrophobic, partitions into PDMS, shrinking the lipid membranes together and forming a bilayer (Fig. 7.2a). Ide and Ichikawa developed a microfluidic device based on successive stacking of a glass slide, plastic sheet, PDMS spacer and agarose-coated coverslip (Fig. 7.2b). Lipid solution is first applied to the plastic aperture and sucked by vacuum to form a thin layer over the coverslip. Electrolyte was then added to the well and the aperture was moved towards 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 and 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 simpler and less time consuming manner than with previous



7.2 Formation of bilayer lipid membranes (BLM). (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 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 of time (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 and Ichikawa, 2005).

techniques. Typical gating phenomena were observed by changes in pH and membrane voltage in the outer membrane protein OmpF obtained from *Escherichia coli* (Kreir *et al*., 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.

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. They can also be used for determination of membrane properties under non-physiological 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 behaviour. Signal transduction has also been enormously researched with many companies having dedicated programmes for signal transduction based drug discovery (Anonymous, 2000). Enzymes such as tyrosine kinase play 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 non-stimulated counterparts. These results could have a marked impact on 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 been envisaged as a potential tool to identify specific targets. Recently, single cell analysis has gained considerable importance in microfluidics-based drug discovery, as these devices are able to perform manipulation, lysis, labelling, separation and quantification of the protein contents in a single cell (Huang *et al*., 2007). Although this technique is not amenable to live cell monitoring, it provides for simultaneous detection of multiple targets, combining higher sensitivity with higher throughput. Using a single cell analysis chip, the number of β_2 adrenergic receptors was determined. The integrated microfluidic chip facilitated cell and chemical handling, cell lysis, electrophoretic separation, and detection of lysate using laser-induced fluorescence (Gao *et al*., 2004; Wu *et al*., 2004). Separation of proteins and peptides has also been achieved on miniaturized electrophoretic cells (Schulze and Belder, 2012; Sikanen *et al*., 2012). Some of these techniques have been dealt with in greater detail in the subsequent section on analysis.

Understanding of the 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 *et al*., 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 particular biochemical event does not necessarily translate into lack of receptor activation. Other cellular components and events, such as second messengers, downstream processes, gene transcription and change in receptor configuration, have to be looked into. This, however, is not possible with the conventional assay procedures (Gurwitz and 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 reduce interaction times, enhance sensitivity and throughput (Kang *et al*., 2008), and aid in separation of complexed and uncomplexed molecules (Bange *et al*., 2005). For these binding studies, receptor or ligand molecules can be immobilized on a PDMS substrate by adsorption (Makamba *et al*., 2005), or covalent bonding (Sui *et al*., 2006), or by microcontact printing as achieved for solution hybridized oligonucleotides (Razumovitch *et al*., 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). The BLMs described earlier have been used extensively for ligand-binding studies over 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 *et al*., 2008). Javanmard *et al*. demonstrated a novel method of coupling a 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 bonding between the interacting molecules by measuring the drag force required to detach the ligand bound to the microfluidic channel when the receptor attached to the surface of microbeads is pressure driven through these channels (Javanmard *et al*., 2010).

7.2.2 Hit identification and lead optimization

After the identification of a particular druggable target, the next step in the drug discovery process is to identify a 'hit', which involves the phases of hit identification (HI), lead identification (LI), and lead optimization (LO). A 'hit' is a particular 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 described as a 'hit-to-lead' process (Goodnow, 2006). Synthesizing and screening the right drugs which can potentially be used, carried forward through a drug development programme, and enter a clinic, starts from correct identification of hits and leads. These steps are imperative, since drug discovery is an expensive process. 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. Drug libraries have been estimated to be in the order of 10^{63} (Bohacek *et al*., 1996). Microfluidic chip-based combinatorial chemistry and high-throughput screening, together aim to result in a paradigm shift, leading to the development of methods of sequential synthesis and testing of thousands of compounds in parallel (Knight, 2000).

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 to generate drug compounds at the 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 have produced enormous drug libraries. This has been further accelerated by the improvement

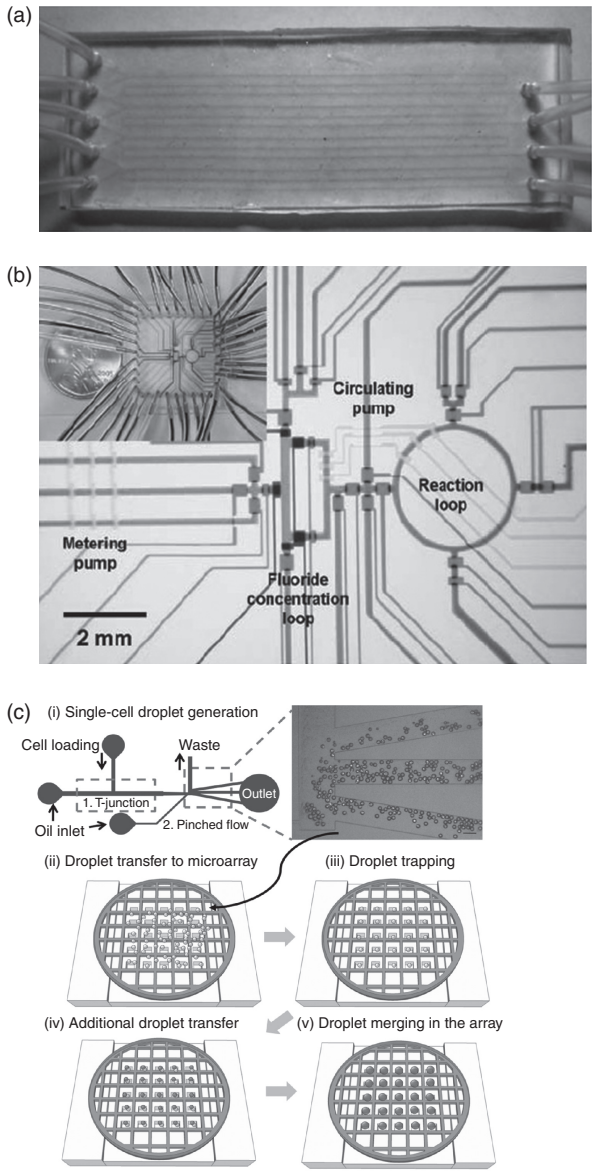
in the design of the microfluidic reactors. These microfluidic reactors can be classified into three types, based on the flow pattern, namely (i) flow-through type, (ii) droplet or slug type, and (iii) batch type. The most common flow-through type enables multiple reagents to be maintained at a temperature, and be pressure driven through the channels. These reactors have been used widely in extraction procedures as well as in multiple chemical syntheses (Keng *et al*., 2012).

Parallel combinatorial synthesis in multiple microfluidic reactors has also been demonstrated utilizing the continuous flow of reagents in microfluidic channels. A multiple microfluidic reactor assembly was fabricated to synthesize carbamates in a multistep procedure (Sahoo *et al*., 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). The complexity and expense of fabrication of this multi-layered device was a concern. Recently, Dexter and Parker exhibited parallel combinatorial synthesis of compounds on a single-layered microfluidic chip (Fig. 7.3a). They fabricated a single layer PDMS chip for synthesizing a 2×2 series of amide formation products (Dexter and Parker, 2009).

However, continuous flow reactors are not suitable for multistep reactions, especially those involving sequential synthesis. A modified technique, termed batch microfluidics, in which specific microvalves control the delivery of reagents in batches, has 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 radiolabelled imaging probe, in nano/microgram scale, was synthesized in five sequential processes involving fluoride concentration, water evaporation, radiofluorination, solvent exchange, and hydrolytic deprotection (Fig. 7.3b).

A newer technology, known as droplet microfluidics, has recently come to the fore. It is based on compartmentalization of each assay in a small droplet, usually in the range of 1 pL–10 nL, surrounded by an immiscible oil, which 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. 7.3c). Due to miniaturization, the device provides high-throughput screening of the droplets (Um *et al*., 2012), but multistep reactions using these devices are still a big challenge.

In addition, these microfluidic reactors have also been used for synthesis of biological molecules, such as DNA. Short synthetic oligonucleotides



7.3 Different types of microfluidic reactors. (a) A continuous PDMS-based microfluidic flow reactor for 2 × 2 parallel combinatorial synthesis. The tubing has been inserted at each inlet and outlet port (Dexter and Parker, 2009). (b) Optical micrograph of a batch type microfluidic reactor with actual dimension (inset) (Lee *et al.*, 2005). (c) 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 *et al.*, 2012).

were joined under thermal cycling in a microfluidic picoArray device to form DNA constructs up to 10 kb instantaneously. 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 acetyl-transferase and luciferase, and reported the yield to be 13–22 times higher than that achieved in microcentrifuge tube, with a 5–10 times longer lasting protein expression. The device is 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 *et al*., 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 poly (methyl methacrylate) 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).

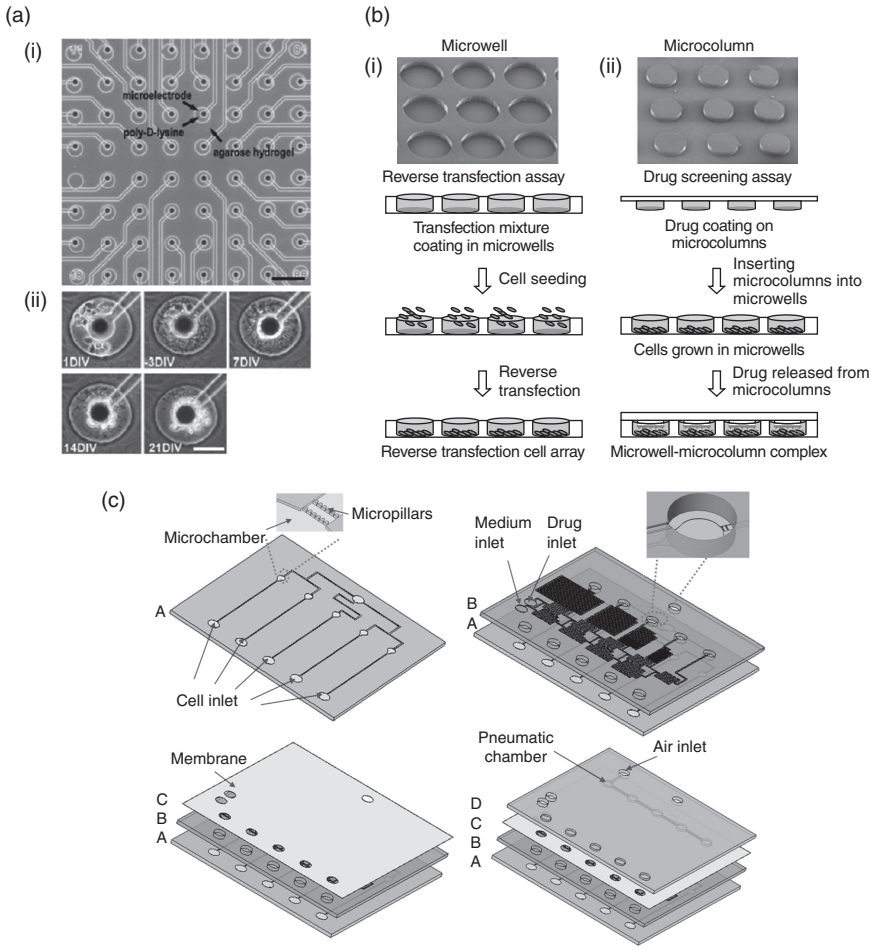
Most of the devices developed use PDMS as the substrate material due to its excellent optical properties as well as its mouldability. However, PDMS is incompatible with many organic solvents and adsorbs many hydrophobic compounds due to its surface properties. Keng *et al*. fabricated a microfluidic platform that is operated by electrowetting-on-dielectric (EWOD). The device was made from inorganic materials coated with perfluoropolymer, 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.

These devices have been put to efficient use to generate drug libraries, which provide a powerful source that needs to be screened to explore new drugs. To screen these large combinatorial libraries of compounds, the pharmaceutical industry has looked at high-throughput screening (HTS) methodologies over the past two decades. 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 ultra-high 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.

High-throughput screening

Traditionally, high density microplates including 96, 384, 1536 and those with >1536 wells have been used extensively for HTS (Battersby and Trau, 2002; Brandish *et al*., 2006). However, liquid handling on a microlitre 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. Also, these platforms can be easily modelled 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 the serial method, compounds are screened successively 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 analysing by a single detector. Parallel analysis is, however, limited by the miniaturization capacity and hence the extent of parallelization (Thorsen, 2004). Nevertheless, both methods have been extensively used in microfluidic HTS.

Microfluidic microwell arrays are versatile tools for cell culture and high-throughput experimentation through cell-based assays, particularly important in drug screening and offering a potential alternative to animal-based testing. 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 of drugs on different cells types. It also offers the potential of testing many compounds on a single cell type in high throughput. A multiwell 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 non-conducting agarose gel layer to isolate the individual neuronal micro-circuits and record individual action potentials of drugs such as bicuculline and *N*-methyl-D-aspartic acid (Kang *et al*., 2009) (Fig. 7.4a). Chen *et al*. developed a complementary microwell and microcolumn system for screening of drugs (Fig. 7.4b). They used microelectro-mechanical 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 would carry the drugs to be topically applied onto the cells. The system was found to be suitable for delivering high-throughput identification of epidermal growth factor receptor inhibitors (Chen *et al*., 2011c). An integrated multilayer microdevice incorporating a drug/medium concentration gradient generator, flow controlling microvalves, and microchambers for

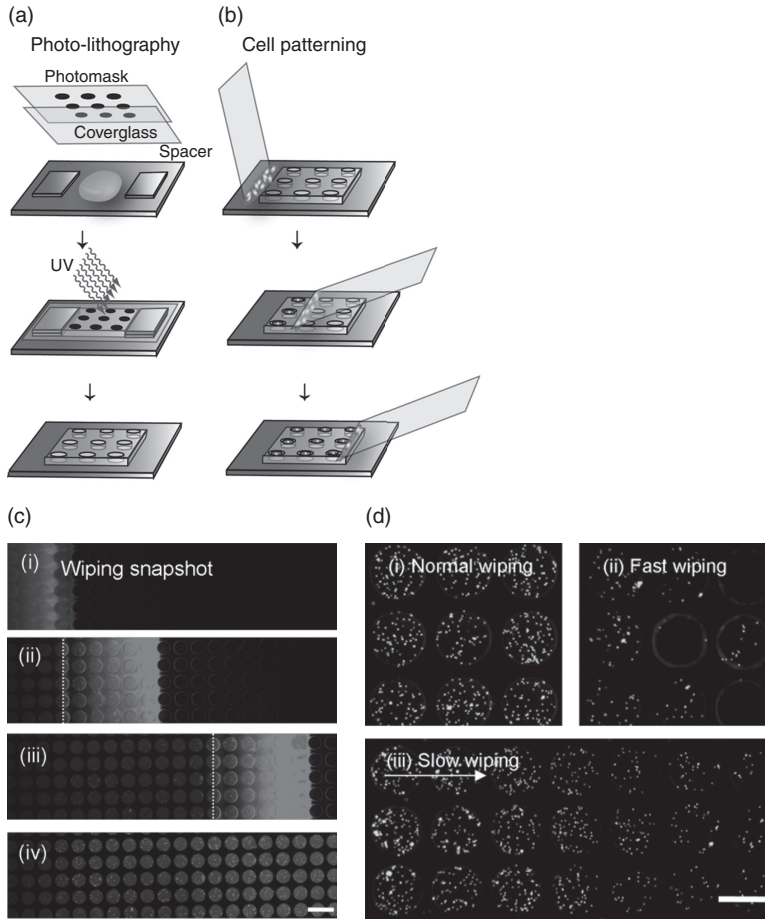


7.4 Microfluidic microarrays for cell based high-throughput screening. (a) A multiwell microelectrode array, (i) phase contrast image of agarose microwells on a micro-electrode array. Each microwell is composed of a microelectrode, poly-D-lysine coated surface, and agarose hydrogel wall, scale – 200 μm. (ii) The growth of neuronal conduits in microwells over a period of 3 weeks, scale – 50 μm (DIV – days *in vitro*) (Kang *et al.*, 2009). (b) Schematic illustration of the microwell and microcolumn cell based assays, (i) workflow for reverse transcription, and (ii) drug screening assay (Chen *et al.*, 2011b). (c) Schematic of a multilayered microfluidic device for drug screening. Layer A consists of pear shaped microchambers for cell culture. The microchambers were surrounded by micropillars to confine the cells inside. Layer B was used to generate concentrations of drug solutions. Layer C was made of PDMS and had microvalves for controlling the drug/medium volume dispensed below. Layer D had an air inlet for distribution of air to pneumatic chambers (Liu *et al.*, 2012).

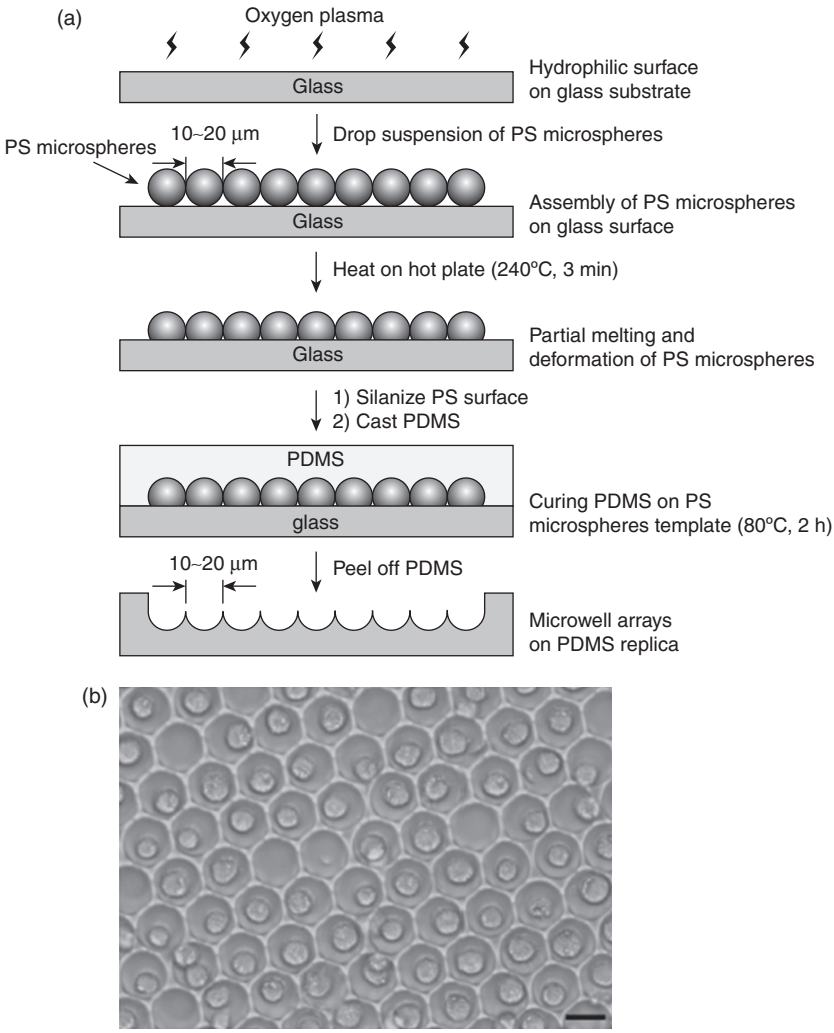
cell culture was recently fabricated by Liu *et al*. for testing the apoptosis behaviour in a cisplatin-resistant cancer cell line (Liu *et al*., 2012). A vertical perfusion mode was adopted in this device, as shear stress due to horizontal fluid flow can adversely impact the cells. Using the set-up, sequential loading of cells, medium, drugs and air was achieved in successive layers of the device (Fig. 7.4c).

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 (Fig. 7.5). 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 *et al*., 2010). They also developed an algorithm and software for automatic counting of cells in a microwell array. The software, named Arraycount, detects the cell count from the fluorescent cell images in high throughput. The results were in close correlation with cell counts from the manual methods (Kachouie *et al*., 2009).

Studying single cell characteristics offers an advantage over observing the behaviour 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 and their progeny over a period of time. One of the first studies pertaining to single cell confinement in microwell arrays for drug screening was reported by Rettig and 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 1 had more than 85% wells with single cell occupancy for both adherent and non-adherent cells (Rettig and Folch, 2005). An interesting round bottom microwell array was recently developed by Liu *et al*. by creating PDMS microwell arrays by reverse moulding using polystyrene microspheres melted on a glass substrate (Liu *et al*., 2010). The size of these microwells could be tuned to 10–20 μm, which is difficult to achieve with conventional soft lithography. The PDMS microwells were then used to confine single cells by pouring excess cell suspension over the microwells, which allowed the cells to settle in. The enzymatic activity of the cells was studied by carrying out the carboxylesterase assay using calcein AM. Fluorescence intensities from single cells could be captured to reveal different kinetic behaviour of entrapped cells, which was related to cell viability (Fig. 7.6). Another novel way of constraining single cells in microwells was demonstrated by Wang *et al*. (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



7.5 Microwell fabrication by photolithography and cell confinement using wiping method. (a) Photolithography: Poly(ethylene glycol) diacrylate was patterned into microwells using a photomask. (b) Cell patterning; a drop of cell solution was pipetted onto a thin glass slide and placed in contact at an obtuse angle with the microwell slide adjacent to the array. The cover glass was rotated to an angle of 45° and moved across the array, spreading the cell solution into the microwells and removing excess solution from the surface. This process localized cells and isolated liquid in the microwells. (c) (i)–(iv) shows the time lapse images of wiping a wedge of rhodamine/cell solution, leaving behind cells in the microwells with no excess liquid on array surface. The dotted line indicates the actual contact of the glass slide on the array surface. (d) (i)–(iii) Effect of wiping speed on cell distribution in microwells, where fast (>10 mm/s) and slow (<0.1 mm/s) wiping resulted in non-uniform distribution of cells, while normal (1.0 mm/s) resulted in uniform distribution (Scale – 400 μm) (Kang *et al.*, 2010).



7.6 Microwell arrays for single cell analysis. (a) Fabrication of PDMS-based microwell arrays using polystyrene microbeads, and (b) micrograph image of Ramos cells in each microwell (Scale 20 μm) (Liu *et al.,* 2010).

amenable to further analytical treatment. 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 co-workers, who devised a plastic microwell array by using economical materials such as shrink wrap film and tape. A carbon dioxide laser was used to cut holes in the tape, which acted as a mask to etch wells in the shrink wrap by oxygen plasma (Lew *et al.*, 2011).

Apart from multiwell arrays, multiplexed screening platforms have also been developed to screen multiple samples in one run. The ability to analyse 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 and 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 and 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 *et al*., 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 analysed. Multiplexed screening of picolitre-sized droplets that could be manipulated using an array of electrodes has also been reported. For example, caspase-3, 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 was compared with 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 digital microfluidics platform that uses the droplet manipulation system instead of pipetting or aspiration of liquids with conventional techniques (Bogojevic *et al*., 2012).

Analysing 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 *et al*., 2001), graphical (Evans *et al*., 2003), electronic (Service, 1995) and physical techniques (Vaino and Janda, 2000) have been developed. An exhaustive review of various encoding techniques has been published by Braeckmans *et al*. (2002). Spectrometric techniques utilize specific wavelengths to analyse 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 a considerable amount of time for fabrication and integration.

Pregibon *et al.* recently developed a novel encoding scheme for multiplexed platforms (Pregibon *et al.*, 2007). In this system, two poly(ethylene glycol)-based monomer solutions, one being a fluorescent dye and the other being an acrylated 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). The 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 in previous methods, the authors demonstrated that the sample volume required was manageable, together with providing higher sensitivity and reproducibility. The system was able to detect DNA at very low concentrations, 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. Post-processing, the cell characteristics can be studied to evaluate the drug effects. Such a novel platform was developed by Rodríguez-Dévora *et al.* (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 rate of cell survival after imprinting. Fast screening utilizing low volumes to assess the effect of three antibiotics patterned together with the bacterial cells could be carried out. This bioprinting approach was compared to the standard micro-pipetting 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 HTS, 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 has to be absorbed across mucous membranes, followed by its distribution to its target site and metabolism to an inactive metabolite to be eliminated from the body. It should also be devoid of any toxic effects. These characteristics, respectively 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 profiles account 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 *et al.*, 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 programme will be increased, as cost escalates with each stage (Kola and Landis, 2004). This is why pharmaceutical companies are nowadays adopting the '*fail early; fail cheap*' approach to identifying the toxicological properties of drug compounds. This is done in lieu of savings in the event that 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 market recalls, as a percentage of approvals, in the United States has fallen from 27.2% in 1980s to 5.2% in 2000s (Qureshi *et al.*, 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 programmes, and the role played by them in drug development.

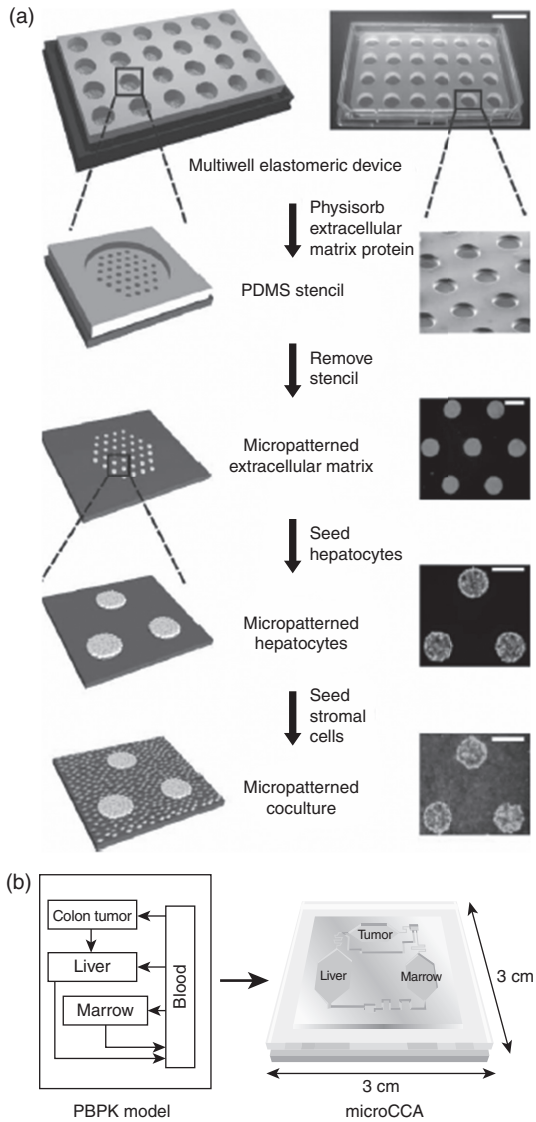
In vitro toxicological testing in cell models provides useful information about the drug candidates, much before the expensive animal experiments and first-in-human clinical trials are conducted. *In vitro* experiments have been long touted to replace animal testing, especially due to the ethical concerns surrounding animal experimentation (Wen *et al.*, 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 a large amount of compound under investigation, which is usually available in limited quantities and may be prohibitively expensive (Muster *et al.*, 2008).

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 *et al*., 2007). An excellent collation of advantages of 3D cell culture over the 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 post launch 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 behaviour when exposed to cytotoxic agents. A multiwell 3D cell culture platform was designed using soft lithography to co-culture primary hepatocytes with mouse 3T3-J2 fibroblasts (Fig. 7.7a). 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, were 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 and Bhatia, 2008).

Kane *et al*. designed a microfluidic 8 × 8 array, composed of PDMS. Each well in the array had two chambers. The primary chamber's bottom was made of glass coated with collagen, for co-culturing rat hepatocytes and 3T3-J2 fibroblasts; the collagen aided selective adhesion of hepatocytes, while 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



7.7 Multiwell culture for *in vitro* toxicity testing. (a) Schematic of the fabrication process (left panel) with photomicrographs of each step (right panel). A PDMS stencil in a 24-well format with through holes at the bottom of each well is sealed to a polystyrene plate, with collagen-I adsorbed on exposed polystyrene. The stencil is then peeled off followed by be application of blank PDMS stencil before cell seeding. Hepatocytes are then seeded which selectively attach to collagen-I, allowing fibroblasts to be seeded in other bare areas (Khetani and Bhatia, 2008). (b) A mathematical PBPK model and a corresponding physical μCCA based on the human body. A μCCA consists of liver, tumour and marrow chambers, interconnected with channels mimicking the blood flow pattern in the human body (Sung and Shuler, 2009).

membrane, was linked to microfluidic channels supplying humidified air with 10% carbon dioxide at 37°C. They also reported similar results, with increased albumin and urea production (Kane *et al*., 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 a particular 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 contributions 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 multi-compartment cell culture platforms 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 modelling (PBPK), having different compartments for different tissues, can assist in understanding parameters such as tissue-to-blood perfusion, enzyme kinetics, liquid-to-cell ratio and physiological stress on a particular tissue/organ (Esch *et al*., 2011).

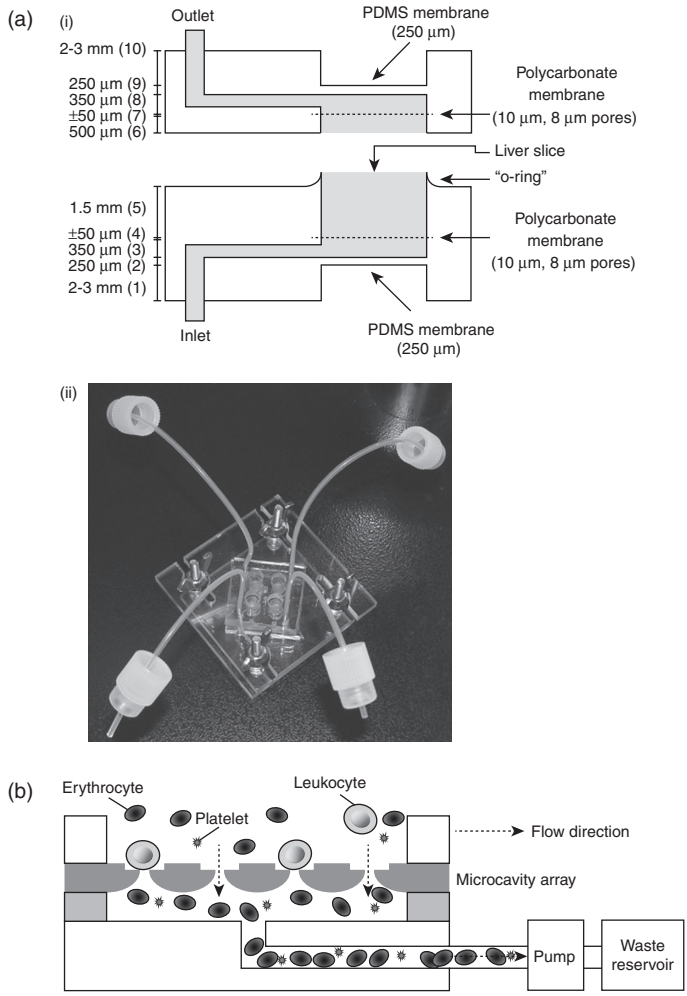
A novel microfluidic system, microscale cell culture analogue (μCCA), has been developed for multiorgan toxicity analysis. A multiorgan culture system, the integrated discrete multiple organ culture or 'wells-within-a-well' system, was designed by Li *et al*. Cells from different organs were cultured in small wells in their respective media 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 towards various organs was also examined. Apart from this, the system offers another advantage in enabling the analysis of the anticancer activity of a drug with respect to its effect on normal tissues. Although the authors did not delve into multiorgan interactions, this in principle can be adapted for this purpose and its capabilities should be further investigated (Li *et al*., 2004).

In a model based on PBPK to emulate the dynamics of the 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 h or 72 h, a selective toxicity towards MES-SA/DX-5 was detected, 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 curves for subsequent *in vivo* animal experiments or clinical trials (Tatosian and Shuler, 2009). 3D hydrogel cultures in μCCA format were developed by Sung and Shuler (Fig. 7.7b). 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 led to the death of hepatocytes, an effect which was unnoticeable in well plate assays (Sung and Shuler, 2009). The literature is replete with tegafur toxicity data, particularly its hepatotoxicity (Maruyama *et al*., 1995). In such a scenario, development of microfluidic systems providing critical toxicity information in *in vitro* models bodes well for preclinical drug testing.

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 analyse 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 *et al*., 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 (Fig. 7.8a). The term 'perifusion' was used instead of perfusion, as the medium flowed around the slices. PDMS membranes were purposely kept thin so as to allow for efficient gaseous exchange. Metabolic activity of 7-ethoxycoumarin observed was comparable to the well plate-based method was observed in this device (van Midwoud *et al*., 2010). Another PDMS-based device was developed to analyse ethanol toxicity in liver explants. Using this device, concentrations as low as 20 mM produced a decrease in mitochondrial



7.8 Microfluidic platforms for *ex vivo* experiments. (a) For culturing liver slices. (i) Cross-sectional view of a PDMS-based biochip for culturing liver slices that were supported on a polycarbonate membrane. The device was termed as a 'perifusion' device as culture medium flowed around the slices. Thin PDMS membranes allowed for efficient gaseous exchange. (ii) A photograph of the device mentioned in (i) (van Midwoud *et al.*, 2010). (b) For leukocyte counting and assessment of haematotoxicity. 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 analysed for toxicity due to various drugs (Hosokawa *et al.*, 2012).

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 *et al*., 2011). Such devices utilizing excised tissues represent clinically more relevant models to replace animal experimentation.

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 haematotoxicity (Fig. 7.8b). In this device, a microcavity array was created by master-moulding PDMS structures to form a sieve-like structure that separated leukocytes from other blood cells. 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 *et al*., 2008; Xu *et al*., 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.

7.3 Microfluidics for drug analysis and diagnostic applications

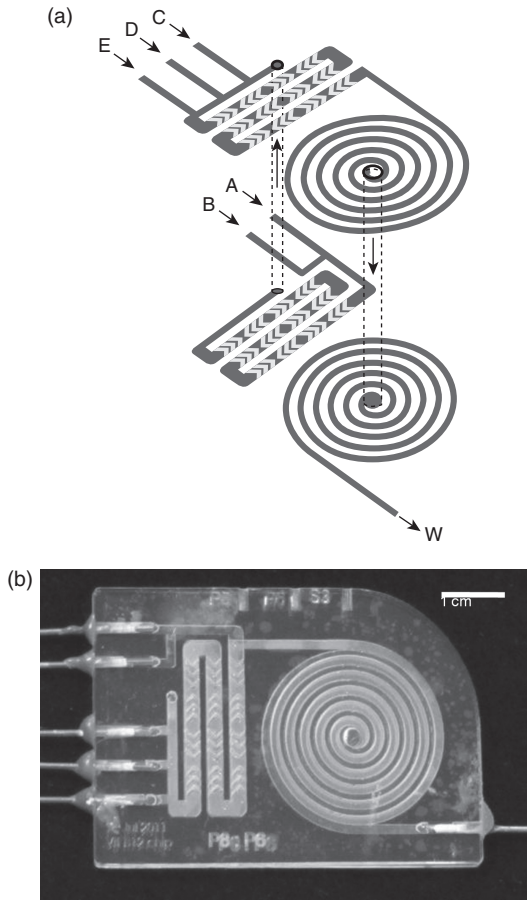
Miniaturization of analytical tools has been propelled by the recent surge in the development and maturation of microfabrication techniques. The better control of physical processes at the micron-scale has further fuelled the interest in micro-analytical systems as new paradigms for pharmaceutical analysis. These systems are aimed at reducing both the sample volume

and time of analysis, besides being amenable to integration with the other platforms and potential for high throughput. High parallelization, thus now possible, had made analysis of multiple compounds fast and easy (Lee *et al.*, 2009). Design modification can provide integrated facilities for handling fluids, and thermal and spatial control for targeting specific detection components to enhance selectivity (DeMello, 2006). With the mass fabrication of micron-sized platforms now made possible through sophisticated instruments, the cost of production of these devices has come down, providing an opportunity to develop single use analytical devices, thereby reducing the possibility of cross contamination (Lion *et al.*, 2004).

7.3.1 Microfluidics for drug 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 choice 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 that 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, helping to increase the assay accuracy. These devices have been more popular in the analysis of biological molecules such as proteins and nucleic acids (Guo *et al.*, 2012; Meagher and Thaitrong, 2012), and have been the subject of other chapters in this book. Here, we would limit our focus to discussion about the application of microfluidics in the analysis of drug entities.

Analysis of pharmaceutical compounds has been carried out using high performance liquid chromatography (HPLC), linked to 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 and chemiluminescence, 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 minuscule working volume microfluidic platforms, it is imperative that the chosen method is able to detect low amounts of the analyte. Chemiluminescence (CL) was reported to be a highly sensitive technique, with vitamin B_{12} concentrations as low as 5 pg/mL being detected easily (Kumar *et al.*, 2009). CL-based methods have

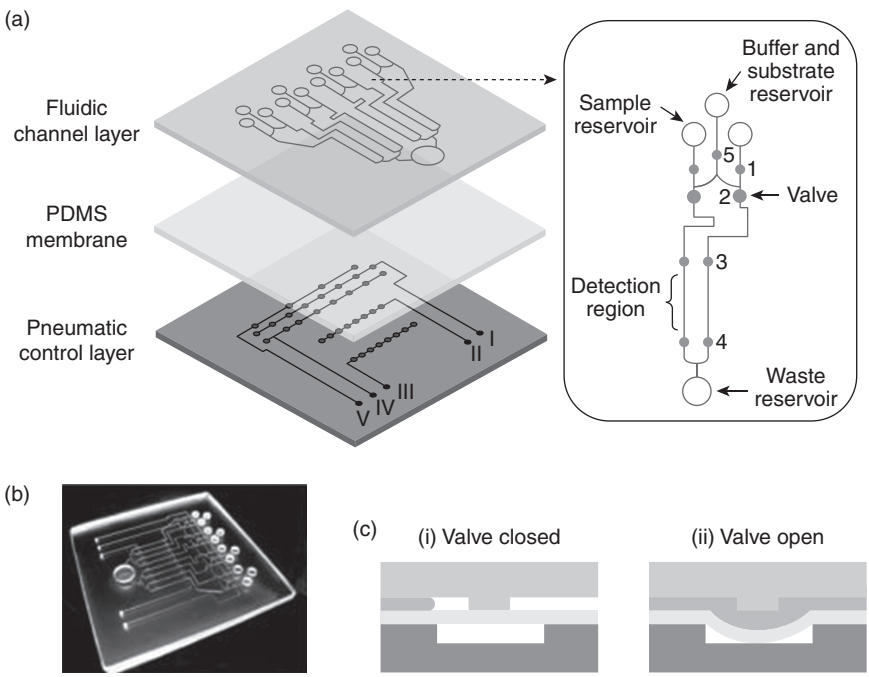


7.9 Microfluidic chip for chemiluminescence based detection of vitamin B_{12}. (a) Schematic of the chip with five inlets, two mixing chambers and two spiral detection units, and the fabricated chip (b) (Lok *et al.*, 2012).

been used for microfluidic detection of vitamin B_{12} and L-phenylalanine. Luminol oxidation by hydrogen peroxide in the presence of externals catalyst ions, such as cobalt (II) and copper (II) and amino acids such as L-phenylalanine under alkaline conditions, is the basis of this test. The resultant product is a blue compound (3-aminopthalate ion) that can be detected at a wavelength of 425 nm (Chen *et al.*, 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. 7.9). The device consisted of a two passive mixing reaction chambers and a double spirally wound microchannel network as an optical detection unit, spanning the three sequentially fabricated layers. Microchannels in the second layer were

covered by the first layer. The second consisted of a mixing chamber and a clockwise spiral detection unit. The third layer had the other mixing chamber and an anticlockwise spiral detection set. The mixing chambers were designed in layers to counter the problem of mixing in the laminar flow. The spirally designed detection channels presented 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 catalyse the reaction. Using the device, up to 0.3 pg/mL of vitamin B_{12} could be detected (Lok *et al.*, 2012). In another CL-based microfluidic chip, using the same principle of oxidation of luminol catalysed by copper sulphate, 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 around 39 picogramme/ml of L-phenylalanine in commercial soft drinks as well as pharmaceutical injections (Kamruzzaman *et al.*, 2012).



7.10 Schematic of a multilayered microfluidic device for analysis of drugs. (a) The device comprised three layers; the top and bottom layer were made of glass and a PDMS membrane was sandwiched between the two layers. (b) Photograph of the device. (c) Cross-sectional view of the microvalves showing closed and open position (Kong *et al.*, 2009).

Won *et al*. developed a microfluidic device on glass slides for simultaneous detection of five sulphonamide drugs (Won *et al*., 2012). The device was provided with modules for pre-concentration and electrokinetic separation of drugs using the field amplified sample stacking (FASS) and field amplified sample injection (FASI) techniques in two parallel channels (Shiddiky and Shim, 2007). Subsequent electrochemical detection of sulphonamides was carried out at the end of the separation channel, which consisted of a silver/silver chloride, platinum wire and aluminium-gold nanoparticles modified carbon paste electrode. The device was able to detect femto-molar level concentrations of these drugs and provide an opportunity to simultaneously detect these drugs in clinical samples. A microfluidic chip integrated to a laser-induced fluorescence scanner was developed for the detection of β_2 agonist drugs such as clenbuterol (Fig. 7.10). 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 *et al*., 1995; Hesketh *et al*., 1992), making it vital to analyse 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). Thus, microfluidic devices can play a crucial role in detection drugs and pharmaceuticals, and can be routinely used in chemical, pharmaceutical and clinical settings with high precision in an economical way.

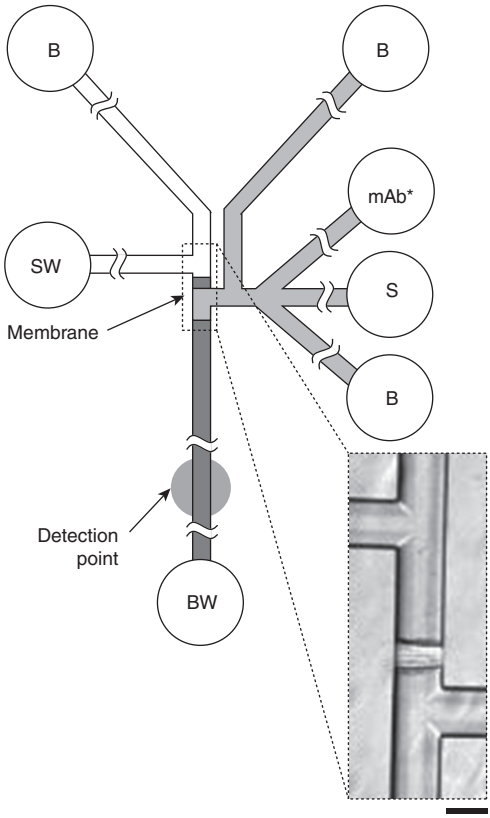
7.3.2 Microfluidics for diagnostic purposes

The conventional diagnostic techniques based on sophisticated macroscopic equipment such as gas chromatography–mass spectrometry are only feasible in big air-conditioned laboratories, which are equipped with trained workforces 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 *et al*., 2010; Yager *et al*., 2006). Miniaturized versions of analytical platforms have been recently conceptualized, primarily based on microfluidic technology, to perform diagnostic analysis, 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 logistical 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 number 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 *et al*., 1997), while in the latter either of the enzyme/substrate/inhibitor needs to be immobilized on a solid surface (Mao *et al*., 2002; Krenkova and Foret, 2004). Mostly, 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, making immobilized assays a preferable choice (Kim *et al*., 2009).

Enzyme immobilization has been mostly carried out on microspheres, also known as microbeads, due to their similar size properties to microchannels, as well as large surface area for enzyme support (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, respectively. In the reaction chamber, microbeads were covalently bound to an enzyme, glucose oxidase, and were supported by microfilters. A poly (ethylene glycol)-based microarray (fabricated by photolithography) encapsulating a horseradish peroxidize, 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 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 offered a simple alternative to other such devices, as no mixing was 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 pre-process the sample. This allowed the serum samples to be directly used for glucose analysis (Sheng *et al*., 2012). Other devices integrating enzyme and immunoassays have been fabricated for simultaneous detection of glucose and insulin (Wang *et al*., 2003).

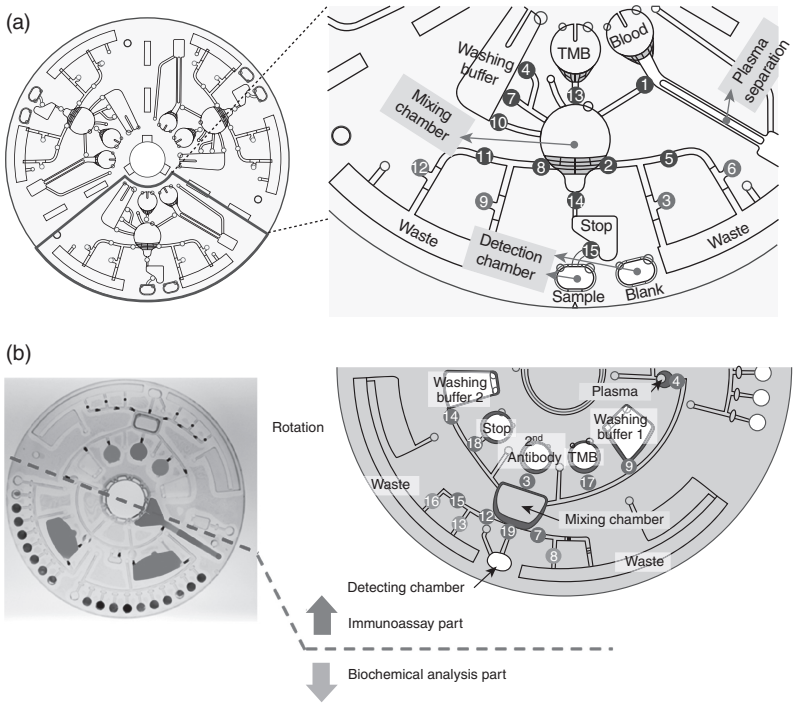


7.11 Microchip electrophoretic immunoassay (μCEI) chip. The device had fluid wells for: S, sample; B, buffer; SW, sample waste; BW, buffer waste; mAb*, fluorescently labelled monoclonal antibody. The device possessed molecular sieves to enrich the sample and electrophoretically separate the molecular antibody that is bound to an enzyme (Herr *et al.*, 2007).

Herr *et al.* designed a microchip-based device for the detection of disease biomarkers in periodontal disease (Fig. 7.11). The multifaceted device had integrated capabilities for sample pretreatment, including filtering, mixing and enrichment of the saliva, which could be withdrawn in a hands-free manner. The device, a 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 the method, they could dispense with 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 antigens and antibodies. However, the

conventional macroscale ELISA protocols are laborious, sluggish, require multiple reagent addition and washing steps, and often result in inconsistent results due to manual glitches. Furthermore, commercially available ELISA kits, as well as the 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 reagent (Cesaro-Tadic *et al.*, 2004; Murakami *et al.*, 2004). Microfluidic ELISA platforms have been researched in great detail in the past few years (Herrmann *et al.*, 2006; Holmes *et al.*, 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,

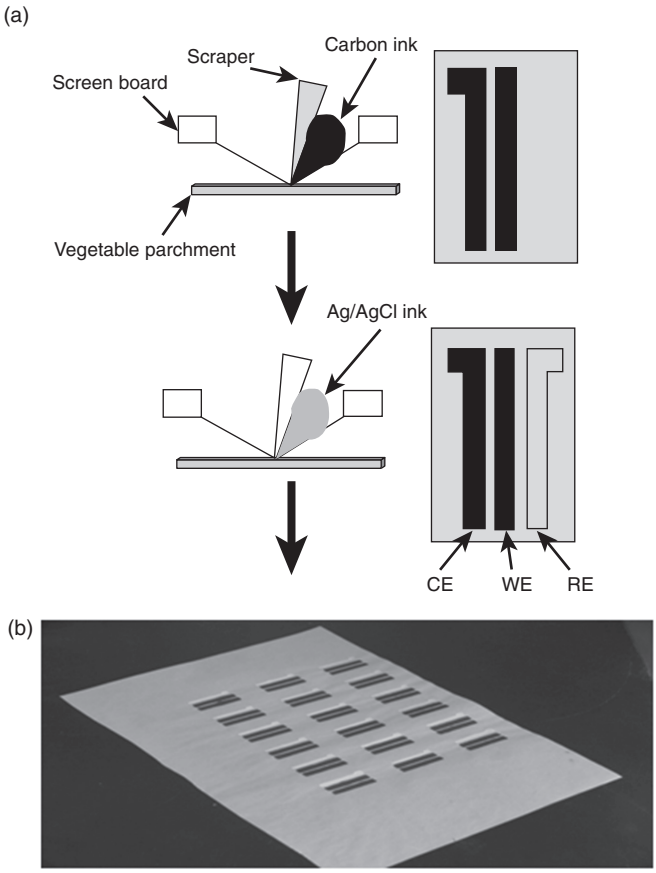


7.12 Microfluidic lab-on-a-disc platforms. (a) Schematic of a fully automatic disposable ELISA platform, with facilities for plasma separation, storage of buffers, reagents, mixing the reactants and collection of waste. ELISA assay could be performed in half as volume and less than 30 min. The numbers indicate the sequence of reaction (Lee *et al.*, 2009). (b) Schematic of an improvised platform with facilities for assessing both immunoassay and clinical chemistry of blood, provided on a single disc. The detection chamber could be used at 10 different wavelengths to accommodate a range of reactions (Lee *et al.*, 2011).

mixing the reagents, and detection of the product. Using just 150 μL of blood, the assay could be performed as opposed to double this volume with conventional methods; the whole assay could be carried out in 30 min, whereas conventional well plate-based ELISA yields results in a minimum of 2 h (Lee *et al*., 2009). Miniaturization did not compromise the sensitivity of the device, and a similar detection limit could be achieved (Fig. 7.12a). Recently, they developed an advanced chip to carry out the immunoassay as well as biochemical assessment of whole blood (Fig. 7.12b). The chip had an 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 measurements at ten 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 *et al*., 2012).

Droplet-based microfluidic approaches have been used for analysis of human physiological fluids such as blood, serum, plasma, sweat and tears. These might be very useful in a point-of-care setting, whereby a patient can use a microdroplet of his/her sample, with minimal training, without much inconvenience, and read the results in real time. A monolithic electrowetting platform consisting of a photolithographically patterned metal electrode array on a glass substrate was designed and the sample was sandwiched between this array and another plate of glass. The sample droplet was encapsulated in silicone oil to prevent evaporation and ensure smooth transport, at frequencies of 20 Hz and voltage less than 65 V. Glucose concentration was determined by a colorimetric assay in various physiological samples and results were found to be comparable to reference values (Srinivasan *et al*., 2004).

Electrochemical methods of detection are recognized to be among 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 their 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 (Wartelle *et al*., 2005; Quinton *et al*., 2011). Screen printed electrodes (SPE) based on carbon are another form of electrochemical analytical device that has been studied for the detection of chemicals, such as nitric oxide (Miserere *et al*., 2006), and biomarkers for cancer (Wan *et al*., 2011). They are useful for electrochemical immunosensor assays, as they offer the advantages of 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, and organic films have been used for electrode fabrication and present an interesting alternative for point-of-care testing (Miserere *et al*., 2006; Schuler *et al*., 2009).



7.13 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 (Yan *et al.*, 2012).

Recently, Yan *et al.* fabricated SPEs on vegetable parchment as a substrate for disposable immunosensor fabrication in detection of prostate specific antigen (PSA) (Fig. 7.13). 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 graphenes, containing the enzyme-linked antibody on gold nanoparticles. The assay could detect PSA as low as 2 pg/mL, and offers a suitable method for detecting potential disease specific biomarkers, allowing for early diagnosis in a disease (Yan *et al.*, 2012).

A microfluidic magnetic disc has been used to separate rare, circulating endothelial cells (CEC) from peripheral blood mononuclear cells (Chen *et al*., 2011b). These CECs have been associated with many diseases, and their low concentration in blood impedes their detection. A magnetic disc was used to trap the cells attached to immunomagnetic beads. Human umbilical vein endothelial cells (HUVEC) were used as a model for CECs and stained with anti-CD146-phycoerythrin antibody, which was tagged to anti-phycoerythrin 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 non-magnetic cells through the connecting channels to the waste reservoir, effectively retaining target cells (HUVECs) in the inlet reservoir, which had a multistage magnet over it.

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 equipments (Mabey *et al*., 2004; Martinez *et al*., 2008a). 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, called 3D microfluidic paper analytical devices (μPAD) (Bruzewicz *et al*., 2008; Martinez *et al*., 2007, 2008b, 2008c).

Microfluidic devices have also been developed for diagnosis of lysosomal storage disorders (Shen *et al*., 2012), cancer (Lien *et al*., 2010; Chen *et al*., 2011a; Tang *et al*., 2012), H1N1 influenza (Lee *et al*., 2012) and herpes simplex virus (Zubair *et al*., 2011), sickle cell disease (Hersher, 2012) and Johne's disease in cattle (Wadhwa *et al*., 2012). In principle, all microfluidic diagnostic devices consist of a molecular sensing unit coupled to a signal converter (transducer) that reads out the results quantitatively. An elaborate review of 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 reader is referred to a few state-of-the-art reviews for more detailed information (Choi *et al*., 2011; Mohammed and Desmulliez, 2011).

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 and Merten, 2011; Foudeh *et al*., 2012).

7.4 Conclusion and future trends

Conclusively, microfluidic devices have shown initial promise in all areas of drug discovery and development, as well as in the domains of miniaturized diagnostic devices. The future of microfluidic devices for application 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 bench-top macroscale equipments, 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 inter-disciplinary effort from engineers and researchers working on fluid dynamics to design micropumps, analytical equipment manufacturers to scale down analysis systems to commensurate with 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 (http://www.fluidigm.com/), 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, 3D structures can be created from elastomers to form integrated valves (NanoFlex™), pumps and channels. Besides this, Caliper Life Sciences (http://www.caliperls.com/) have also developed several automated/semi-automated 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 sizeable 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 check-ups, to adopt such self-usable devices. In addition, it has to be ensured that such devices are safe to use and dispose, without causing any serious environmental hazards.

7.5 Sources of further information and advice

Although we have tried to encompass the major developments on the drug discovery and analysis front in this chapter, the reader is referred to the following for further details. A review reflecting on the origins and the current status of microfluidics by George Whitesides is highly recommended (Whitesides, 2006). Another comprehensive review, by Yeo *et al*., that describes the role of microfluidics in various biological applications is also recommended (Yeo *et al*., 2011). Other important works that appear in literature and circumscribe the two areas of interest in this chapter include (Imming *et al*., 2006; Yager *et al*., 2006; Kang *et al*., 2008; Wen and Yang, 2008; Lombardi and Dittrich, 2010; Livak-Dahl *et al*., 2011).

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