CHAPTER

Lab-on-a-chip technology and microfluidics

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"Everything in excess is opposed to nature"
Hippocrates (460–377 BC)

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1 INTRODUCTION

Since the introduction of a miniaturized gas chromatography analyzer on a silicon wafer in the 1970s by Terry et al. [1] and most prominently since the conceptual work on a miniaturized total chemical analysis systems by Manz et al. in 1990 [2], the field of micro total analysis systems (μTAS) or lab-on-a-chip (LOC) technology has been under intensive development in many biotechnological areas spanning from basic theoretical models and academic proof-of-concept studies to commercial applications. LOC are ideally described as miniature versions of their macroscale counterparts and therefore usually integrate all the component units of a complete laboratory essay [3]. The term microfluidic is generally used to describe the precise control and manipulation of small volume of fluids on a micrometer scale, which is the basis of LOC systems. The attractiveness of such miniaturized systems can be attributed in large part to its size effect, which allows portability, low consumption of sample/reagents and power, and short assay time. Further, it is associated to some unique physical phenomena that emerge at such scale and bring numerous benefits in pharmaceutical applications from the early drug discovery and screening stage to the final targeted and controlled delivery stage, as will be addressed later [4]. The high interdisciplinarity of this technology has received inputs from a large spectra of researchers from different areas of expertise in order to develop and apply microfluidics in a wide range of (bio)technological applications, such as clinical diagnostic [5], proteomics [6], cell and tissue engineering [7], pharmacology [8], and environmental monitoring [9], among others [10,11]. The value of this technology is demonstrated by the growing number and improved quality of published papers [12]. According to the ISI Web of Science, about 45,000 of documents related to microfluidics have been published since 2000 being almost 10% related to pharmaceutical applications (Fig. 1.1).

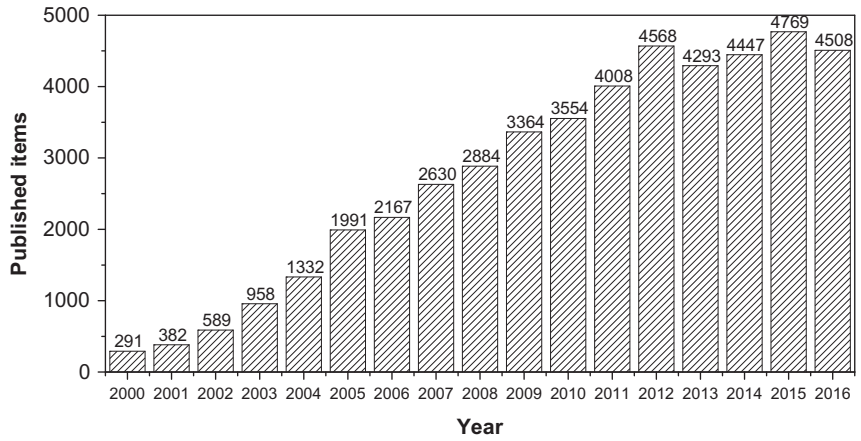

FIG. 1.1

Published items related to "microfluidics" by year since 2000.

Data from ISI Web of Science.

This strong growth over the years along with the potential to produce revolutionary and practical miniaturized devices has led to the emergence of a number of companies dedicated to microfluidics and LOC for different application areas, being approximately 274 worldwide in February 2016 [13]. Some examples are Abaxis (diagnostics), Advanced Liquid Logic (research instruments), Biosite (diagnostics), Chiral Photonics (packaging, prototyping, and manufacturing), Aixtek (consulting), FlowJEM (prototyping), Microfluidic Imaging (imaging), Cepheid (diagnostics), Cytonome (therapeutics), Micronics (custom development, manufacturing, and research instruments), Microflow Laboratory (consulting and prototyping), Medtronic (medical devices), Luna Innovations (contract R&D), ALine Inc. (development and components), and i-STAT (diagnostics). This technological boom led MIT Technology Review to nominate microfluidics as one of the 10 technologies that will change the world, with particular relevance in the life science area [14].

The present chapter provides a general description of the essential components and properties of LOC systems, including concepts of materials and fabrication techniques. Further, their applications in relevant biotechnological fields are presented and discussed. In particular, the applicability and advantages of microfluidic technologies in the pharmacological area will be highlighted. The main objective is to provide an overview to scientists and engineers on the possibilities and potential offered by microfluidic technologies to develop innovative and improved products for drug discovery and development.

2 DEFINITION, MAIN CONCEPTS AND CHARACTERISTICS

As briefly described above, LOC systems are based on a broader technology called microfluidics, the science and engineering of manipulating and processing small volumes of fluids (typically from 100 nL to 10 μL of samples and reagents) in microchannels that have at least one dimension (e.g., channel width, depth, or diameter) with length scale from 10 to 100 of micrometers. LOC are often described as miniaturized versions of their macroscale counterparts. This means that successful operation of technically complex assays on chip is designed to include all or most of the components and stages of a complete laboratory procedure in an integrated, automated, and small platform (Fig. 1.2) [5]. These stages can include sampling, sample pretreatment, chemical reactions, product separation and isolation, detection system, and data analysis [15]. Therefore, different kinds of components such as filters, pumps, valves, actuators, heaters, motors, and other functional units have been miniaturized. Likewise, detection systems, such as sensors and detectors, including optical, magnetic, and electric detection, and all the associated electronics have been developed, integrated, and successfully applied in LOC [16].

The strong decrease in the length scale gives rise to unique, important, and sometimes nonintuitive phenomena at the microscale that are not present at the macroscopic scale and are essential for many biotechnological applications. In this context, fluid flow can be typically characterized by two regimes: laminar or turbulent, which is defined by the relative contribution of inertial and viscous forces

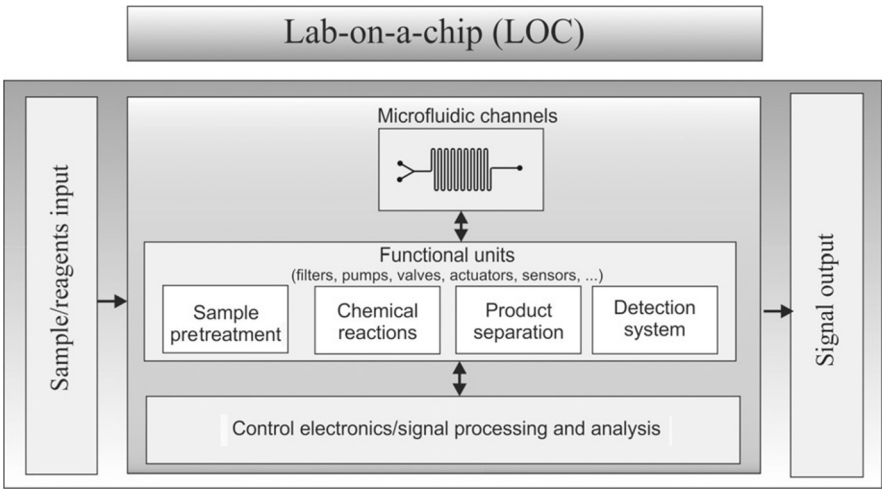

FIG. 1.2

General components of a LOC.

on a fluid flowing in a channel. It is usually described by the Reynolds number (*Re*), a dimensionless parameter defined by the density and viscosity of the fluid, plus the average velocity of the fluid flow and the characteristic length scale (e.g., diameter of the channel) [14,17]. The transition between laminar and turbulent flow typically occurs above a *Re* of 2000 in internal flows [14]. Inertial forces dominate at larger *Re*, while viscous forces govern at low *Re*. Therefore, reducing the characteristic length scale has the same effect on the fluid behavior in terms of *Re* as increasing the viscosity of the solution. This means that in microfluidic systems, flows are well below *Re* of 100 or even below unity, and so, the flow is truly laminar, dominated by viscous forces. Thus, the fluid velocity is invariant with time at all locations when the boundary conditions are constant [17]. As a consequence, fluid streams flow parallel to each other, and mixture between them occurs just through convective and molecular diffusion. This enables the design of separation and detection devices on laminar fluid diffusion interfaces [18–20]. However, this fact has also important implications in many applications requiring the mixture of fluids, especially when low diffusion coefficients are present. Nevertheless, to overcome this limitation, powerful passive and active mixers have been developed and successfully integrated in microfluidic systems [21–23]. Another critical issue to consider in microfluidic system is the fluid transport system, that is, sample introduction and/or extraction. In fact, flow rates ranging from hundreds of microliter per minute for high-volume throughput to picoliter per minute for applications requiring micron- to submicron-sized channel must be obtained using precise fluid drivers [24,25]. To achieve such requirements, two main methods have

been employed. Microfluidic channels made of materials that are charged under experimental conditions are used to induce the well-known phenomenon of electroosmotic flow (EOF). In this case, a blunt fluid flow profile is obtained (Fig. 1.3A), being however susceptible to variation of channel wall coating and fluid composition, limiting its use as generic pumping system [26,27]. In turn, pressure-driven flow by using mechanical positive displacement pumping shows the advantage of very little compliance, which allows controlling the exact volume of pumped fluid and knowing the exact location of the fluid meniscus within the microchannel. A particularity of this system is that the fluid flow exhibits a nonuniform velocity profile, which is usually pseudoparabolic, that is, maximum at the center of the microchannel and decreasing to zero velocity immediately near to the channel walls (Fig. 1.3B) [28,29]. Such systems are mechanically complex and hard to miniaturize, and very low flow rates are generally difficult to obtain. Nevertheless, these fluidic transport systems have demonstrated their suitability in many biotechnological applications, being the system of election by most of the researcher in this area [14].

In view of the above and from a technological point of view, it is possible to claim that LOC technology offers many unique benefits when compared with larger-scale conventional systems that include the following: (i) miniaturized devices, allowing portability, in situ measurements, and development of point-of-care systems; (ii) minute consumption of fluids, ideal for handling costly and difficult-to-obtain samples and reagents; (iii) reduced production of waste, making them environmental friendly; (iv) reduced energy consumption; (v) ability to perform high-throughput analysis by processing several assays in parallel; (vi) quick reaction and fast analysis, allowing the results to be obtained within seconds or minutes, instead of hours or days; (vii) improved sensitivity/precision; (viii) versatile and controllable processing of the microfluidic systems at dimensions from micrometers to nanometers; and (ix) widely applicable building materials including plastic to produce microfluidic systems at very low unit cost, allowing them to be disposable and avoiding any type of cross contamination [6,21,22].

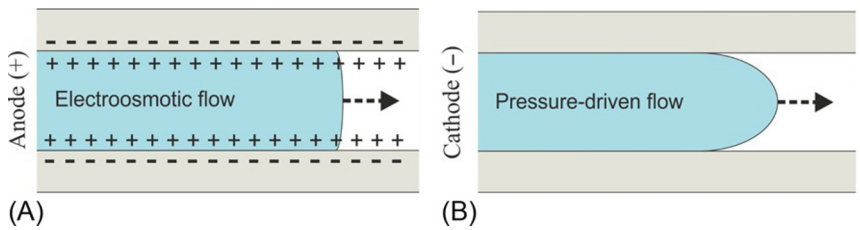

FIG. 1.3

Schematic representation of (A) electroosmotic flow and (B) pressure-driven flow.

3 MICROFLUIDIC TECHNOLOGY FOR PHARMACEUTICAL APPLICATIONS

From the pharmaceutical application point of view, microfluidic systems offer a better representation of the realistic physiological and pathological conditions of complex systems for both fundamental research and drug development comparatively with conventional macroscale in vitro assays that continue to give misleading and nonpredictive data for in vivo response [30,31]. In fact, microfluidic systems allow to model biological environments and physically mimic the complex cell-cell and cell-microenvironment interactions found in biological tissue and organs (such as the liver, lung, gastrointestinal tract, kidney, and heart), usually referred as "organ-on-a-chip" [32–34], or at least some of the physiologically relevant processes related to the so-called adsorption, distribution, metabolism, and elimination (ADME) processes in the body, which have an important role in expediting early stages of drug discovery and help to bypass animal testing [35,36]. This is because microfluidic systems can provide a precise control of the fluidic microenvironment, which is particularly relevant and representative, as many important biological processes in cells and other biological entities take place and have sizes at the micrometer scale, matching microfluidic channel dimensions (Fig. 1.4).

Fluid flows are an important part of both healthy and pathological conditions, including not only the more obvious flow of blood and lymph in the circulatory system but also the interstitial flow of blood in nearly all soft tissues. The accurate manipulation of fluid flows in microfluidic systems, with high surface-area-

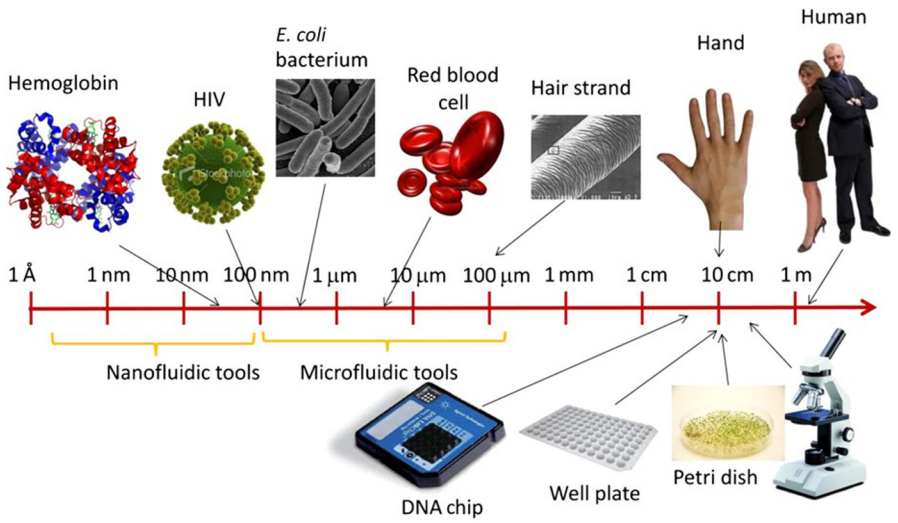

FIG. 1.4

LOC technologies as tools at molecular and cellular scale [30].

to-volume (SAV) ratio, allows to replicate blood circulation in three-dimensional (3-D) microenvironments, with microvascular perfusion and diffusion between mimicked microvessels and 3-D cell culture providing a continuous supply of nutrients and oxygen, which is closer to what cells encounter in real tissues or organs, alleviating the translational barrier to in vivo expectations [37,38]. Moreover, a uniform thermal field and precise temperature control are reached due to the excellent heat transfer properties [39,40]. However, high SAV is also usually associated with high protein adsorption depending mostly on the wetting properties of the microfluidic system (that can be physically important to the cultured cells). To overcome these limitations, specific surface modifications of the microchannels by plasma treatment or coating with specific chemical compounds have been adopted [41,42]. Therefore, key aspects of the biological setting include both micrometer structures and properties, as well as controlled fluid flow over the spatiotemporal environment, which can be simulated in microfluidic systems. A high degree of architectures and biocompatible materials, well-developed and well-characterized microfabrication technologies, also provide researchers with a large toolbox to produce specific and tailored designs in a reliable and reproducible manner. Another aspect is the fact that real-time monitoring of cells or tissue-specific response using standard microscopy techniques are also possible since microfluidic systems made of transparent materials, such as glass or polymers, can be designed to fit on top of a standard microscope slide [15,17,30,39]. Finally, microfluidic system allows to significantly save cell and drug sample volumes from 10- to 1000-fold less than the conventional counterparts, facilitating systematic high-volume testing in various stages of the drug discovery process that could be prohibitively expensive otherwise since the quantities of tested drugs or cells are normally very limited in pharmaceutical research and development [37].

Therefore, with mimicked close-to-in vivo microenvironments and organ-on-a-chip designs, 3-D microfluidic cell culture systems will increase the in vitro drug screening accuracy that in turn would reduce failing rate through clinical trials in the near future and facilitate the development of safer and more effective drugs, namely, in terms of controlled and targeted delivery, at a reduced cost [37]. The next step is to connect various organs-on-a-chip devices in order to create "body-on-a-chip" that will allow not only to study the effects of drugs in individual organs but also to simulate the interactions between various organs, providing a more complete and comprehensive analysis that would ultimately revolutionize how drugs are developed [32,43,44]. Current works on organs-on-a-chip involve intestine-liver [45], liver-kidney [46], and intestine-liver-skin-kidney cocultures [47] and neurosphere and liver spheroid cocultures [48], among others. Details of different types of organ-on-a-chip, with attention brought to their design, materials, objectives, and results, are further discussed in Section 5.3 or can be found in excellent reviews related to this matter [32,35,43,44]. In addition, the unique advantages, compactness, and controllability of LOC have allowed the development of implantable smart microfluidic drug delivery systems consisting of a number of biocompatible microscale components that can regulate and monitor

the delivery of the right amount of drug into a specific target site. Such microdevices have been developed for the treatment of cancer, cardiovascular disorder, eye and brain diseases, stress, and diabetes [49–51].

Important from a technological point of view, microfluidic systems are applied not only for assay development and disease treatment/diagnosis but also for templating nano- and microparticles during their fabrication for various pharmaceutical applications and, in particular, for drug delivery purposes. Droplet microfluidics with precisely controlled production of droplets to be used as templates for reproducible and scalable particle fabrication allows significant improvements in tuning sizes (with minimal deviation from mean dispersity values), shapes, and morphologies of the materials when compared with traditional bulk techniques. Typically, particle fabrication comprises three consecutive steps: (i) formation of droplets in microfluidic generators; (ii) shaping of these droplets in specially designed microchannels; and (iii) solidification by chemical, photochemical, or physical methods to form final particulate emulsions [52]. Passive or active droplet generation methods are adopted [53] according to the desired design and final application, for the production of spherical and nonspherical particles, microcapsules, and vesicles of both organic and inorganic origin, based on single and double multiemulsion templates. The latter are expectedly more challenging to manufacture, due to the requirement of using two-phase systems and their precise control to achieve complex shapes, such as the core-shell design [54]. On-chip fabrications of drug delivery systems have been recently reported, achieving complexity in drug carriers coupled to their precise size and composition that contribute to better prediction and tunability in the drug release profiles [55]. Efforts for advancing manufacturing and control of drug delivery particulate-based systems are excellently reviewed in Riahi et al. [56].

4 MATERIALS AND PROCESSING TECHNIQUES

The materials that have been employed for the fabrication of microfluidic systems range from silicon, glass, and ceramics to polymer-based materials that include elastomers, thermosets, thermoplastics, and more recently paper. Depending on the application, required function, and degree of integration, special attention should be paid on choosing the correct material for the fabrication of the microfluidic system as it determines both the inherent properties of the device and the possible fabrication technologies that can be used [57]. Characteristics such as flexibility, air permeability, electric conductivity, solvent compatibility, optical transparency, and biocompatibility may be important when selecting a material [58]. Another important factor is the cost that must be minimized in order to fabricate cost-effective products and single-use disposable devices to avoid cross contamination between essays.

4.1 SILICON AND GLASS

Silicon and glass are typically processed by well-known fabrication methods from the semiconductor industry (Figs. 1.5 and 1.6) such as bulk micromachining using wet and dry etching, although silicon structures can also be fabricated by surface micromachining [59]. Bulk micromachining produces structures within the substrate, that is, substrate is selectively etched, using photolithography to transfer a pattern from a mask to the surface. In turn, surface micromachining allows developing structures on the top of the substrate, which means that thin layers of silicon are subsequently deposited using chemical deposition methods. Silicon is transparent to infrared light, but not in the visible spectral range, making fluorescent detection or fluid imaging challenging.

On the other hand, glass is optically transparent and shows low-background fluorescent. Further, glass modification chemistries are silanol-based, such as for silicon. Favorable properties of silicon and glass come from their thermostability and solvent compatibility. Therefore, nonspecific adsorption can be reduced or cell growth improved through chemical modification of the surface. Nevertheless, the hardness of silicon and glass, the higher cost and time of fabrication, and the difficulty to seal the microfluidic structure and to fabricate and integrate functional units, together with the nongas permeability, have prevented their use in many microfluidic applications and motivate the use and development of other materials that can be easily fabricated and are compatible with a broader range of biological applications [60].

4.2 POLYMERS

Polymer-based microfluidic systems appear as an interesting alternative, in particular for being relatively inexpensive, suitable for mass production processes, and adaptable through formulation changes and chemical modification [61,62]. An additional benefit is the wide range of available polymers that offer a large flexibility in the selection of material with specific properties. According to their physical properties, polymers can be classified into thermosets, elastomers, and thermoplastics. Thermosets such as SU-8 are normally stable even at high temperature, resistant to most solvents, and highly biocompatible and usually show proper transparency and mechanical properties. SU-8 allows the fabrication of high-aspect ratio and free-standing microstructures using soft lithography [63,64]. When properly heated and exposed to specific UV light using high-resolution photomasks with an inverse pattern (as the resist is negative), the parts exposed become cross-linked, while the remainder is soluble and removed during development process. Therefore, SU-8 has been often used as structural material for the fabrication of functional units (e.g., microelectromechanical systems) and often as permanent building template for microfluidic systems based on poly(dimethylsiloxane) (PDMS), the most popular elastomer in microfluidics (Fig. 1.5). In the latter case, during the process, generally called replica molding, PDMS liquid prepolymer is cast on photoresist templates, thermally cured at mild temperature (40–70°C) and peeled off easily due to its

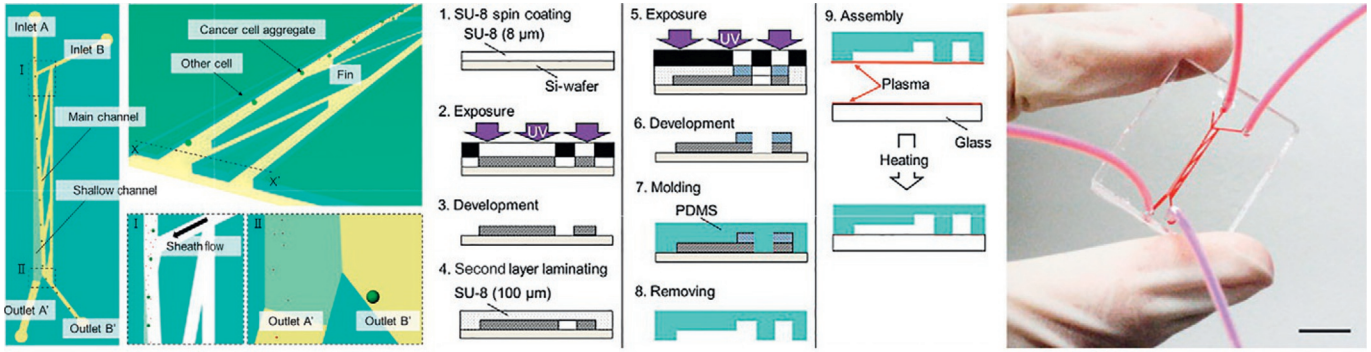

FIG. 1.5

PDMS microfluidic chip for cancer cell separator using size-dependent filtration. The PDMS microchannel was produced by replica molding using an SU-8 master mold fabricated by photolithography. (A) Concepts of the microfluidic chip for filtering ultralow concentrated cancer cells in patient's peritoneal washes. (B) Fabrication process of the microfluidic chip. SU-8 sheet and two-step exposure were used to make a mold of the chip. The two-step exposure was performed to fabricate a precise uneven PDMS channel for cell filtration. (C) Photograph of fabricated microfluidic chip. The main channel and shallow channels had heights of 100 and 8 μm, respectively. Bar is 10 mm.

Adapted from T. Masuda, M. Niimi, H. Nakanishi, Y. Yamanishi, F. Arai, Cancer cell separator using size-dependent filtration in microfluidic chip. Sensors Actuators B Chem. 185 (2013) 245–251.

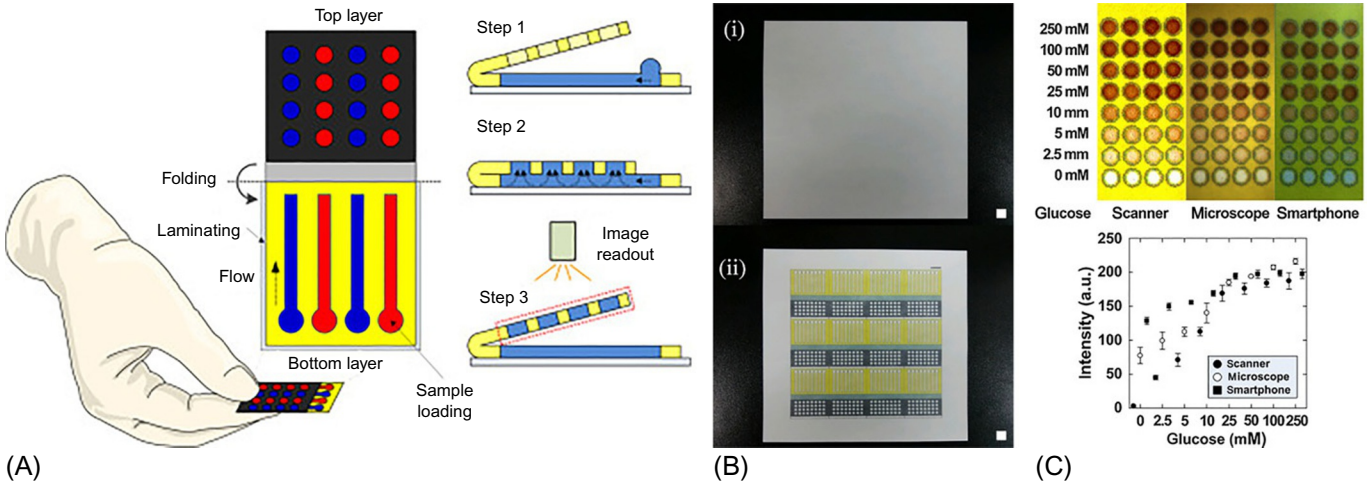

FIG. 1.6

Three-dimensional paper-based microfluidic device that enables vertical flow multistep assays for the detection of C-reactive protein based on programmed reagent loading. (A) Schematic representation of the paper-based device. The device consists of two layers. The priming and reagent solutions for colorimetric protein and glucose bioassays were preloaded to each reservoir of the top layer. (Step 1) The test solutions were loaded to each injection zone of the bottom layer. (Step 2) The chemical reactions in folded paper-based 3-D microfluidic device through tip-pinch manipulation of the thumb and index fingers. (Step 3) Air dry and image readout after unfolding. (B) Paper-based 3-D microfluidic devices (i) before and (ii) after wax impregnating. Hydrophobic patterns could be clearly observed in the back view after wax impregnating. Scale bar = 10 mm. (C) Colorimetric bioassays and intensity analyses of glucose concentrations with three image readout instruments. Calibration curves for glucose concentrations of 0–50 mM were $R^2 = 0.9781$ for the scanner, 0.9686 for the microscope, and 0.9658 for the smartphone.

Adapted from S. Choi, S.K. Kim, G.J. Lee, H.K. Park, Paper-based 3D microfluidic device for multiple bioassays. Sensors Actuators B Chem. 219 (2015) 245–250.

low surface tension [65]. To enclose the obtained open microfluidic channels, PDMS can be bonded reversibly to PDMS, glass, or other substrates by simply making contact or irreversibly by using oxygen plasma treatment or a thin mildly cured layer of PDMS as glue.

PDMS shows very interesting properties for the fabrication of functional units (e.g., valves and pumps) or/and for the fabrication of PDMS microfluidic systems for biotechnological applications (e.g., for long-term cell culture, cell screening, and biochemical assays in sealed microchannels) that include high biocompatibility, porous matrix allowing permeation of gases, high elasticity and reasonable cost, rapid fabrication, and ease of implementation [66,67]. However, the nonspecificity and permeability by hydrophobic molecules into the channel walls due to the hydrophobicity of the PDMS surface along with the water evaporation through channel walls can cause a change in the concentration and composition of the fluid. Several strategies such as chemical surface modification along with using continuous flow can often be addressed to overcome these issues [68]. Regarding thermoplastics, because of their wide use in the industry, their processing by thermomolding is well known. In this case, a large number of structures can be produced at high rate and low cost using metal or silicon templates and high temperatures. However, the fabrication of this kind of templates is time-consuming and expensive and therefore is not widely used for prototypes, being excellent for commercial production [57]. Typical approaches for sealing open microchannels include thermobonding and glue-assisted bonding [69]. Moreover, surface grafting or dynamic coating can be used to modify the surface of thermoplastics, and electrodes are easily integrated [70]. Thermoplastics show the interesting ability of being reshaped multiple times by reheating, which is appropriate for molding and bonding. Poly(methyl methacrylate) (PMMA), polystyrene (PS), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) are typical thermoplastics used for the fabrication of microfluidic systems [57]. Although thermoplastics show slightly better solvent compatibility than PDMS, they are barely permeable to gases, and their rigidity makes the fabrication of functional units difficult. In turn, although their melting temperatures are high (i.e., over 280° C), perfluorinated polymers, such as perfluoroalkoxy (commonly known as Teflon PFA) and fluorinated ethylene propylene (Teflon FEP), show good gas permeability, enough softness to fabricate functional units, excellent inertness to chemicals and solvents, antifouling, low nonspecific protein adsorption compared with PDMS, cellular compatibility over 5 days, and good optical transparency [58].

4.3 PAPER

Paper is a cellulose-based material recently introduced as a promising substrate for the development of flexible, disposable, biocompatible, and low-cost microfluidic systems. In addition to generating flow to transport aqueous liquids due to its porosity and hydrophilicity and allowing further filtering and separation, paper can be

chemically modified and conjugated with many biomolecules, including peptides and nucleotides [58]. Based on these properties, paper-based microfluidic systems have been mainly developed for diagnosis purposes since the white background provides a contrast for color-based detection techniques (Fig. 1.6).

Based on the results obtained in this field, it is believed that paper could provide an advantageous platform for accomplishing in vitro precompound screening steps, offering a solution to many economical obstacles inherent in the pharmaceutical industry [71]. Therefore, it is shown that a large set of materials and processing technologies are currently available for microfluidic system development, and new ones are emerging at a rapid rate. Nevertheless, although different materials can be modified or combined to fabricate powerful devices for specific applications, current trends demonstrate that for laboratory research, the proper selection of materials typically implies ease in prototyping and high performance of the system, while in the industry, the major concerns rely on the cost of production and the reliability in use [57].

5 REPRESENTATIVE APPLICATIONS

During the last decade, a significant amount of studies has emerged taking advantage of the characteristics of microfluidic systems for simple sample handling, reagent mixing, separation, and detection of the complex biological environments. Along with this, recent improvements in fabrication techniques allow the manipulation of difficult samples and reagents, while still reducing overall costs. Important for pharmaceutical testing, modern microfluidic devices require between 0.1 and 10 μL of sample, significantly decreasing sample and waste volumes. Initial attempts are already carried out to industrialize the fabrication and design of parallel flow of several fluids, meaning multiple samples scanning on a single and portable device. In addition, recent technological advances in material science led to even more obvious reasons for pursuing microfluidics for pharmaceutical applications. Indeed, fabrication of microfluidic systems on plastic or paper materials allows for mass production at low costs, and these devices can even be disposable. Meanwhile, the investigation toward optimizing designs for high-throughput screening multiple assays will considerably reduce time and human effort compared with standard in vitro and in vivo analysis. Meanwhile, additional investigations are still necessary for confirming the credibility of highly complex LOC capable of sampling, processing, separation, detection, and waste handling on a single chip. Such fast and continuous progress makes microfluidics the technology of choice for future drug discovery/development; pharmacokinetic evaluations and toxicity screenings; drug delivery; diagnostics; and, lately, developing of in vitro 3-D and whole-body models for analysis. To this end, this section is aimed to present some of the most representative examples of microfluidic systems for pharmaceutical investigations, from commercialized simple LOC devices to novel, highly complex miniaturized designs.

5.1 MICROFLUIDIC TECHNOLOGY FOR DRUG DEVELOPMENT, DRUG DELIVERY AND DIAGNOSTICS

5.1.1 Protein expression and enzyme activity/kinetics

Gaining deeper insights of relevant targets for drugs, such as membrane proteins and enzymes, is of paramount importance for advances in pharmaceutical concepts, which will also lead to better understanding the effects of drugs on biological systems and to profile the effects on the metabolic pathway level [72]. Thus, the development of microfluidic devices for cell-free screening is of particular importance in drug discovery for a clear in vitro view on drug-target effects. One such study aims to significantly reduce consumption of reagents in drug discovery by the development of a strategy for parallel high-throughput modules for cell-free expression of functional cell proteins [73]. The disposable device is compatible with 96-well microplate readouts and couples a reaction microchamber with adjacent loading ports and the feeding chamber. The tested membrane-associated proteins were bacteriorhodopsin and apolipoprotein A, both expressed in a single reaction, whereas soluble luciferase and β-lactamase were also cosynthesized.

Related to advances in drug-target research and early-stage toxicity screens, the information of kinetic data on the reaction of enzymes with small molecules are gaining significance for drug discovery and development [74]. Here, the additional challenge that the developed microfluidic platforms has to meet is the rapid (within minutes) determination of enzyme activity and automated measuring [75]. To do so, enzymes are typically immobilized on solid supports in microchannels that are subjected to a continuous flow of reagents. The same design is also employed for the determination of enzyme inhibition in microchannels, this time from the generated fluorescence data. Of especial relevance is that the results in a microfluidic approach are obtained after just 2 min, compared with 15 min necessary for the same data in the standard plate approach [76].

5.1.2 Diagnostics

Although of secondary interest for the pharmaceutical industry, there is a strong focus in developing microfluidic systems for early diagnosis, particularly relevant for difficult-to-treat diseases and conditions, for example, malignant tumors or nosocomial infections. Together with their simplicity and improved diagnostic speed compared with time-consuming off-site laboratory tests, microfluidic devices are being developed aiming at better sensitivity and portability. An example is the development of compact disk-based microfluidic systems able to automatize biochemical assays and immunoassays that are eliminating human errors and allow minute reagent consumption during detection [77]. The type of samples for testing and their collection are not affected by the device design due to the fact that the sample collection remains external, as in the case of any other testing. Examples of the device fabrication range from simple microfluidic immunoassays for rapid saliva-based clinical diagnostics [78] to simultaneous multidetection of hepatitis B, hepatitis C, and HIV biomarkers in blood serum [79]. In line with the latter, research focus is

directed to develop point-of-care testing devices for infectious diseases (in particular HIV), of paramount interest for public health (Fig. 1.7) [80,81].

Regardless of the sample, multimodal detection is of specific interest for developing competitive immunoassays and simultaneous detection of biomarkers with preference of measuring their fluorescence signals. Nevertheless, label-free immunoassays are also being conducted in microfluidic systems thanks to the coupling

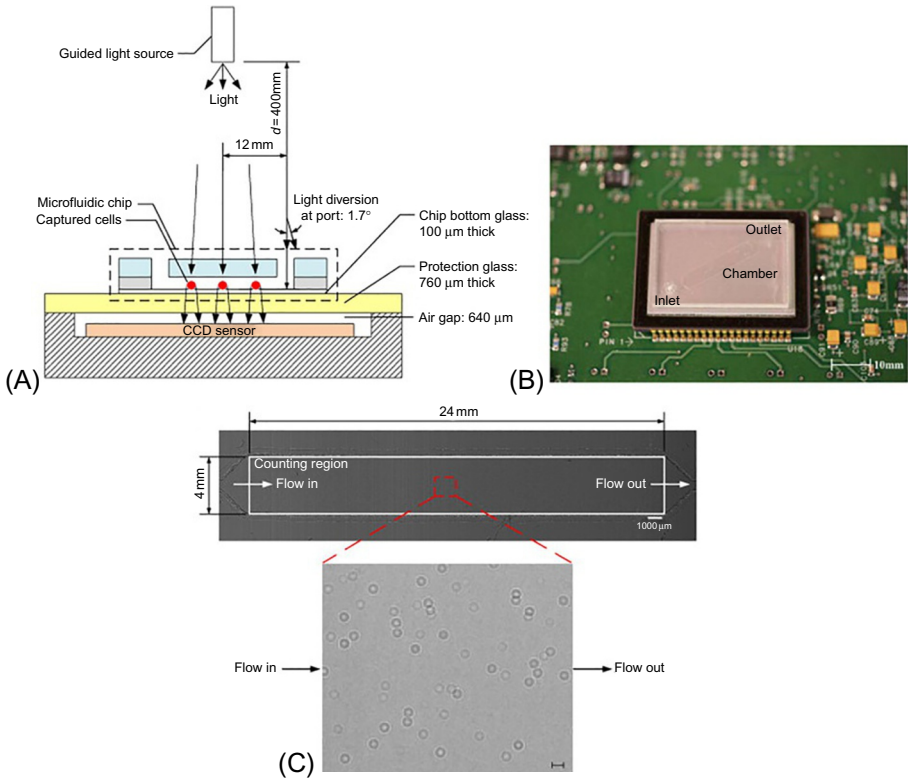

FIG. 1.7

Imaging platform for detection captured cells with a disposable microfluidic device. (A) When light is incident on the captured cells, cells diffract and transmit light. Shadows of the captured lymphocytes generated by diffraction can be imaged by the device in 1 s. Image is obtained with the lensless imaging platform. (B) Photograph of the microfluidic chip and the imaging platform. The entire microfluidic device can be imaged without alignment by simply placing the microfluidic channel on the sensor. (C) Image taken with the imaging platform and the shadow image of the cell in the microfluidic channel is shown. The image is obtained by diffraction. Scale bar = 100 μm.

Adapted from S. Moon, H.O. Keles, A. Ozcan, A. Khademhosseini, E. Hæggstrom, D. Kuritzkes, U. Demirci, Integrating microfluidics and lensless imaging for point-of-care testing. Biosens. Bioelectron. 24 (2009) 3208–3214.

with robust and sensitive detection methods, such as surface plasmon resonance [82] and imaging ellipsometry [83], generating consistent results with widely accepted ELISA tools. One of the newest paradigms in cancer diagnosis and treatment are exosomes, released from both normal and cancer cells, however with a different footprint and role in remote cell-to-cell communication and signaling [84]. These large extracellular vesicles could serve as carriers for bioactive proteins and different RNA molecules, which means involvement in tumor progression, metastasis, and even drug-resistance mechanisms [85,86]. In this context, an initial tumor could be detected by identifying exposing exosomes in related body fluids (e.g., sputum, blood, and serum), released at a very early step in tumor progression. Logically, they became not only targets for new drug discovery and development but also biomarkers for the diagnosis of cancer or even seen as transport vehicles for drug delivery [87,88]. Thus, exosomes are being targeted by future microfluidic systems that should feature design and dimensions accommodated to the size of these vehicles. The use up to date is still in its infancy and concerns replacement of processes of ultrafiltration and/or ultracentrifugation for the isolation of exosomes from cell culture supernatants. The traditional centrifugation protocols are limited to isolation based on the size of bioentities and cannot distinguish between different exosomes, those from healthy cells and those from tumor. By using microfluidic channels with especial patterns, similar to that used to isolate rare circulating tumor cells from blood [89] and similar dimensions to small vesicles, it is anticipated that such shortcoming might be resolved that will allow improved handling, analysis, and manipulation of exosomes. Overall, a broader use of microfluidic platforms is yet to be established in diagnostics, as both reliable point-of-care home/clinical devices and separation/purification tools.

5.1.3 Microfluidic high-throughput screening

Systematic screens and large data processing became an integral part of pharmaceutical research that facilitates the evaluation of complex reactions, interactions, and systems. Systematic screens are useful to resolve massive data for chemical [90], biochemical [91], and cell-based assays [92]. Since global screens in pharmacy lead to improved reliability of the developed treatments, today's existing libraries are counting up to tens of thousands of elements. Microfluidics-based systematic screens are likely to be more frequently used in the near future. First progress was achieved by Caliper Life Sciences with their generic platform employed for various types of high-throughput screening (HTS) applications [93]. Its primary use is carrying out enzymatic assays on a glass microchip with integrated capillaries that drag examination fluids from plate wells, at the same time continuously drawing enzyme and substrates from wells integrated on chip. The mixtures are transported in a microchannel to a detection point where fluorescence readout is performed. The chips can transport a large number of examination fluids, intercalated with buffer flushing steps to clean the system between the readings. This microfluidic network is capable of assaying with considerably higher throughput and significantly less consumption than

conventional plate-based screening devices. The platform is currently used in a large number of pharmaceutical companies in HTS applications.

5.2 CELL-BASED DEVICES

5.2.1 Simple cell-based devices

LOC technology is nowadays being increasingly investigated for developing in vitro models of different diseases/conditions or for carrying out more predictive toxicity studies. Due to the nature of biological systems, of particular interest are cell-based models, both single-cell arrays and complex 3-D cell culture systems. In in vitro models, cell type and source are the key factors influencing accurate representation of the (patho)physiological states found in vivo and thus reliability and validity of in vitro studies. To date, successful examples include primary cells of the liver, heart, and brain, among others. However, culturing of primary cells usually results in a reduction in specificity due to important alterations of the environment in cell culture vessels [94]. The current choice of microfluidic systems is owed to better mimicking the cellular microenvironment and improved cell handling, positioning, and analysis. Nevertheless, some challenges still remain, such as poor small-volume liquid handling ability, large consumption of reagents, and high cost of operation. Liu et al. [95] suggested an integration of combinatorial mixer to 3-D microfluidic device able to culture and screen the combinatorial effects of multiple-compound exposure on cultured cells. A 1 cm $\times$ 1 cm chip with a three-input combinatorial mixer and eight individually isolated microculture chambers was fabricated to proof the concept (Fig. 1.8).

Of particular importance is culturing stem cells in microfluidic platforms, since nowadays adopted cell isolation and culturing techniques are not able to predictively direct their differentiation toward relevant cell types in order to produce in vitro models of, for example, disease for drug-target evaluation or toxicity studies [96]. Microfluidic assays appear as alternatives for cell fate in vitro control by improving the spatiotemporal cell behavior, especially due to better control of biochemical and biophysical extracellular factors [97,98]. Nevertheless, the development of such platforms is still in its infancy, and more intensive focus to such investigations is necessary to propose an optimal design to control cell fate by environmental factors.

One important application of microfluidic cell culture systems is analysis of DNA damage in cancer models, an alternative to the widely accepted multistep comet electrophoresis assay, which analyzes damage and repair on a single-cell level. A microwell array patterned in an agarose layer of Wood et al. [99] not only allows single-cell trapping and high-throughput analysis of DNA damage but also overcomes impeded cell analysis because of overlapping cells. The device is fully automated and enables analysis of captured ones in microwell single cells in a single focal plane (and not overlapping). The technology is transferable to study DNA damage or drug screening in a high throughput, because the micropatterned array (microwells) can be sandwiched between a substrate and a microliter plate. Another noteworthy development is in droplet technology for drug discovery, which further elaborates

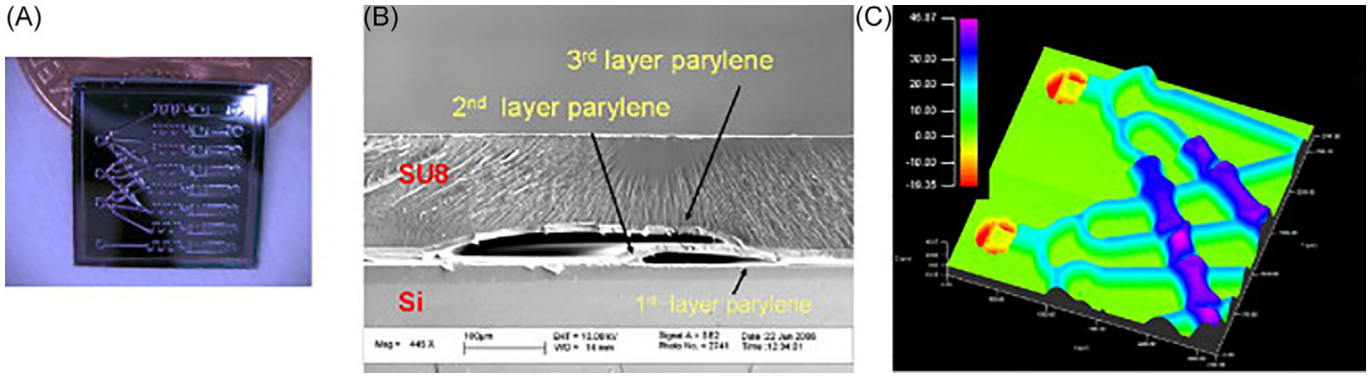

FIG. 1.8

Microfluidic multiple cell culture array with an integrated combinatorial mixer. (A) Fabricated 1 cm × 1 cm chip. (B) SEM image of the cross section of the microfluidic overpass. The overpass has two-level microfluidic channels, and such structures allow that two fluidic streams are separated spatially at the overpass. (C) Surface height profile scan of the overpass region. The overpass structure is about 15–20 μm higher than the first-level microfluidic channels.

Reproduced from M.C. Liu, D. Ho, Y.-C. Tai, Monolithic fabrication of three-dimensional microfluidic networks for constructing cell culture array with an integrated combinatorial mixer, Sensors Actuators B Chem. 129 (2008) 826–833.

encapsulation of single cells within droplets being again compatible with high-throughput screening and cell sorting [100]. For example, a droplet viability assay that allows the quantitative scoring of cell viability was developed to allow screening of drug libraries for cytotoxic effects on cells [101]. A cell-containing droplet is combined with a fluorescently encoded one and with varied concentrations of drug, whereas the system enables their incubation during ~15 min before analyzing cell viability.

5.2.2 Toxicity

In case of toxicity evaluation, the information obtained from these models serves to predict the safety aspects of the potential drug candidates, where absorption, distribution, metabolism, elimination, and toxicity properties are evaluated. Accordingly, the undesirable toxicity of drug is nowadays a leading cause of drug development failures [102]. The established assays for cytotoxicity, genotoxicity, drug-drug interactions, and metabolite-mediated toxicity are microplate- and micronucleus-based ones. These evaluations are also possible to be carried out in microfluidic platforms [103–105]. Similar designs were used to go beyond the evaluation of toxicity in cells/organs, to reconstitute the metabolism of a drug, and to evaluate systemic responses in in vitro models. The provided pharmacokinetic information for early toxicity evolved into a holistic view of toxicology in vitro, to be carried out prior to more expensive in vivo investigations. One example is to evolve from cell culture analogue [106] to a microscaled "animal-on-a-chip" system that integrates cocultures of different tissues and fat cells with physiological fluid arrays on a single chip [107,108]. The chip is a model to create a surrogative predictor of animal experiments for chemical exposure assessment, something only achievable with time-consuming and costly multiple conventional toxicity studies. This chip represents a step closer to progresses that will enable robust in vitro toxicology models for testing pharmacokinetic profiles of drugs and systemic toxicity specific to humans, instead of animals [109]. Of course, for the viable adoption in pharma, both high-throughput and high-content assays must be conductible, which means properly designed and integrated microfluidic setup. Additionally, research and development efforts should also be directed to microfluidic systems for functional 3-D cell cultures.

5.2.3 Three-dimensional cell culture

Growing cells in 3-D brings about several advantages to flat-layer culturing: (i) altered cell morphology, (ii) more realistic drug response due to increased resistance, (iii) captured phenotypic heterogeneity, and (iv) mimic of the tumor environment. Also, in addition to controversies related to in vivo testing, many cancers still lack qualified animal models. Compared with flat cell layers, 3-D cultured cells more closely resemble cell-cell interactions and tumor heterogeneities in vivo [110]. On the minus side, 3-D cell elaboration is more complicated and costly than already adopted and reasonably reliable monolayer cultures. Nevertheless,

new developments in chip technologies and microfluidic platforms are now paving the way for more feasible and affordable 3-D cell cultures and experiments.

For example, micropatterned substrates can be used to precisely control the size and shape of the multicellular aggregates (microtissues), which enable the optimal supply of nutrients to cells and the removal of undesired metabolites. In this context, the fabrication of temperature-responsive microarrays for the formation of artificial tissue of controlled size and shape as tissue models for drug discovery is particularly interesting [111]. Thus, temperature-responsive poly(*N*-isopropylacrylamide) polymer micropatterns provide reversible states of cell-adherent (37°C) and cell-repellent (24°C) behavior. Alternatively, microtissues can be processed in microfluidic devices via photo encapsulation of up to 1000 cells in polyethylene glycol hydrogels, so that the microtissues could be studied with a multiplexed microfluidic approach [112]. The mechanically stabilized microtissues of 250–350 μm have been used in high-throughput flow sorting and analysis, as an example of quantitative statistical analysis. On the other hand, strategies using continuous-flow lithographic encoding can be used in similar methods as an attempt to improve multiplexing capabilities [113]. Such attempts are directed to the standardization of high-throughput tests to evaluate the response of 3-D models to different drugs and their combinations. In light with the aforementioned, new microfluidic platforms provided with increased functionality are developed to improve data quality from in vitro assays [114,115]. However, besides providing deeper insight into the spatiotemporal cues that govern cell fates, the key point is to turn these platforms into high-throughput devices with sufficient accuracy for drug discovery platforms. Chi et al. excellently reviewed how the scientific advances turned out into more matured 3-D biomicrofabrication technologies and exemplified several companies offering chips for reliable biochemical assays; cell/biomarker and infectious disease diagnostics; and 3-D models for drug discovery, screening, and delivery [37].

5.3 ORGANS-ON-A-CHIP, A STEP TOWARD WHOLE BODY MODELS

5.3.1 Organs-on-a-chip

Going beyond cell-cell interactions in 3-D chip models, efforts are being carried out to optimize the fabrication of microfluidic devices that combine several cell types in 2-D or 3-D designs in order to simulate human organs on a single chip [116,117]. Such chips resemble more closely complex interactions between different tissues and organs, in contrast to mimicking interactions between cells of the same type in 3-D models [34]. Thus, these systems hold enormous potential to reduce or even completely replace animal testing because they circumvent the major limitation of in vivo tests, that is, metabolic differences between humans and animals. It is expected that the obtained information on drug efficacy and toxicity on an organ level will allow more accurate early-phase decisions in future drug development.

From a technological point of view and in order to successfully simulate tissue-tissue interactions, a model with different cell types cultured in 3-D designs but using separate chambers is proposed, where the microtissues were connected by a

microfluidic network [118]. The connective network allows for reproduction of the pharmacokinetic profiles of drugs simultaneously in cancerous and healthy tissues, which were by far different to the effects observed in a conventional microplate assay. It is worth mentioning that additional experimentation should be made for adjusting tissue geometries and channel profiles so that fluid residence times correspond to physiological residence times of body fluids in the targeted organs. In a similar approach, a compartmental design has been proved to be successful for the fabrication of a bilayer silicon microfluidic structure, aimed to mimic alveolar-capillary interface of a human lung while integrating mechanical cell actuation [119]. The mechanical actuation was simulated by applying vacuum at the separate ends of a middle channel with cultured human alveolar epithelial cells in the air on the upper side, whereas the lower channel contained a fluid with vascular endothelial cells. The resulting stretching of the silicone membrane enhanced cellular uptake of nanoparticles, influencing inflammatory responses similarly as in in vivo studies. In a more complex approach, the reconstitution of a small artery on a microfluidic chip was achieved by Gunther et al. [120]. A triple microchannel system for the fixation, perfusion, and superfusion of the vessel was used for long-term culture and investigation under the influence of phenylephrine or acetylcholine. This system may thus present a ground-breaking achievement for enabling pharmacological and toxicological screens and evaluate the effect of new drugs in the arteries. Readers are further encouraged to search the references from Table 1.1, containing some of the most relevant examples of organs-on-a-chip.

Besides toxicological response in 3-D model platforms that comprised healthy cells to mimic human organs, modeling microfluidic platforms that resemble in vivo cancerous situations is of especial interest for pharmacological monitoring of drug effects. The so-called tumors-on-a-chip are models aimed to screen the behavior of cancer cells, for example, anti-angiogenesis, during the treatment with drugs; investigate interaction between healthy and tumor cells; or focus on improving the detection of cancer at the disease onset. For the latter, microfluidic devices are designed with lateral channel structures in which circulating tumor cells or relevant biomarkers in small concentrations are collected and quantified [133,134]. More complex structures are necessary to be developed for detailed recapitulation of tumor microenvironments, such as recently developed in vitro model with 3-D structure of microfluidic channels where tumor cells and endothelial cells are cultured within extracellular matrix under perfusion of interstitial fluid [135]. The system allows simulation of complex drug transport around the tumor and is tested with nanoparticulate-based therapeutic agents aiming at targeted delivery of therapy. The information obtained from this tumor-microenvironment-on-chip model is particularly relevant as it provides guidance for design of nanoparticles (including perception about optimal size and morphology) in the associated therapeutic approaches.

Although the principles for developing tumor models or hybrid tumor models (comprising both tumor and healthy cells) are similar for different cancer types, only individual investigations on each particular model can give specifics regarding the

Table 1.1 Examples for organs-on-a-chip for drug testing and delivery

Model organ	Experimental model	Means of testing	References
Heart	Anisotropic cardiac microtissues and soft elastomers in thin film	Effect of isoproterenol on cardiac contractility	[121]
	Micromolded gelatin hydrogel	Cell metabolic function over 4 weeks for chronic studies	[122]
Liver	Microfluidic endothelial-like barrier of hepatocytes	Effect of diclofenac on hepatotoxicity	[123]
	Multiplexed microfluidic channels each one with 3-D microenvironment	Five model drugs for dose-dependent on-chip testing	[124]
Lung	Two microchannels one with epithelial cells and another with endothelial cells, separated by a poly(dimethylsiloxane) membrane	Organ-level responses to bacteria, inflammatory cytokines, and silica nanoparticles	[119]
	Two side channels for vacuum-mimic breathing	Effect of several drugs for the prevention of interleukin-2-induced pulmonary edema	[125]
Brain	Microfabricated low-stress silicon nitride membranes	In vitro model of blood-brain barrier based on the effect of protein treatments to cell seeding	[126]
	Molded transparent polymers in a multicompartment cell culture platform	Culturing neurons of the central and peripheral nervous systems	[127]
Kidney	Multilayer device integrating a polydimethylsiloxane microfluidic channel and a porous membrane	Culturing and analysis of renal cells while mimicking tubular-like stress environments	[128]
	Extracellular matrix-coated polyester membrane separating the main channel into luminal and interstitial space	Albumin transport, glucose reabsorption, and brush border alkaline phosphatase activity in epithelial cells	[129]
Intestine	Two channels separated by a semipermeable membrane for cell inoculation and culturing	Long-term (2 weeks) culture and monitoring of polarized transport activity of Caco-2 cells	[130]
	Same as the above with cocultured epithelial cells and normal intestinal microbe	Tests for mimicking complex structure and physiology of living intestine	[131]

Table 1.1 Examples for organs-on-a-chip for drug testing and delivery—*cont'd*

Model organ	Experimental model	Means of testing	References
Blood vessels	Lithography-engineered microvascular networks in 3-D collagen scaffold	Characterization of morphology, mass transfer processes, and long-term stability of the endothelium	[132]
	Soft lithographic definition of triple microchannel network of poly(dimethylsiloxane) on a single plane	Smooth muscle and endothelial function and investigation under the influence of phenylephrine or acetylcholine	[120]

effectiveness of therapeutic approaches, regardless of their nature. Thus, it is of paramount importance that the microenvironments of specific tumors are matched in those models. Studies are continuously reported, while here, we highlight the progresses in the development of lung [136,137], breast [138], prostate [139,140], pancreas [141], and brain [142] cancer models, for various purposes in cancer research.

5.3.2 Multiple organs-on-a-chip

A further step not only toward ultimate realistic investigation models but also toward complexity is culturing multiple organs-on-a-chip (Fig. 1.9) [144–146]. Particularly challenging for providing reliability in these authentic whole-body-on-a-chip systems is the necessary allometric scaling effect of the microorgans. Thus, scaling must involve mass allometrics of the organs, coupled to mimicking different blood circulation and pulse through the organs. In addition, the preservation of the important organ functions is paramount during scaling, all connected to raising challenges of the microfluidic design. Finally, interconnecting of organs-on-a-chip should follow the real interaction of organs in humans, that is, which organs must interact with others and which must not come into contact with the common medium. The common medium must be "friendly" for all cells in the model.

As a standout work on a multichannel 3-D system, integrating microorgans of liver, lung, kidney, and fat tissue was presented by Zhang et al. [147]. A common medium was maintaining the functions of four different cell lines (i.e., microorgans), coming from one reservoir with connections to an inlet directly attached to each cell group. Such design enables testing the multi-organ implications in drug delivery and toxicity. In an ideal scenario (without a drug), all cells were able to function above 90% of their usual activity. When tested with a growth factor, growth was promoted in three out of four cell lines, with a significantly lower viability of the fourth line. In a further experiment involving protein microspheres to control the growth factor release into only one cell line group, the results showed that it is possible to selectively stimulate one particular cell line without adverse effects to other cells.

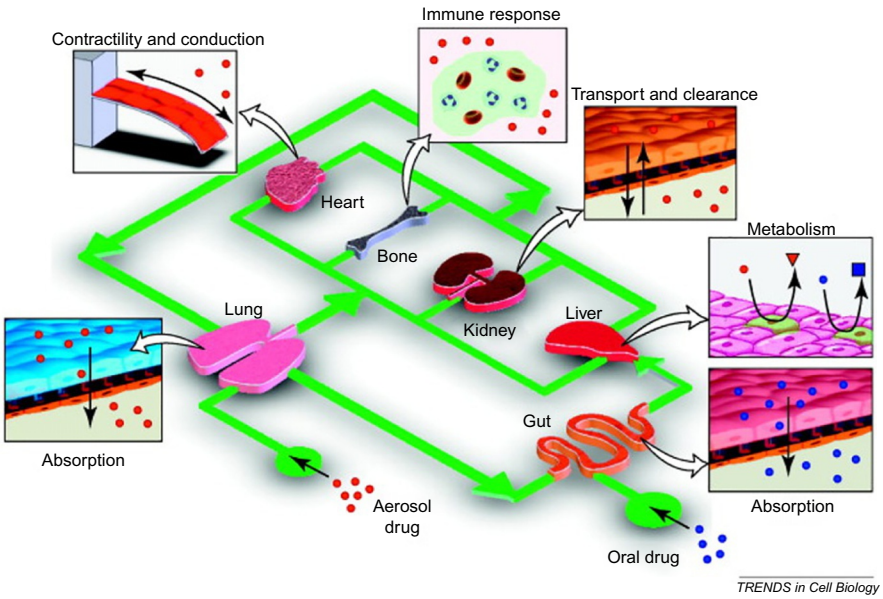

FIG. 1.9

The human-on-a-chip concept. Biomimetic microsystems representing different organs can be integrated into a single microdevice and linked by a microfluidic circulatory system in a physiologically relevant manner to model a complex, dynamic process of drug absorption, distribution, metabolism, and excretion and to more reliably evaluate drug efficacy and toxicity. As shown in this example, an integrated system of microengineered organ mimics (the lung, heart, gut, liver, kidney, and bone) can be used to study the absorption of inhaled aerosol drugs ("aerosol drug") from the lung to microcirculation and to measure their cardiotoxicity (e.g., changes in heart contractility or conduction), transport and clearance in the kidney, metabolism in the liver, and immune-cell contributions to these responses. Drug substances ("oral drug") also can be introduced into the gut compartment to investigate interplay between orally administered drugs and molecular transporters and metabolizing enzymes expressed in the various organs [143].

Another multiple organs-on-a-chip was designed to test drug toxicity in three different cell lines separated in three culture chambers, which enabled mapping drug pharmacological effects [148]. The device was tested with healthy liver cells, colon tumor cells, and leukemic cells. A reservoir containing suitable medium was connected to the three cell culture chambers, whereas the medium flow rate was controlled by gravity. An anticancer drug was used for testing, showing a decline in viability between all three cell groups.

Besides culturing tissues and multiple organs, microfluidic devices are increasingly investigated for improved screening and data synthesis of animal organisms.

Small vertebrates are very useful in drug discovery for testing on a systemic level; however, with conventional methods, the testing is usually highly complex, dependent on many low controllable factors, and time-consuming. Automated microscopy [149] and especially microfluidics are significantly simplifying and accelerating the testing. Microfluidic platforms simplify the handling, positioning, orienting, and manipulating of the entire organisms and analyses, proved on different organisms, such as not parasitic worms [150], embryos of a fly [151,152], or zebra fish [153]. Exemplified, microfluidic arrays are able to order and vertically orient, for example, fly embryos on a large scale to enable quantitative imaging of the embryos in a position not possible with traditional coverslip-based approaches. A microscale meandering manifold injects and transports the embryos to intersections with cross-flow channels where the similar in size embryos are vertically trapped in a cylindrical cutout controlled by the injection pressure. Then, the embryos can be securely transported in the orientation essential for high-throughput screening systems, for example, to allow quantitative analysis of different patterns within the biosystem [151]. The model was also successfully translated to high-throughput assays and monitoring developmental responses of embryos to external stimuli [154]. Another example is a high-throughput system for automated investigations, proved for chemical and genetic screening of zebra fish larvae [153]. All the steps of the system were automated, from loading the larvae, optical detection, positioning and rotation in a rotatable capillary, image focusing and acquisition, laser manipulation, and dispensing step back into a multiwell plate. The system is able to provide confocal imaging and laser-based microsurgery of oriented zebra fish larvae within less than 20 s and set the basis for similar investigations [155,156].

6 SUMMARY AND OUTLOOK

An overview of the main topics related to microfluidics for pharmaceutical applications has been provided demonstrating the technological ability to miniaturize many larger-scale complex processes and giving rise to the LOC concept.

With respect to pharmaceutical applications, microfluidic systems, together with the inherent advantages of miniaturization, low cost, fast processing, and low sample quantity, allow to obtain high-quality, high-throughput data, which is particularly relevant for drug development and testing. Microfluidics-based drug testing platforms allow evaluating time-dependent dynamics, toxicity, and multicell interactions. Thus, performance and eventual side effects of drugs not only are detected but also allow evaluating the underlying mechanisms of drug response. Particularly interesting is the mimicking of the in vivo microenvironments and the organ-on-a-chip approach that will, eventually, lead to realistically mimic characteristics and functionality of a whole-body response. This will represent a major breakthrough not only in the pharmaceutical area but also in the whole biomedical field.

Finally, it has been shown that the major future trends and needs in this field are the development of high-performance microscale research and development

platforms and the fabrication of low-cost, portable analysis systems. In this context, a strong interdisciplinary approach is needed, ranging from the essential knowledge of the physical-chemical aspect of materials and processes to the development of suitable materials and production technologies, taking into account the biomedical aspects of the final application. All these together will allow overcoming the present limitations and needs of these microtechnologies with huge macro implications.

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