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A Comprehensive Review on Perfusion Cell Culture Systems

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Abstract: The enormous cost and time required for launching of a new drug on the market request a redesign of testing approaches and validation strategies. Here, microfluidics, micro and nanotechnologies can play an important role, impacting the cell culture model or the delivering strategies. We will review the recent lab-on-a-chip strategies for cell culture models with potential application for drug screening platforms. Moreover we will overview also the materials involved in the microfluidic assisted cell culture models.

Keywords: microfluidics; cell culture; bioreactors; biomaterials

Sistematičen pregled pretočnih sistemov za celične kulture

Izvleček: Uvedba novih zdravil na trg zahteva veliko razvojnega časa in je povezana z ogromnimi stroški. Za znižanje stroškov in časa se nujno pojavlja zahteva po preoblikovanje pristopov testiranja in strategij za validacijo ustreznosti zdravil. Tukaj lahko mikro in nanotehnologije ter uvajanje mikrofluidnih pristopov odigrajo pomembno vlogo pri izgradnji modelov celičnih kultur ali pa so v pomoč pri razvoju strategij za vnosa zdravil. Pregledni članek predstavlja določene nove strategije, ki temeljijo na lab-on-a-chip mikrofluidnih pristopih in njihovo praktično uporabnost pri predkliničnem testiranju zdravil. Poleg tega je v članku podan tudi pregled biomaterialov, ki se uporabljajo pri izdelavi mikrofluidnih platform, namenjenih raziskavam modelov celičnih kultur.

Ključne besede: mikrofluidika; celične kulture; bioreaktorji; biomateriali

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1 Introduction

The cost of developing of a new drug is rising exponentially along with every phase of development, reaching US$800 million per drug [1]. In this direction, the identification of the drug potential toxicological profile in the earlier development stage became a necessity. On the other hand, combinatorial chemistry as well as molecular biology and genomics understanding have led to a rapid growth of the group of novel compounds [2]. As a result, *in vitro* drug metabolism testing platforms are gaining increasing importance compared to animal model counterpart in the early stage drug screening given the high throughput testing capacity. It is not surprising to find that tremendous efforts have been put into developing suitable *in vitro* tissue model for the perusal of drug development. The main focus is on liver, the main organ involved in drug metabolism. *In vitro* models such as isolated perfused livers or liver tissue slices are difficult to use in high throughput applications despite their close imitation to *in vivo* hepatic tissue. The isolated primary hepatocytes, strike a balance between high throughput and intact cellular architecture [3]. However, isolated primary hepatocytes rapidly lose their differentiated functions when cultured using standard cell culture conditions [4]. Therefore numerous culture models have been developed

to prolong their functions. The cell culture models can be divided in two major groups based on the modality of media refreshing: static culture models and perfusion culture models. For the perfusion culture models the media is continuously replaced. As such, O_2 and nutrients transport, as well as waste removal from cellular local environment improved [5]. For example, it has been shown that under perfusion the viability, life span and metabolic performance of primary hepatocytes improved [6]. The phase I and phase II enzymes also showd long term stability in perfusion culture [7]. Perfused-cultured hepatocytes responded well to inducer and have shown stable induction of CYPs up to 7 days [8]. However, the main drawback of the perfusion culture system relies in the shear stress induced by the flow. A high value of the shear stress could be detrimental to cell viability and cell functions *in vitro* [3, 9].

Used on a large scale for application related chemical synthesis [10-12], cell manipulation and analysis [13-19], or drug discovery [20-23], microfluidics can be an interesting support for application related tissue engineering [24-26].

This article gives an overview on microfluidic related cell culture models and focuses on the system dedicated to drug screening. It also succinctly presents the materials involved in the construction of the microfluidic bioreactors.

2 On chip cell culture models

'Organ-on-a-chip' models allow restatement of *in vivo* tissue-tissue interfaces, biochemical cues and mechanical microenvironment. These models offer the opportunity of *in vitro* drug screening and could be alternatives to animal experimentation [27, 28]. On-chip models present the advantage of using less cells and reagents. In the next sections we will review the main cell culture models underlining the contribution of microfluidic and microtechnology in this direction.

2.1 Cell lines

Cell lines are well-established cell culture model. Under suitable conditions the cells will proliferate indefinitely. Cell lines are not restricted by limited number of cell divisions due to mutations. The limitation is also known as Hayflick's limit [29]. Liver cell lines are a popular choice for studying liver function and toxicity mechanism *in vitro*. They are, however, not suitable for drug metabolism and toxicity predictions because not all metabolizing enzymes are present in cell lines and the ones present are not at their normal physiological levels. One merit of human cell lines is that they can be used to gather information relevant to human body functions. Moreover, they are easy to handle and can help reduce the use of animals. Disadvantages occur because their dependence of gene expression, on passage number, unstable cells and dedifferentiated cells with phenotype no longer resembling that of the cells *in vivo*. Cell lines are also prone to contamination by other cell types, which happens with 15-20% of cell lines [30].

2.2 Liver cell lines

HepG2 cell line is the most commonly used human liver cell line. It is derived from hepatocellular carcinoma. Compared with isolated primary hepatocytes, its level of CYP is lower. Another commonly used cell line is HepG2/C3A, it is selected for its improved differentiated hepatocyte phenotype. Both of these cell lines have been cultured on chip [31-33]. Another liver cell line HepaRG was recently generated. It is reported to be more metabolically competent, however it has yet to be studied in microfluidic devices [34]. HepG2 was first integrated into microfluidic device in 2003 by Leclerc *et al.*[35]. They showed that the cells function properly for at least 12 days on their perfusion device. In microfluidic studies of HepG2 cells, the cells were treated with various compounds of different concentrations to study toxicity. Their viability was determined by live dead staining and optical imaging [36-38]. By using microfluidic devices, it is possible to achieve multiple incubations in one chip and generate concentration gradients easily. For high throughput screening of cells, this is especially useful. For instance, the PDMS chip developed by Ye *et al* [38], (Figure 1), incorporated eight identical structures with integrated gradient generator based on the principle reported by Jeon *et al* [39]. Two inlets are present on chip, for medium and for drug mixed with medium respectively. The two liquids were mixed in a wide channel then split multiple times to generate mixture having different concentration ratios with the initial solution. The HepG2 cells can therefore be exposed to various concentrations of drugs, and are able to be observed directly under a microscope. Eight identical structures ensured that eight different compounds can be tested on chip concurrently. The device was set up in an incubator at 37 °C with 5% CO_2. PDMS is gas permeable such that a stable microenvironment can be established.

However the expression of metabolic enzyme in HepG2 is low, making it unsuitable for toxicity prediction. Both biotransformation process of drugs and toxicity profiles are altered compared with *in vivo* situations [40]. In the mean time it is generally accepted that, cell lines can be used to investigate molecular pathways due to

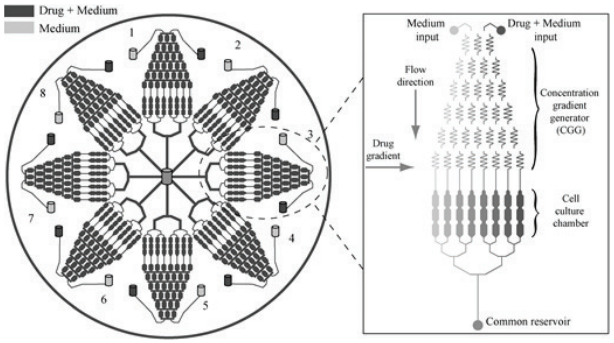

Figure 1: Schematic of the chip developed by Ye *et al* [38], with eight identical structures and gradient generators. (Copyright 2007 Royal Society of Chemistry).

their robustness. Another example is the system developed by Sung *et al* [41] to monitor CYP activities optically. It has a green light emitting LED for excitation and a photodiode for detection. HepG2/C3A were cultured in Matrigel on chip. It has been reported that cells maintain their functions better in a 3D configuration [42]. Metabolic activities of CYP1A1 and CYP1A2 were assessed by ethoxyresorufin and were shown to have improved functions compared with conventional monolayer cultures. Continuous perfusion of medium was applied to the cells, to ensure that the cells are constantly exposed to fresh medium with fixed nutrient concentration. This device is useful for real time monitoring of CYP activities for primary hepatocytes as well. Moreover, Carraro *et al* [43] developed a PDMS device to mimic the human hepatic microvascular bed. HepG2/C3A cells were maintained up to 10 days. Phase I and phase II metabolites were detected during this period. The incorporation of primary hepatocytes was also feasible. The hepatocytes were not exposed to medium flow directly as is the case of in vivo situation and the exchange of medium took place by diffusion through polycarbonate membrane with pore size of 0.4 mm.

2.3 Primary cells

Primary hepatocytes are generally accepted as a better *in vitro* model to predict *in vivo* metabolism than cell lines [44]. They can be isolated from liver tissue by collagenase perfusion, which digests the connective tissue [45]. In primary hepatocytes, metabolizing enzymes are present at their natural physiological levels. Thus they can be used to predict hepatic metabolism quantitatively. Although metabolic enzymes are initially at their physiological levels, CYP-mediated metabolism gradually decreases during extended cultures. To prevent this, the cells can be cultured in Matrigel with supply of inducers. Alternatively, non-parenchymal cells can be co-cultured with primary cells [46-48].

Furthermore, liver anatomy was mimicked by Lee *et al* [49] who fabricated a PDMS device (Figure 2a). The device featured an artificial liver sinusoid with an artificial barrier layer mimicking endothelial barrier layer. Primary rat and human hepatocytes were maintained for 7 days. A similar structure was used by Nakao *et al* [50] for bile canaliculi formation. The microfluidic structure allowed the rat primary hepatocytes to align, to form two rows like a hepatic cord. This way the bile canaliculi can be formed at the interface between cells (Figure 2b).

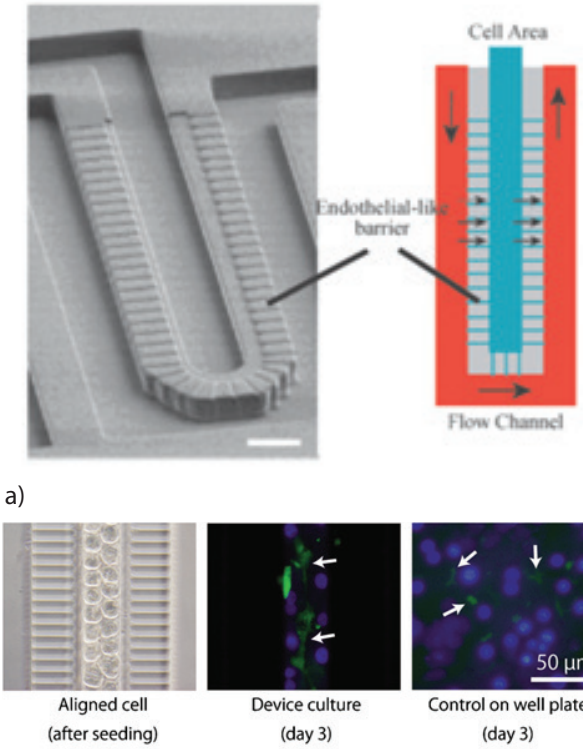

Figure 2: a) Optical image and schematics of the device resembling a liver sinusoid, cells are cultured in the cell area, medium flows around outside of the barrier. (Copyright 2007 John Wiley and Sons, Inc.) [49] b) Bile caniculi formation in a microfluidic structure: aligning of the cells in two lines like a hepatic cord, bile caniculi (green color) formation, control in static cell culture [50].

Another primary hepatocyte culture chip was fabricated by Griffith lab using microfluidic techniques. Hepatocyte metabolic activities was tested with the chip [51]. The 3D culture scaffold was fabricated in silicon with deep reactive-ion etching. Primary hepatocytes were cultured in the bioreactor for 2 weeks. The level of mRNA expression of CYP enzymes, transcription factors and phase II drug metabolizing genes were retained. A higher throughput version of their device was recently developed. It incorporates a pneumatic micropump

and fluidic capacitor to achieve pulseless flow (Figure 3) [52]. Hepatocytes remained viable and retained capacity for albumin synthesis during culture. The device was placed in a humidified incubator with controlled pH and O_2 content. The flow rate was set to 250 µL/min.

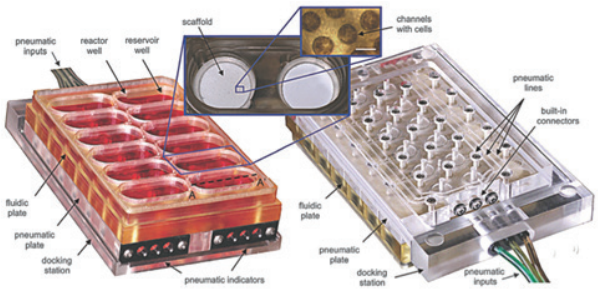

Figure 3: Perfused microwells for culture of hepatocytes with integrated pneumatic pump (Copyright 2010 Royal Society of Chemistry) [52].

2.4 Spheroid cell culture model

Hepatocytes form aggregates when they are weakly adherent or non-adherent to the culture substrate. The presence of the 3D cytoarchitecture through the re-establishment of 3D cell-cell contacts, together with the secretion of extracellular matrix material within the spheroid, had been hypothesized to contribute to better maintenance of differentiated function compared with the traditional matrix overlay [53] and matrix monolayer culture [54]. Studies have shown the maintenance of the drug metabolizing enzymes in extended cultures of spheroids [55] as well as induction of some key enzymes in response to prototypical inducers [4]. However the presence of necrotic/ hypoxic cells in the center of the spheroid due to oxygen diffusional limitations in large sized spheroids [56] as well as the difficulty to handle floating spheroids in conventional wells have limited their used in long-term metabolism, enzyme induction and cytotoxicity studies [57]. An overview of the methods to achieve 3D cell culture models using microfluidic systems was presented by Choudhury *et al* in [58]. Currently, different techniques are used for cell assembling into spheroids. Their key point is promoting cell-cell interaction and limiting cell-substrate interaction. A well known technique is hanging drop method [59]. This method is relatively simple, but the exchange of cell media is challenging [60]. Moreover, the limited volume of the drop (50 µL) made this culture method less suitable for drug screening applications and difficult to be translated into large-scale production. Another commercially available method (AggreWell™ by Stem Cell Technologies) consisted in centrifugation of the well-plate [61-63]. Despite the relatively high cost of the well-plate, the method also required incubation for spheroid formation. Rotational bioreactors (spinner flasks) can also be use for spheroid formation, but the large shear stress generated limits its application to primary hepatocytes [64]. Another classical method, liquid overlay involved cell culture on a low adhesive layer. The method was simple and inexpensive, but induced a large variation of the spheroids' diameter [65-67]. Another method consisted in micropatterning of selective-adhesive structures on a non-adhesive substrate [68, 69]. The main advantage of the method was the uniform size and distribution of the 3D cellular aggregates. Other microfluidic methods involved cell trapping barriers [70], bubble or droplet-based methods [71], microwells in which rotational flow of a cell suspension was induced [72, 73], and cell assembling by ultrasonic actuation in microwells [74]. An ultrafast microfluidic method for cell aggregation in spheroids was recently reported by Alhasan *et al* in [26]. The method consisted of combining surface acoustic wave (SAW) microcentrifugation with the use of fast gelling hydrogel. The method was demonstrated with human mammary gland carcinoma cells (BT-474) and with mesenchymal stem cells (MSCs). It is relevant to mention that the formation of spheroids was performed in standard tissue culture plasticwear. Moreover, the size of the spheroid can be simply tuned by selecting the power input to the SAW. Another relevant approach in spheroid cell culture is the concept of "constrained spheroids"(CS) presented by Tong *et al* in [25]. The CS cell culture model overcame one of the most relevant problems related to spheroid cell culture. In the static culture, due to the turbulence generated by culture media change, the spheroids lose their adhesion on the substrate. Under perfusion, due to their relatively large diameter and fluid velocity, the spheroids are exposed to a momentum generated by the Stokes force. This momentum removes the spheroid from the substrate causing cell loss. In order to overcome this problem, in the CS model, the spheroids are trapped and stabilized by sandwich configuration between a PEG-AHG-modified glass and an ultra-thin Parylene C membrane. This allowed to maximize mass transfer, and to overcome uneven cell count and spheroids size-related issues. The glass substrate was modified for more uniform and rapid hepatocytes spheroids formation within 1 day, allowing for earlier drug testing and perfusion culture initiation. The membrane was specifically modified so that the hepatocytes in the spheroid will preserve their cytoskeleton distribution. The results showed not only a better conservation of the cell count but also an improvement of the cell function.

2.5 Intact tissue

Primary cells can be co-cultured with non-parenchymal cells to mimic the natural hepatic architecture after isolation from intact liver tissue. Instead of using isolated

cells, it is also possible to collect intact tissue directly from the body and perform *in vitro* assessments. Compared with isolated cells, intact tissues have intact cell matrices as well as all cell types and their enzymes, cofactors and transporters. Thus, they highly resemble the *in vivo* architecture. Intact tissues can be obtained from animals or humans by surgery. Two ways have been exploited so far, namely liver biopsies and precision-cut liver slices. Liver biopsies can be obtained by cutting liver sample by hand or using biopsy punch, whereas precision-cut liver slices are obtained by using Krumdieck tissue slicer or Brendel-Vitron tissue slicer.[75] Tissue slices of thickness from 100µm can be obtained by tissue slicers, the thickness of the slices obtained are usually small enough for nutrients and oxygen to diffuse to inner regions. During culture period, the level of metabolic enzymes also decreases gradually, as in the case of primary hepatocytes. The rate of decline is slower compared with primary hepatocytes [76]. Recently, several groups have incorporated precision-cut liver slices [77] and biopsies [78].

2.6 Biopsies

For biopsy, Hattersley *et al* [78] designed a device which consisted of a Y shaped channel with two inlets and one outlet. Three chambers with inner diameter of 3 mm were present for insertion of the biopsy. Tissue chambers were located on top of microfluidic channels to avoid the direct exposure of tissues to media stream. The nutrients were delivered mainly by circulating medium. Regarding the tissue biopsy, the cells further away from the medium flow are exposed to lower concentration of O_2 and nutrients. Since, the hepatocytes in vivo are located just a few microns from the blood stream, it becomes difficult for cells to survive when they are more than a few hundred microns from the blood stream [79]. To regulate pH and O_2 content, the chip was put into an incubator. Lactate dehydrogenase and DNA were measured through the outlet of the device. Morphologies of the cells were also assessed.

2.7 Precision-cut liver slices (PCLS)

PCLS was first integrated to micro-bioreactor in 1996, PCLS were first fixed on a microscope slide with plasma clot, while perfusion was performed directly on the slide [80]. Fluorescence confocal laser cytometry, facilitated the assessment of cytochrome P450 distribution in PCLS. However, only one side of the slice was exposed to medium, which hindered the transport of nutrients and gases. In addition, enzyme activities were not quantified with the help of this device. Consequently another microfluidic device fabricated by Khong *et al* [81] was used to perfuse thick liver slices of 0.3-1 mm thick. The tissue slice was placed directly in medium flow and 7 needles were inserted to the tissue slice to facilitate mass transport inside the tissue slice. CYP1A and UGT were reported to be stable for up to 3 days. This device could be used for induction and inhibition studies with PCLS. More recently, van Midwoud *et al* [77] developed a micro-perfusion bioreactor to study the rat liver metabolism. The device was fabricated out of PDMS with incorporation of polycarbonate filter and PDMS membranes. In each chamber, PCLS of 3 to 4 mm diameter were cultured in a continuous flow of medium. The PCLS functionalities remained for 24 hours, human PCLS were integrated and tested, the metabolism and viability were comparable to those of conventional well-plate system. Thus, microfluidic bioreactor helps to reduce the use of animals for preclinical testing by using scarce human material.

The continuous flow applied to the slices ensured that direct analysis of the outflow. An HPLC device equipped with UV detection was coupled with the bioreactor to achieve real time detection of metabolites [82]. Metabolites could immediately be measured upon exposure of a slice to medium. Retention of viability could be demonstrated. By increasing substrate concentration over time, the device was also used to measure inhibition constant. Only three tissue slices were used, which would allow studied to be performed with scarce samples. Moreover, the device can detect unstable metabolites instantaneously. This is difficult to achieve in conventional well-plate system.

2.8 Organs-on-a-chip

Conventional 2D and 3D cell culture models have demonstrated their values in tissue specific biomedical research. However they may not accurately predict *in vivo* tissue behavior and drug activities due to their difficulties in recapitulating multi-scale tissue architecture, tissue-tissue interface and mechanical cues. Microfluidic organs-on-chips have the possibility of overcoming these limitations [83]. Organ-on-a-chip devices also enable high resolution, real-time imaging and various assays of biochemical, genetic and metabolic activities. The first major step in organ-on-chips for drug development happened in 2004, when the Schuler group designed a microfluidic chip for pharmacokinetic studies of multiple cell types interconnected by microchannels [84]. The device featured three cell culture chambers for lung, liver and other cell types on a single silicon chip. It targeted the examination of the adsorption, distribution, metabolism, elimination and toxicity (ADMET) profile of chemicals *in vitro.* By achieving physiological liquid-to-cell ratio, shear stress and liquid residence time, this device paved the way for using microfluidic devices to reduce or even replacing animal testing in the pharmaceutical industry. An-

other organ-on-a-chip devices to investigate crosstalk between different organs was designed by Zhang *et al* [85]. This multi-channel 3D microfluidic cell culture system features compartmentalized microenvironments for drug screening. Liver, lung, kidney and adipose cells were simultaneously cultured in 4 compartments. The four cell types represent the drug-metabolizing and storage capabilities in the human body. This kind of multiorgan system can potentially be used for drug testing, food safety testing as well as pathogen testing.

Over the past decade, a lot of devices have been developed to support PK-PD modeling. Acetaminophen is one of the commonly studied drugs in microfluidic devices. In a study done by Mahler *et al* [86], HepG2 cells were coupled with intestinal cells. They demonstrated that administration of acetaminophen caused glutathione depletion in intestinal cells. A dose dependent hepatotoxicity response was also observed. The result obtained from the microchip was similar to *in vivo* experimental results.

In spite of the swift advances of microfluidic devices, certain hepatic functions such as bile duct clearance or sustained production of metabolic enzymes (as compared with the 1-year lifespan of hepatocytes *in vivo*) still cannot be completely modeled using chips. The presence of flow might not always be beneficial either, some metabolites accumulate in small static microenvironments that are undetectable in flow conditions due to sensitivity issues [87].

2.9 Fish-on-a-chip

Zebrafish and especially its embryo, is a vertebrate model for study in embryogenesis, development biology, cell biology and genetics and is becoming an important model for preclinical drug discovery applications. The overall drug toxicity in Zebrafish embryo is comparable with that observed in mammals [88]. Due to shorter development time and cheaper maintenance, Zebrafish model is cost-effectiveness. Zebrafish embryos are small, easily obtained in large numbers, accessible immediately after fertilization, they are optically transparent and pigmentation mutants exhibit extended period of transparency [89]. The embryos are permeable to peptides, drugs and dyes. Also, specific genes can be inhibited or mutated and the entire genome of Zebrafish has been sequenced and can be accessed online [88, 90]. The drug studies on embryos, mostly performed on 96 well microtiter plates [91] were not suitable for dynamic long-term culturing and imaging of embryos. For this reason "fish-on-chip" solution are desirable. Martin *et al* [92] proposed a high-throughput vertebrate screening platform (VAST) in which the fish embryos were manipulated and oriented for cellular resolution imaging. Their platform permitted large-scale chemical screens. Drug studies on Zebrafish and Medaka embryos [93],[94] have already found their way into microfluidic systems. A study related to the delivery of foreign compounds into the embryos by electroporators is presented in [95]. Research on Zebrafish embryonic development using microfluidic devices are presented in [96] and [97]. A programmable and automated chip-based platform, which facilitated the accurate and reproducible *in vivo* drug dynamics and studied Zebrafish embryos is presented in [98]. Akai *et al* [99] proposed a 3D microfluidic embryo array for real-time developmental analysis of transgenic Zebrafish embryos. The PMMA chip allowed automatic loading, docking and exposure to micro- perfusion treatment of the embryo. An optomicrofluidic device that combined a light modulation system with a microfluidic circuit was developed to detect the oxygen consumption rate of a single developing Zebrafish. It was presented by Huang et al in [100]. Erickstad *et al* [101] proposed microfluidic system to observe different behavioral responses of Zebrafish larvae to different levels of hypoxia. A review of fish on chip platforms is presented in [102].

3 Materials for bioreactor fabrication

A key point in the correct design of the microfluidic bioreactor is the correct selection of the materials involved in its fabrication. A detailed analysis of the materials involved in cell culturing can be found in [103]. The selection of these materials is critically connected with the application. For drug screening applications, for example, fabrication of the microfluidic reactor in glass/silicon technology can be more suitable due to the low absorption of drug and metabolites. Otherwise, for application such as cell proliferation or cell migration polymeric materials are more suitable. Three main groups of materials can be identified: polymer, silicon-based materials and metals.

3.1 Polymers

Poly(methyl methacrylate) (PMMA), polycarbonate (PC), polystyrene, polyurethane, and poly(dimethyl siloxane) (PDMS) are common polymers found in microfluidic technologies [104, 105]. PDMS is the most used polymer. Soft lithography, developed by the Whitesides group [106] is usually used to fabricate PDMS devices. Advantages of PDMS include cost effectiveness, fast prototyping ability, good adhesion to glass, good gas permeability and transparency [107]. On the other hand, PDMS is a hydrophobic material. This makes it easy to absorb organic solvents, hydropho-

bic drugs and metabolites. The aspect ratio achievable with PDMS is 2:1. There are methods to enhance the surface properties of PDMS. Some of the approaches are: surfactants modification, polyelectrolyte modification, covalent modification, chemical vapor deposition, phospholipid layer modification and protein coating modification [108, 109]. Consequently, various PDMS microbioreactors have been developed for hepatocyte culture. Leclerc *et al.* developed a PDMS microbioreactor for perfusion culture of fetal human hepatocytes [110]. During the one-week perfusion period, the cells showed good attachment and proliferation. The albumin expression was higher than that of static culture by about 4 times. PDMS bioreactors have been demonstrated as a good option for large-scale hepatocyte culture due to its good gas permeability. One of the first PDMS perfusion bioreactors demonstrating large-scale culture of HepG2 was developed by Leclerc *et al* [111]. They achieved culture of HepG2 with density similar to that of a macro-scale bioreactor [49]. Cyclic olefin copolymers (COCs) have been used by Raasch *et al* [112] for manufacturing of a microfluidic devices for endothelial cell culture in order to overcome limitations of PDMS material. Besides PDMS devices, PMMA material is also commonly used in MEMS fabrication. Patterns and microchannels can easily be fabricated onto PMMA surfaces using electron beam lithography [113] or laser ablation [114].

3.2 Silicon-based materials

Silicon-glass technology is one well established process for microfluidic devices [115, 116]. Their biocompatibility and applications in cell culture have been studied extensively. Silicon [117], silicon dioxide [117], silicon nitride [118-120], silicon carbide [121, 122] and SU-8 substrate [123] have all been shown to be non-cytotoxic. Amorphous silicon, for example, has been demonstrated as a good substrate for growth of renal proximal tubule cells [124, 125]. After pretreatment of ECM proteins, single-crystal silicon and polysilicon chips are shown to promote attachment of renal tubule cells. Cell functions and behaviors are also similar to cells cultured in plastic cell culture flasks. Renal cells cultured on silicon chip showed good expression of tight junction proteins like ZO-1 and high level of trans-epithelial resistance (TER), a measure of tight junction formation function [126]. Porous silicon is also frequently used in cell culture and cell adhesion studies. The surface of porous silicon can be modified by oxidation, salinization and collagen coating to promote cellular attachment. Porous silicon also has unique biodegradable property compared with single-crystal silicon, property that makes it useful for a number of *in vitro* and *in vivo* applications. For instance, porous silicon films can induce hydroxyapatite growth and promote bone healing *in vitro* [127]. Silicon nitride and silicon carbide are deposited with CVD or PECVD techniques respectively [128]. The hydrophilic property and small thickness of silicon nitride made it a good option for the study of cell-cell interaction *in vitro.* Ma *et al* developed a silicon nitride membrane for the study of blood-brain barrier (BBB) model [129]. In this model, they co-cultured endothelial cells and astrocytes on different sides of an ultra-thin silicon nitride membrane. The close proximity of the two cell types promoted cell-cell interactions and led to formation of tight cell barrier.

3.3 Metals

Metals are also frequently utilized in biodevices and microfluidic bioreactors, especially for devices with electrodes and electric circuits [130]. Gold, platinum and titanium were commonly used metals for electrodes. Their biocompatibility made them safe for *in vivo* applications [131]. To enhance cell survival and tissue regeneration, Kim *et al* designed an implantable electrical bioreactor [132]. It provided electrical stimulation to the human mesenchymal stromal cells (hMSCs) seeded in the device. Cells stimulated with electrical currents showed increase in proliferation.

4 Conclusions

We presented an overview of cell culture models, which in conjunction with microfluidic setup can further move the *in vitro* cell culture models towards replicas of *in vivo* environment. As practical experience, the selection between static and perfusion models is driven by the application. For liver based models for example cell functions are similar in the first week for both static and perfusion models. The difference becomes relevant after 2 weeks culture. As a result, for applications that require up to one-week cell culture the static model is more suitable. Otherwise for long-term cell culture the perfusion system is more relevant. The perfusion system is more complex and in most cases its use requires special skills. Meanwhile, the cost of the perfusion system cannot be neglected.

For the cell culture model the organization of cell in spheroids is a better mimic of *in vivo* environment. The spheroid model presents more cell-cell interaction than cell-surface interaction (characteristic of 2D models). Organ-on-a chip models start to be more and more attractive for drug screening.

The main requirements of the perfusion chip can be summarized as follows:

- conserved the cell count over the testing period,

- good mass transfer allowing diffusion of O_2 and nutrients from media to the cell culture model to remove the metabolites and by-products,
- low shear stress to the cells,
- low risk of contamination (reduced number of microfluidic connections, tubes and fluidic elements),
- maintenance of stable temperature and pH,
- ease to handle,
- low drug and metabolites absorption.

Coupling the spheroids cell culture model with microfluidic setup is for our point of view the future step for the long term drug screening platforms with main application on chronic toxicity testing.

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