Chapter 6
Biomaterial-Based Microfluidics for Cell Culture and Analysis

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Abstract Microfluidic devices can integrate drug injection, bioreactors, sample separation and detection on a individual platform with properties of fluidic incubation, programmable three-dimensional (3D) channels in micro scale, low reagent consumption, and on-line detection, which makes them popular in in vitro cell culture and analysis. Since the appearance of the initial generation of microfluidics in 1970s, materials for microfluidics experienced the age of inorganics such as silicon and glasses, and now have entered the period of organics, in which main materials used in microfluidics are polymers. Also, polymers constructed the chips gradually undertake much more functions rather than a basic supporting plane through applying functional and smart biomaterials. In this chapter, biomaterials utilized in microfluidics will be elaborated from the perspectives of the fabrication of microfluidics, cell culture and analysis on chips.

Keywords Biomaterials · Microfluidic chip · Cell culture · Scaffolds
Cell analysis · Cell observation

6.1 Introduction

Passed through 20-year development, microfluidic chips now occupy a significant position in cell biology and cell analysis. Comparing with traditional plate culture methods, the on-chip cell culture has incomparable superiorities. As an important example, microchips can realize the simulation of in vivo microenvironment, which makes on-chip cell experiments reliable and accurate. However, the application of microfluidic chips in cell research still has some limitations. Various methods have been developed to increase the feasibility of microfluidic devices, such as employing functional biomaterials or optimizing the design of chip structures to

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realize better manipulation of cells and fluids. Making use of appropriate biomaterials is a very effective and flexible way of improving the biocompatibility of microfluidic devices and offering functions for on plane and three-dimensional space (3D) cell culture and analysis, which has great significance for drug evaluation, diagnosis and therapy, biological research and fields based on cells. In this chapter, we will introduce materials used in the microfluidic chips construction and on-chip cell culture and analysis.

6.2 Materials of Making Microfluidic Chips

Microfluidic devices have programmable channels and chambers with the lowest resolution in micrometer, which makes them helpful in chemical analysis, clinical evaluation, environmental monitoring, and biology. In particular, due to the dimensional consistence of cells and micro-scale channels, microfluidic devices are extraordinarily suitable for cell research to realize cell culture, manipulation, and analysis at a continuous flowing state [1]. Also, microanalysis on microfluidic platforms only requires a small reagent and analyte volume, which can determine cellular and extracellular secretions at low amount, even for a single cell.

To achieve biofunctions for cell culture and analysis on microfluidics, choosing appropriate materials is the key point, because materials have direct contact with cells and will certainly influence cell state and behaviors such as cell viability, cell proliferation and cell metabolism. Moreover, functional groups on biomaterial surfaces can also be utilized to immobilize proteins, aptamers, or DNA in microfluidics, which further assist in cell adhesion, capture, and analysis. Here, materials used in microfluidics establishment will be elaborated in three parts, the inorganic materials that are mainly employed in the first generation microfluidics, and organic materials containing plastics, elastomers and hydrogels and papers.

6.2.1 Inorganic Materials

With the maturity of micro-electromechanical system (MEMS) preparation technology, some inorganic materials, such as silicon and glass, were employed in constructing the first generation microfluidic chips. The initial microfluidic device was a gas analysis system mainly fabricated in silicon by photolithography and chemical etching techniques [2]. This portable gas chromatography successfully separated gaseous hydrocarbon mixtures within 10 s, which brought a revolution for micro-scaled manipulation and analysis of chemical micromolecule and biological macromolecule from then on. In the fabrication of MEMS devices, four main techniques are involved, which are thin-film deposition, lithograph, etching, and packaging in turn.

The thin-film deposition is the procedure to create a layer on inorganic substrates for further lithography and etching [3, 4]. Chemical vapor deposition (CVD), physical vapor deposition (PVD), electrodeposition and substrate oxidation are some commonly used methods. In the lithography step, channel patterns are designed on a photomask by computer-aided design and then transferred on the deposited layer of inorganic substrates to act as a mask in the next etching step [5]. The etching process can ordinarily be divided into wet etching [6] and dry etching [7]. Wet etching is based on chemical reactions between the corrosion solution and substrates. The etching process has the same reaction rate in all directions in a homogeneous medium, thus, the wet etching is always isotropic. Due to the isotropy, channels with vertical sidewalls can be hardly obtained on amorphous substrates, such as glass. To solve this problem, single-crystal silicon wafers are commonly adopted in anisotropic etching because silicon etchants have anisotropic corrosion along the crystallization direction. As shown in Fig. 6.1, the wet etching was performed on single-crystal silicon surfaces and grooves with different cross-sections shapes were obtained when applying diverse etchants. Compared with wet etching, the dry way has fewer limitations and better anisotropic corrosion, which makes the fabrication of micro-scaled patterns on multiple substrates possible. High-pressure plasma etching, reactive ion etching, and ion milling are some frequently-used techniques in the dry etching. Besides, microchannels with good vertical sidewalls can be obtained at a high aspect ratio through a dry etching process [8]. The final step, packaging process, which is also called the bonding process, ordinarily employs an adhesive layer or thermal fusion treatment to seal the microchannels with substrates [9].

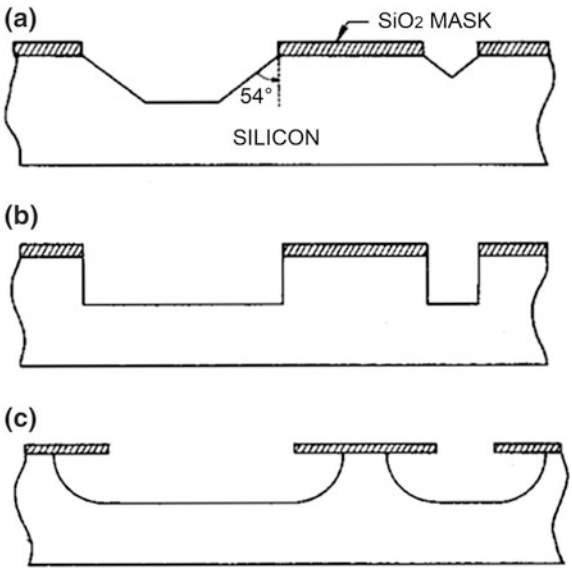

Fig. 6.1 Cross sections of grooves etched in silicon. **a** KOH etchant in (100) silicon, **b** KOH in (110) silicon, **c** HF-HNO_3 etchant. (Reprinted with permission from Ref. [2])

In recent years, silicon and glass are gradually replaced by polymers in constructing microchannel structures. In spite of this, glass is still the main substrate material used in microfluidic devices, because it is widely used in biological monitoring which requires high transparency and good surface modification activity. Except for the glass, other inorganic materials also have good performance as substrates. For instance, quartz-based microfluidic devices were proved to be efficient in cell identification and protein separation. In Dochow's work of Raman-activated cell sorting (RACS) [10], when at near-infrared laser excitation, quartz substrates exhibited fairly low Raman background in the fingerprint region, which solved the problems in RACS when adopting glass as substrates in cell identification. Moreover, quartz also improved protein separation in the way of acting as a substrate with good heat dissipation that leads to larger electric fields in the isoelectric focusing (IEF) of proteins [11].

6.2.2 Polymeric Materials

Inorganic materials are efficient in chemical analysis but have some formidable defects when applied in biology. For instance, necessary gases in cell incubation, such as oxygen and carbon dioxide, are difficult to penetrate through the inorganic layer and produce negative effect on cells. Also, opaque materials like silicon wafers are not suitable for cell observation using microscopes. Meanwhile, polymeric materials have developed rapidly in both diversity and multi-function that can meet the demands of microfluidic devices. Because of the lowe Young's modulus, polymers became the main material used for microfluidic chip construction in biological applications. Besides, polymers can easily be processed in various ways with low cost, and the diversity of polymeric materials makes the surface modification flexible and facile. According to the classification of polymers, polymeric materials constructing microfluidic chips can be divided into two categories, that is, plastics and elastomers.

6.2.2.1 Plastics

Plastic is defined as the polymeric materials with low elastic deformation ability, which can further be divided into two categories, thermoplastics and thermosetting plastics, based on whether the materials can melt and be repeatedly molded when heated or dissolved in solvents.

Thermoplastic materials always have linear molecular chains and low glass transition temperature (T_g), which results in good thermal processability, such as hot embossing, thermal molding and bonding. Several kinds of thermoplastics are commonly used as commercial products in biology. For instance, polystyrene (PS) is the most widely adopted material in culture dishes; polymethyl methacrylate (PMMA), and polyethylene terephthalate (PET) are ideal candidates for cell

observation on microfluidic platforms due to their good biocompatibility and transparency, as evidenced by their extensive applications in agriculture, food industry, and medicine [12–14]. Battle et al. fabricated a PMMA microfluidic solid-phase extraction (μSPE) device through hot embossing. In this device, abundant micropillars integrated in the main channel had high efficiency in enriching and purifying membrane proteins from whole blood lysate [15]. However, the linear polymers could easily release low molecular weight compounds into the solution and can be dissolved by organic solvents. Also, the low gas permeability of plastics limits their employment in constructing the principal parts of microfluidic chips. In Midwoud's work, as shown in Fig. 6.2, biocompatibility of PMMA, PS, polycarbonate (PC), and cyclic olefin copolymer (COC) were tested to determine their applicability as the principal material of microfluidic devices [12]. As a result, compared with polydimethylsiloxane (PDMS) which is the chief materials in constructing microfluidic devices at present, no apparent improvement was observed on these thermoplastics.

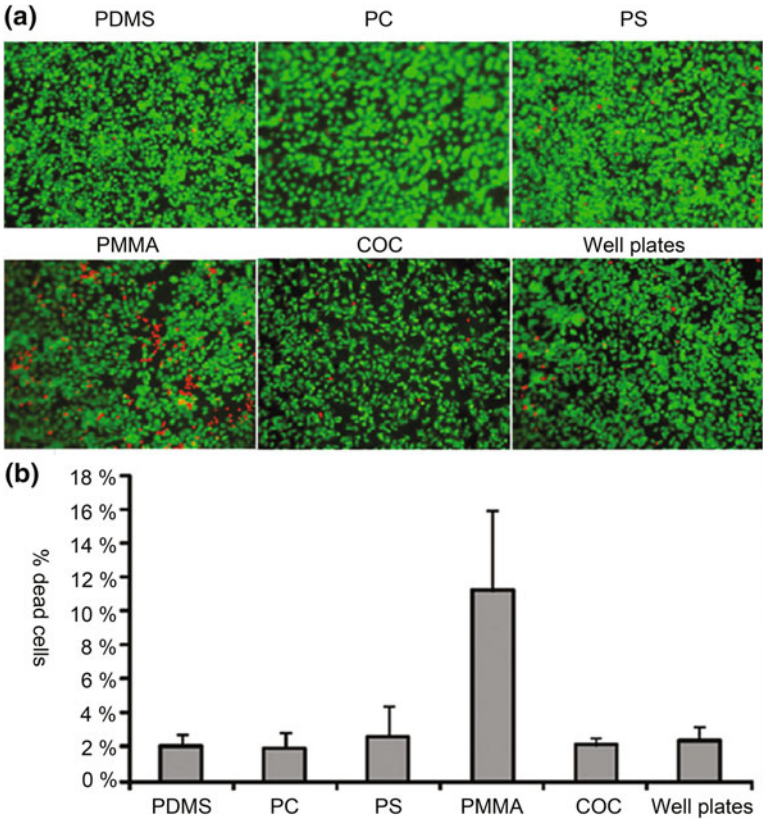

Fig. 6.2 HepG2 cells cultured on different thermoplastics and PDMS after UV-ozone treatment and collagen coating. **a** Fluorescent photographs stained by Line/Dead kit; **b** Cell mortality. (Reprinted with permission from Ref. [12])

In case of thermosetting plastics, molecular chains cross-linked into network structures give the material with resistance to heating and solvents. Once formed the network, stiffness of the thermosetting plastics will increase and it becomes difficult to remold the material. Therefore, few thermosetting plastics are considered to be employed as the principle material for microfluidic chips. However, in microfluidic chips fabrication, the thermosetting plastic SU-8 negative photoresist is frequently used and can be utilized to establish a free-standing microstructure [16].

6.2.2.2 Elastomers

Elastomers are materials that can stretch more than half of the original length along the force direction when sustaining strong external load, and all deformations can recover after the force is removed. The high elastic deformation ability of elastomers makes them flexible and soft. Elastomers are ideal candidates for cell culture and analysis, because soft surfaces lead to better cell viability than rigid surfaces [17]. At present, PDMS is the most commonly used polymer as the principle materials in fabricating microfluidic chips, because it is a flexible material with high transparency, good gas permeability and biocompatibility, which is beneficial to cell activities. Among fabrication methods of PDMS microfluidic devices, the soft lithography technique has developed into a routine way. In particular, glass/PDMS hybrid microfluidic devices, in which PDMS constructs the main part of microfluidic chips and glass is employed as the substrate, are popular in cell culture and analysis. The soft lithography contains replica molding and rapid prototyping. Once a mask is prepared, PDMS casting can be repeatedly performed using the mask.

The process of PDMS microchannel preparation is illustrated in Fig. 6.3. In the first step, a microchannel mask is prepared through UV illumination on photoresist covered by a printed photomask with designed channel patterns. Then, a mixture of PDMS prepolymer and curing agent is poured on the microchannel mask with a convex plain form. Before baking, removing air bubbles is necessary. The principle part of microfluidic chips, microchannels, can be achieved after baking, peeling, and punching holes in turn. The obtained PDMS microchannels can be bonded to other silicon-based surfaces (e.g., silicon, glass, quartz, PDMS) though covalent bonding.

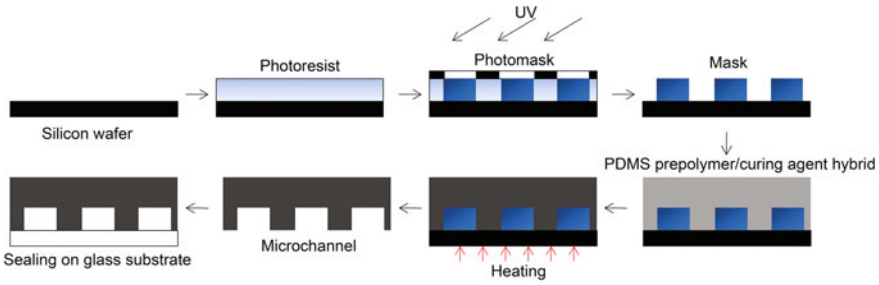

Fig. 6.3 Schematic diagram of PDMS-based microchip fabrication through soft lithography

Although PDMS-based microfluidic devices are widely applied in biological research, their hydrophobic surfaces will lead to non-specific adhesion of proteins or analytes. To address the issue of non-specific adhesion and integrate functional biomolecules, a hydrophilic treatment is always needed. Generally, the hydrophilization is achieved through convenient surface modifications [18], for instance, plasma cleaning [19], silanization treatment [20], and surface coating [21]. After surface modification, PDMS-based microfluidic devices have better performances in biomolecular separation, protein/cell enrichment and release, and cell culture.

In addition to PDMS, other synthesized elastomers are also used in the fabrication of microfluidic devices. For example, Roy et al. replaced PDMS with styrenic thermoplastic elastomers (TPEs) in building multilayer microfluidic devices [22], because PDMS is inadequate for industrial-scale fabrication and application. Human cells were successfully cultured on surfaces of the isothermal TPE microdevice after sealing with DNA and protein solutions. Perfluoropolyether (PFPE) can also be used to build microfluidic chips. Rolland et al. [23] reported a preparation method of microfluidic devices with resistance to organic solvents by using the photo-curing "liquid Teflon" material, which extended the application of microfluidic devices to novel fields.

6.2.3 Hydrogels and Papers

Although hydrogel and paper are essentially polymer materials, by the reason of their particularity and great development potential, here we purposely pick them out as an individual portion to introduce.

6.2.3.1 Hydrogels

Hydrogels have network structures consisted by coadjacent polymeric chains formed under certain conditions, in which the liquid padding the interspace acts as dispersion media. This kind of material containing hydrophilic groups and cross-linked macromolecular networks leads to both high water content and the ability of shape preserving. Different from absorbent materials such as cotton and sponge that the water absorbing capacity comes from the capillary phenomenon, water molecules in hydrogels bond with the hydrophilic groups on networks through chemical bonds, which gives them strong water retention capacity with few water loss even under pressure [24–26]. Therefore, hydrogel is a stable high water-content material with plenty holes between the polymeric chains. The character resulted in hydrogels possessing properties such as easy mass transportation, good cell viability and proliferation and making them an ideal candidate in native extracellular matrix (ECM) imitation.

Both natural and synthesized hydrogels are employed in preparing microfluidic chips. Natural hydrogels such as collagen, calcium alginate, cellulose, gelation, and chitosan are commonly used biomaterials in microfluidics [27, 28], however, due to their low mechanical durability and microstructure reproducibility, few of them were employed in constructing the main part of microfluidic devices. They are frequently used as scaffolds in microchips for 3D cell culture and organ simulation, which will be described in the section "3D culture" (see Sect. 6.3.2) and "Organ-on-chip system" (see Sect. 6.4). Although artificial hydrogels are rarely used in fabricating the main part of microfluidic chips neither, the polyethylene glycol (PEG)-based hydrogel is an attractive material in bioanalytical devices because PEG has strong non-biofouling characters and versatile processability with tunable mechanical strength to support the structure stability. The idea of adopting PEG hydrogels in the fabrication of microfluidic devices comes from a frequently used surface modification method in which PEG is modified on silicon-based surfaces to solve the problem of non-specific adsorption of biomolecules. Though the modification can be realized through physical adsorption and chemical immobilization, inadequacies such as inherent defects on the modified layer and loss of resistance to biofouling in long-term fluid flush gradually invalidate the modification and bring back the hydrophobicity. The fabrication of PEG microfluidic chips mainly use soft lithography, which is similar to that of PDMS devices. Kim et al. [29] employed PEG, PEG-diacrylate (DA) and PEG-dimethacrylate (DMA) in making microchannels through UV curing. In the result, low molecular weight of PEG-DA (M = 258) and PEG-DMA (M = 330) had good resistance to water swelling, thus were used for the microchannel fabrication. Finally, a PEG microfluidic device entirely made up by cross-linked PEG chains were fabricated within merely 10 min and can be sealed to another PEG layer irreversibly by UV irradiation. In addition of using pure natural or synthesized hydrogels, recently, composite materials of natural and artificial hydrogels have been widely applied in biology. Cellulose hydrogel composites can be used as the principle material to fabricate microfluidic devices after blending with synthesized polymers or changing the solution concentration, structure, and properties of the cellulose hydrogel. For instance, as shown in Fig. 6.4, Pei et al. [30] succeeded in fabricating cross-linked cellulose (RCC) hydrogel and cellulose–collagen (RCC/C) hybrid hydrogel-based integrated microfluidic chips, which has well-controlled pore size, high transparency, good mechanical durability, and good biocompatibility in both 2D and 3D cell culture.

6.2.3.2 Papers

Paper is a sheet consisted of wood fibers deposited layer by layer. This close packing of layered structure of fibers gives paper integrated multi-functions, such as foldability, filterability, hydroscopicity and surface modifiability. Also, the capillary action of papers makes paper-based microfluidic devices have wide applications in rapid diagnostic tests [31]. In paper-based microfluidic chip fabrication, choosing

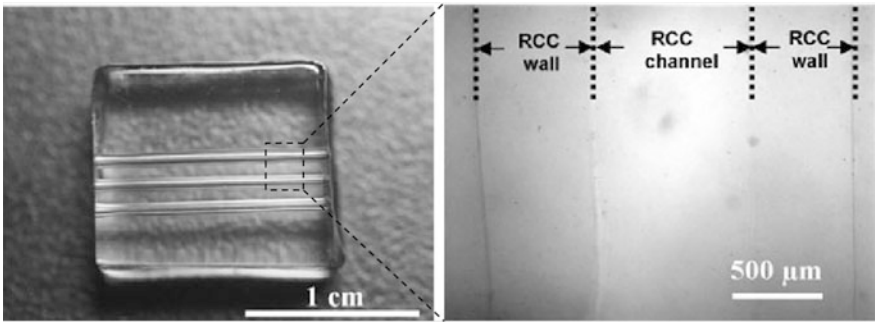

Fig. 6.4 Microchannels fabricated by cellulose hydrogels. Digital images of RCC hydrogel with three channels (*Left*) and optical images of channels in bright field (*Right*). (Reprinted with permission from Ref. [30])

an appropriate paper that can accomplish the requirements of assays is of great significance, because different kinds of papers have obvious differences in chemical and physical properties, such as capillary flow rate, pore size distribution and porosity, and some other factors that have great influence on paper performances. Cellulosic material is the most frequently employed paper in microfluidic devices [32–34], which includes filter paper, chromatography paper, and nitrocellulose paper. The generation of fluidic channels on papers is another key point in preparing a paper-based microfluidic chip. Through making hydrophobic barriers in horizontal or vertical directions, the reagents can be leaded into separated channels for further detections. The channel fabrication can be realized on papers easily and flexibly through combining hydrophobic substance coating (e.g., wax pattering, alkyl ketene dimer printing, PDMS plotting) and paper shaping (e.g., embossing, fold, clipping). Thuo et al. [35] built up a microfluidic device using omniphobic papers through embossing and "cut-and-stack" assembling methods, as illustrated in Fig. 6.5. Fluids in this device behaved similarly as they did in open-channel devices. Laminar flow and droplet generation can be realized using this paper chip, which can be utilized in environmental monitoring and droplet-based synthesis and analysis.

6.3 Cell Culture

Before human trials, preliminary assays should be performed on animal models. However, animal experiments are expensive, cumbersome, restricted, and the obtained results are suspectable for human data due to genetic diversities existed between different species. The emergence of cell clone technology deals with the problems in animal experiments and cell incubation plays a very important role in pharmacology, medicine, tissue engineering, and so on. Commercial culture dishes

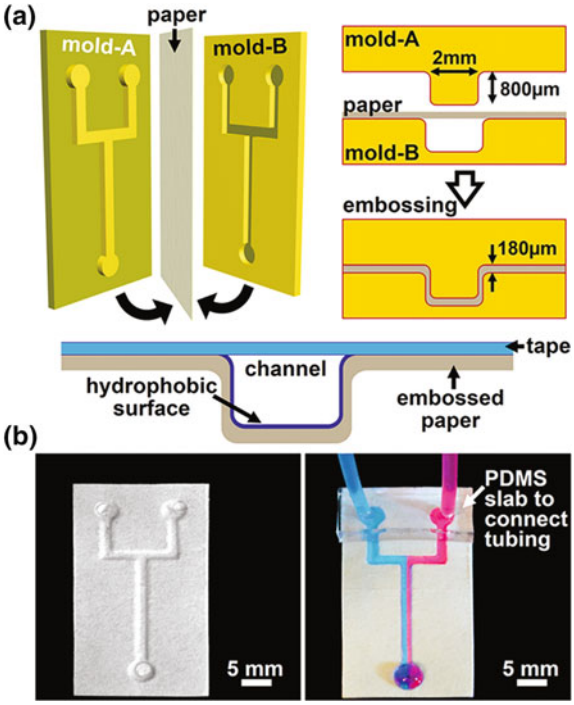

Fig. 6.5 An open-channel paper microfluidic device. **a** Schematic diagram of the construction; **b** Photographs of laminar flow in the device. (Reprinted with permission from Ref. [35])

and bottles merely support static culture in general. However, providing cells an environment similar to their in vivo microenvironment which contains fluid stimulus, substance exchange and cellular interactions is of great importance and has powerful impacts on cellular behaviors and functions. Therefore, microfluidic chips are the most appropriate candidate for microenvironment simulation, because controllable culture medium supply, 3D cell culture, drug stimulation, metabolite extraction and analysis can be integrated on this programmable fluid platform. With microfluidic chips, some tough missions, such as constructing the important nidus structures, now find possible solutions.

6.3.1 Plane Cell Culture

Cell culture performed in microfluidic devices are primarily on plane microchannel surfaces for experiments focused on investigating fluid impacts on cells. Most of the materials for on-chip cell culture are polymeric materials, such as PS, PC, TPE, and PDMS. Although these materials can be applied for cell incubation, manipulation and analysis, their inherent hydrophobic surfaces will lead to irreversible adhesion of abundant albumin, which in turn prevents cell adhesion and causes adverse effects on cells. Accordingly, the hydrophilic modification is essential. Numerous

methods have been developed to convert the hydrophobicity [36–38], which includes physical protein adsorption, chemical modifications, and modifying biomaterials.

The physical protein adsorption can produce a protein matrix layer on polymer substrates. In general, hydrophobic surfaces of polymer substrates can non-specifically adsorb serum proteins easily from the culture medium, which decreases the affinity of substrates for cell adhesion. However, extracellular matrix (ECM) proteins have good affinity with cells, thus importing ECM protein solutions in microfluidics is one of the most convenient way to achieve protein modification. Fibronectin (FN), laminin, collagen are some frequently employed proteins in physical modification to improve cell adhesion and proliferation. The forces between proteins and substrate surfaces are mainly some weak interactions, therefore, the adsorbed protein can easily detach from the interacting surface. As a result, the physical adsorption of proteins is only valid for a limited duration to maintain cell adhesion.

The chemical modification is capable of creating powerful covalent bond linkages between proteins and the substrates, which is more effective and stable than the physical adsorption. Convenient plasma cleaning can used to introduce hydrophilic groups on substrate surfaces, but the generated hydrophilicity is not very stable and will soon recover to hydrophobicity after a certain duration time or heating [39]. Chemical modifications formed by covalent bonding between proteins and substrate surfaces are very stable. Through this way, ECM proteins are efficiently immobilized on material surfaces. For instance, Kuddannaya et al. [40] succeeded in modifying FN and collagen type I (C1) on PDMS by the chemical reaction with (3-aminopropyl) trirthoxy silane (APTES) and cross-linker glutaraldehyde (GA), as exhibited in Fig. 6.6. After surface modification, mesenchymal stem cells (MSCs) were incubated to evaluate the validity of the modification. Results of cell adhesion and cell viability proved that the surface modified with APTES, GA, and proteins

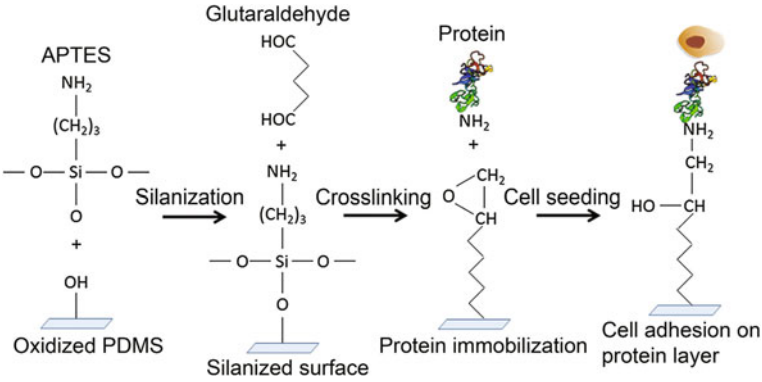

Fig. 6.6 Schematic diagram of PDMS surface modification. (Reprinted with permission from Ref. [40])

effectively reduced the hydrophobicity of PDMS, which is beneficial to cell adhesion and proliferation.

In addition to natural materials (e.g., ECM proteins, peptides, carbohydrates), synthesized biomaterials can also be utilized in improving the hydrophilicity of substrates. Among those, smart biomaterials draw much more attentions because they have response regulation of the surface wettability to environmental changes. The wettability can be controllably adjusted through such as pH value, temperature, light, and solvents. These factors have their advantages under different use requirements, but for on-chip cell culture, temperature is thought to be the most appropriate environmental factor to adjust the hydrophilicity. In many thermal-responsive systems developed for cell culture, poly (*N*-isopropylacrylamide) (PNIPAAm) is the most frequently employed biomaterial [41, 42], because the PNIPAAm chain undergoes a reversible conformational transition near its lower critical solution temperature (LCST) [43] that is acceptable by cells. As illustrated by Fig. 6.7a, when environment temperature is below LCST, a large number of complex H bonds are exposed on surfaces of molecular chains and arrange along

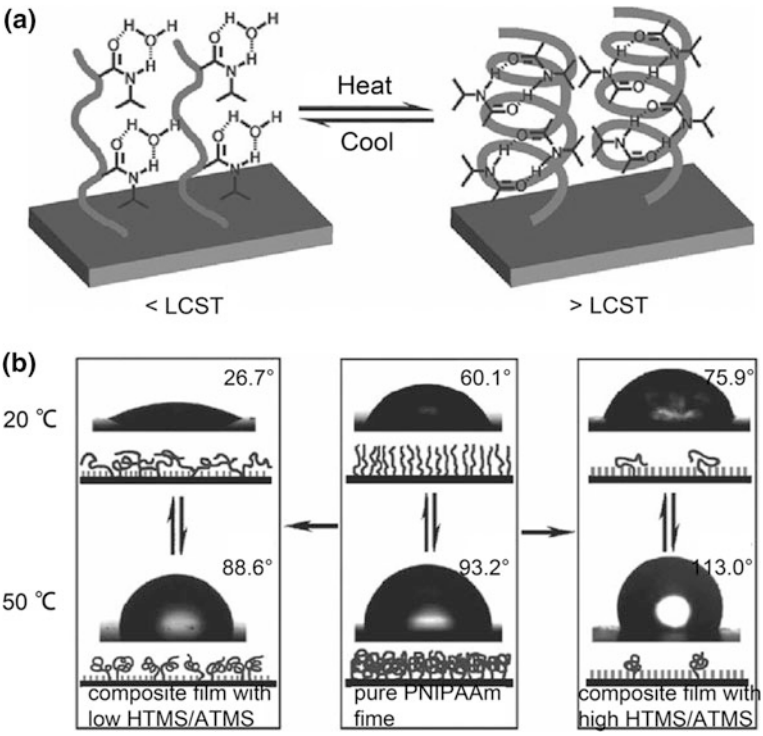

Fig. 6.7 Temperature-responsive PNIPAAm surfaces. **a** H-bonding mechanism for the coil–globule transition of PNIPAAm film at its LCST (Reprinted with permission from Ref. [43]). **b** Schematic diagram of the relationship between PNIPAAm configuration and responsive wettability. (Reprinted with permission from Ref. [44])

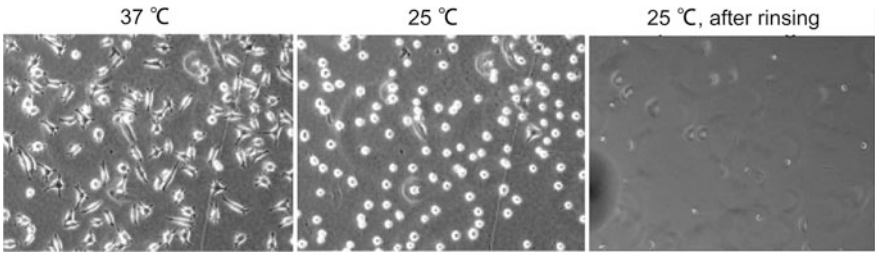

Fig. 6.8 Temperature-responsive cell culture surfaces. Phase contrast microscopy images of L929 mouse fibroblast cells after incubation at 37 °C on PNIPAM microgel films and 20 min after cooling the surface to 25 °C. At 25 °C gentle rinsing nearly removed more than 90% of the cells from the surface. (Reprinted with permission from Ref. [45])

the chains. However, when above LCST, molecular chains distort and cover a part of the H bonds, making wettability of the surface changed (Fig. 6.7b) [44]. As shown in Fig. 6.8, Schmidt et al. achieved cell adhesion conversion on PNIPAA microgel films [45]. Moreover, physicochemical parameters and the cell adhesion performance of the PNIPAA films before and after temperature variation were investigated. The results proved the efficiency of PNIPAA microgel film in regulating cell adhesion and release through temperature control, and the temperature slightly above LCST was found to be the optimum temperature for cell adhesion and proliferation.

6.3.2 3D Cell Culture

Simulation of the in vivo microenvironment on cell culture is expected by drug evaluation, oncotherapy, and biological research. In spite of the plane cell culture can easily be proceed on various substrates, extensive studies proved that the spatial arrangement of cells has significant influence on cellular behaviors and functions [46–48]. In this background, the proposal of developing 3D cell culture system on chip was put forward. Generally, there are two mainly ways achieving spatial cell culture on microfluidic chips. One is through multi-layer chip design, and the other one is making use of scaffold materials, especially hydrogels. In order to achieve better results, the multi-layer structure design and scaffold employment are often used in combination.

Using the multi-soft lithography technique, microchannels with different width, depth, and shape can be integrated on a single microfluidic device. 3D cell culture in these microdevices can be realized through placing cells on target channel surfaces at different layers under control. The spatial cell culture in microfluidic chips can be achieved in various ways through delicate design, such as preparing microwells, height-gradient chambers, and making use of intercellular porous films. The microwell and height-gradient structures are often fabricated by multiple

ultraviolet exposures with a group of photomasks. Figure 6.9 exhibits Kang's research of developing a multilayer microfluidic device with concave microwells and plane chambers to culture embryonic stem (ES) cells and regulate the formation of uniform-sized embryoid bodies [49]. Lin's group also did many researches of on-chip 3D cell culture and analysis. For example, Wu constructed a 3D microfluidic chip with height-gradient chambers to imitate the diffusion process between blood vessels and tissues [50], and cytotoxicity of quantum dots (QDs) was investigated on this established drug evaluation platform using HepG2 cells. As another way of building 3D cell culture models, sandwich-structured systems with a porous thin film used as the intercellular film are commonly adopted in 3D cell co-culture to investigate cellular interactions between two or more types of cells [51]. Figure 6.10 exhibits the typical structure of the "sandwich" coculture microfluidic platform. Normal mouse embryonic fibroblasts (mEFs) (without inactivation) and mouse embryonic stem (mES) cells were cultured on two sides of a PDMS porous layer for feeder-separated coculture, which was proved in the tomography by laser confocal microscopy (Fig. 6.10c). The mEFs resulted in the formation of mES cell colonies with excellent undifferentiated state confirmed by the expression of Nanog, octamer binding protein 4 (Oct-4) and alkaline phosphatase (ALP), which demonstrates the significant advantages in efficiency and simplicity of the microfluidic device.

Above methods achieved spatial cell culture to some extent, however, they are not the very real 3D cell culture, because cells cultured on these platforms still attach to microchannel surfaces or the porous membranes that present plane culture state. To fill microchannels with cells, introducing scaffold materials in channel space is necessary to assist in cell proliferation and migration. Meanwhile, porous structures of scaffold materials can guarantee the mass transportation in chip. Generally, biomaterial hydrogels can be used as ideal scaffolds [52, 53], which has been mentioned in the previous portion (see Sect. 6.2.3.1). Some ECM proteins, such as collagen, fibrin, matrigel, FN, and mixture of these proteins were primarily considered as the principal part of hydrogel scaffolds to encapsulate

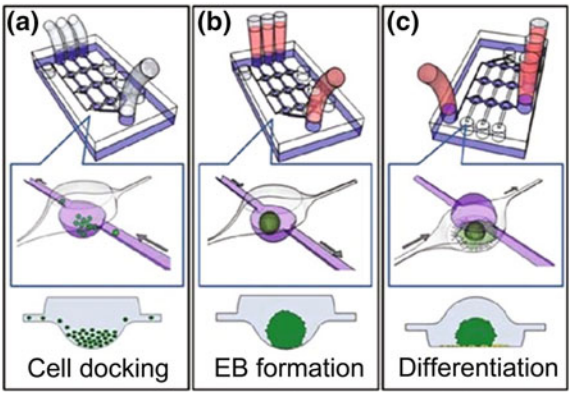

Fig. 6.9 A multi-layer microfluidic device containing concave microwells and flat cell-culture chambers. **a** Schematic of the cell docking in concave microwells; **b** Schematic of the EB formation; **c** Retrieval of EBs from the concave microwells after converting the microfluidic device. (Reprinted with permission from Ref. [49])

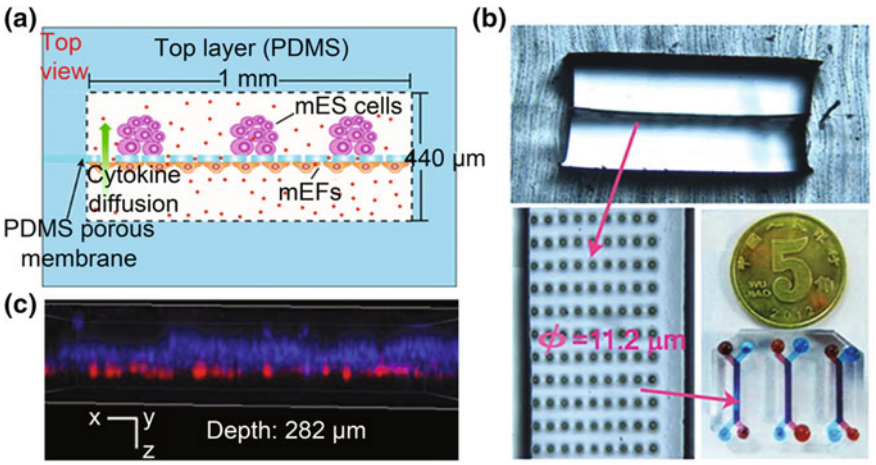

Fig. 6.10 The typical "sandwich" coculture microfluidic platform. **a** Cross section of the device; **b** Photographs of the assembled microchannel, PDMS porous membrane and fabricated microdevice; **c** Confocal image showing the mES cells/mEFs coculture system. (Reprinted with permission from Ref. [51])

dispersed cells. For example, Kamm et al. accomplished 3D cell culture with collagen [28, 54–56]. They fabricated a 3D microfluidic assay filled with collagen gels to determine the influence of endothelial KLF2 expression on smooth muscle cell (SMC) migration [54]. SMC cells co-cultured with endothelial KLF2-expressing endothelial cells (ECs) showed an apparent decrease in migration. They also adopted collagen as 3D scaffolds in investigating the neurite transforming under a growth factor gradient generated by microfluidics [28]. More remarkably, they introduced physiologically relevant 3D capillary morphogenesis on hydrogel microfluidic platforms stimulated by growth factors [55] and cells [56]. Calcium alginate and gelatin also have good biocompatibility as scaffolds in cell culture. For instance, Choong Kim succeeded in embedding fibroblast cells in alginate beads [57]. The cell beads can influence the EC monolayer cultured in collagen scaffolds and leaded to the formation of circular lumen-like structures. In addition to ECM proteins, cellulose, the most abundant biomaterial in natural, shows great potential in fabricating 3D microenvironment for cells on chip. Cellulose is one of the crystalline structural polysaccharides, a linear polymer connected by β-1, 4-D-glucoside bond, which can be produced by wood, tunicate, and bacterial. Among celluloses from different sources, the bacterial cellulose has wide applications in personal care, pharmaceutical, bioscience [58], due to its very special structures and properties (nanometer networks, high water retention, good biosynthesis controllability and biocompatibility). The nanostructure and morphology of bacterial cellulose make it very suitable for being used as the scaffolds for cell immobilization and migration [59, 60] (Fig. 6.11), and forming ECM. Also, as shown in Fig. 6.12, hydrogels of bacterial cellulose is an ideal material for tissue

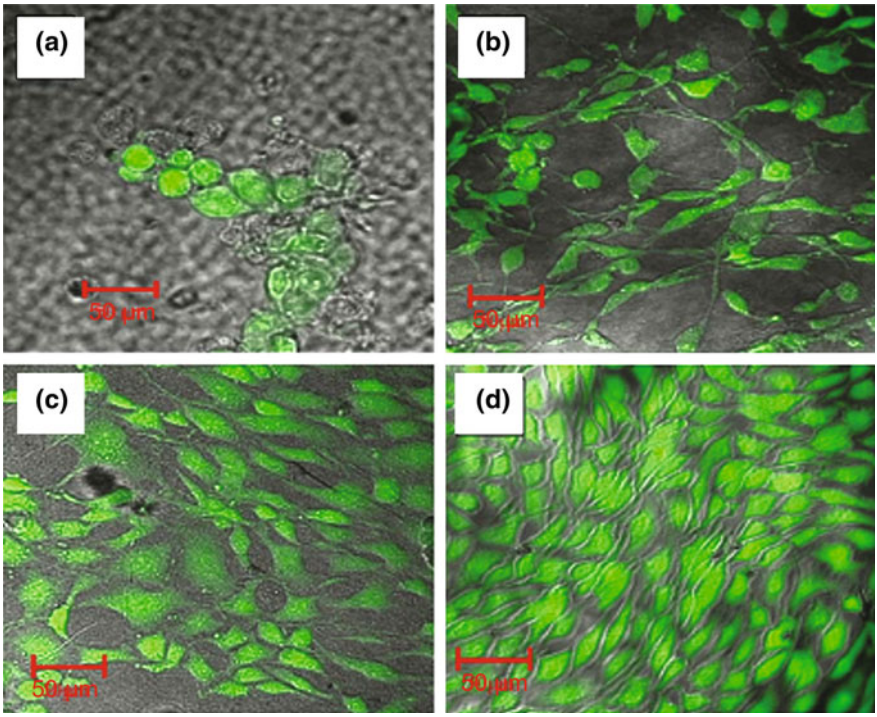

Fig. 6.11 Bacteria cellulose in cell culture. Confocal laser microscopic images of 6 days old fibroblast NIH/3T3 cells grown on **a** AGM_Ca, **b** BC gel, **c** BC_M and **d** GTA_GM. (Reprinted with permission from Ref. [60])

engineering[61], which has been applied in skin repair, wound dressing, tissue regeneration and periodontal treatment. As a famous work, artificial blood vessels made of bacterial cellulose have been employed in clinical experiments [58]. This kind of artificial blood vessel has stable inner cavity, good stitching stability and good permeability of water, ions, and small molecules. Besides, with the development of nanoscience, several techniques are capable of processing cellulose into cellulose nanofibril (CNF), such as TEMPO-mediated oxidation followed by mechanical disintegration treatment [62], and the mechanism and obtained nanofibers are exhibited in Fig. 6.13. Nanocelluloses also exhibit great potentials in biomedicine [63]. For instance, NFC has shear-thinning property, and based on this feature, Bhattacharya et al. prepared a type of injectable hydrogel by making use of plant-sourced CNF [64]. Cytotoxic tests of the CNF hydrogel using HepaRG and HepG2 cells proved the biocompatibility of this material, and mitochondria activity of the cells cultured in CNF hydrogels had no apparent distinction compared with standard 2D culture cell incubation on CNF. Also, a gyroid scaffold consisted of

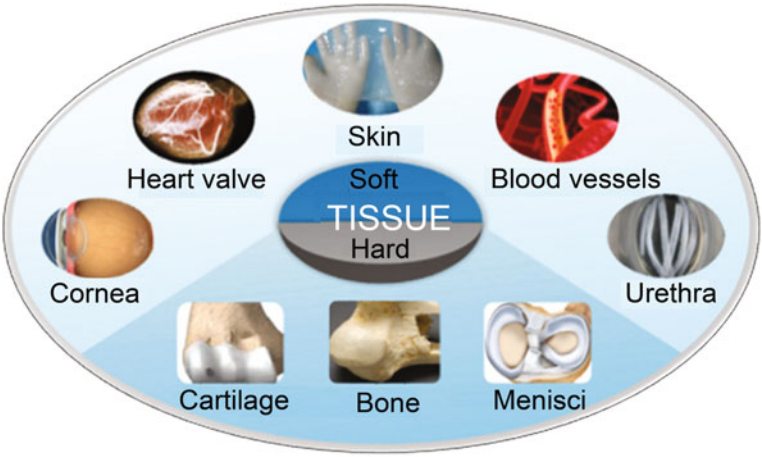

Fig. 6.12 Biomedical applications of BC-based materials in tissue engineering. (Reprinted with permission from Ref. [61])

nanocellulose and nanochitin hydrogel was fabricated using a template-assisted method [65]. The hydrogel possessed shape recovery behavior even after supercritical drying. In investigations of the effectiveness of the produced hydrogel as bone tissue scaffolds, human muscle satellite cells (HMSCs) showed good adhesion and spread on CNF, which may due to the electrostatic interactions between CNF surfaces and collagen 1 at pH 7.4 in phosphate buffer solutions (PBS). Consequently, the cellulose nanomaterial hydrogel is a promising material that can be used in microfluidic devices as scaffolds for 3D cell culture.

Hydrogels, especially natural hydrogels, have some drawbacks in 3D cell culture. For instance, materials of different batches exhibit inconsistent properties. Also, the transportation of nutrients and generated oxygen concentrations have gradient distributions along the thickness of hydrogels. Furthermore, some experiments require dense cell concentrations, such as a multicellular tumor ball, which can hardly be constructed in hydrogel scaffolds. Therefore, gel-free 3D culture was put forward to settle the problems. One way is employing intercellular polymeric linkers to generate cell aggregations[66]. As shown in Fig. 6.14, Mo et al. used a positively charged dendrimeric intercellular linker in assembly of C3A cells. As a consequence, the fabricated cell aggregation had high viability, proliferation, and 3D cell morphology. In other ways, microwells [67] and microbubbles [68] fabricated in chips can assist in forming 3D cell culture structures. For example, Liu et al. prepared quasi-spherical microwells on PDMS substrates using an ice lithography-based benchtop method [69]. The concave microwells can generate dense and homogeneous multicellular tumor spheroids that can be removed from the microwells during cell incubation. Moreover, the long-term in-chip culture can be achieved.

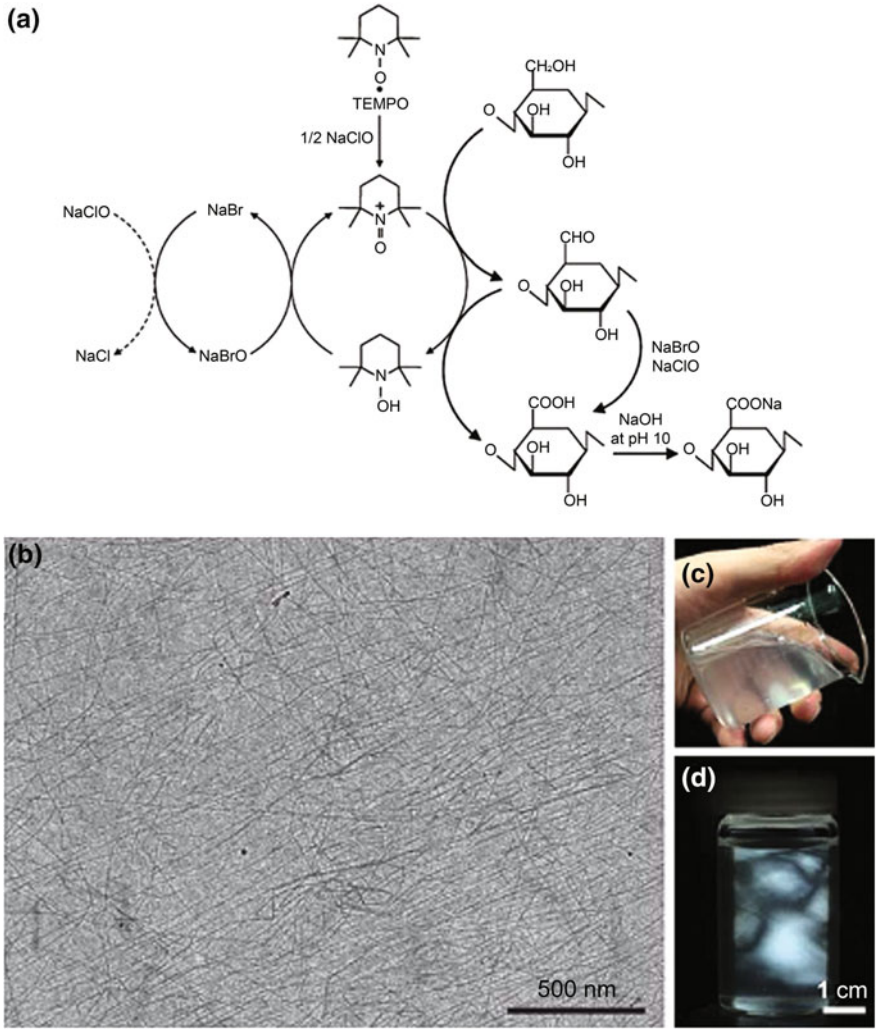

Fig. 6.13 Cellulose nanofibers prepared from wood pulp by TEMPO-mediated oxidation. **a** The mechanism of TEMPO/NaBr/NaClO oxidation in water at pH 10–11; **b** TEM image of a dried dispersion of TEMPO-oxidized hardwood cellulose; **c** Photographs of a typical dispersion and **d** when observed between cross polarizes. (Reprinted with permission from Ref. [62])

6.4 Organ-on-Chip System

Realizing artificial organs for clinical therapy and medical research services is always a hot topic. Cells are the fundamental unit to maintain functions of life activity and metabolism. Basically, organs are the aggregates of various cells that orderly arrange and distribute in a certain space. Therefore, the organ fabrication is

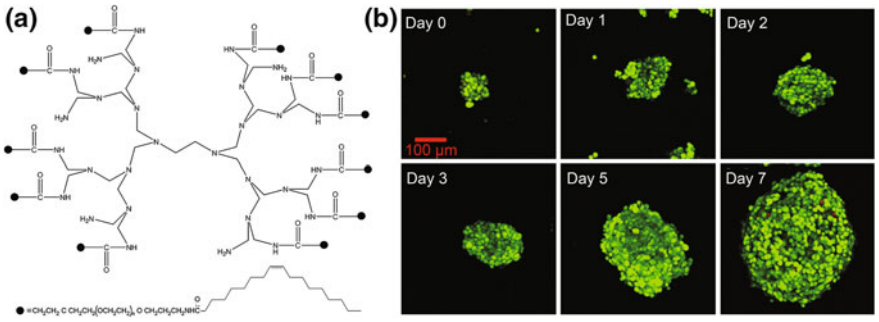

Fig. 6.14 Linker—engineered multi-cellular structures exhibit good viability and proliferation. **a** Schematic diagram of synthesis of oleyl-PEG conjugated DAB dendrimeric linker; **b** Confocal images of C3A cells stained by Live/Dead kit in multi-cellular structures during 7-day culture. (Reprinted with permission from Ref. [66])

essentially a simulation of the microenvironment and the composition and spatial structure of cell aggregations in organs. However, constructing an organ system is of great challenge, because the manipulation of different cells and maintaining their 3D structures with sustaining supplements is a harsh task. The emergence of microfluidic chips settled the issue of creating a blood supply system by designable microchannels. Also, the development of on-chip 3D cell culture makes microfluidic chips promising in fabricating organs, which has great significance in pharmaceutical research, tissue engineering, medicine and biology.

Organ-on-chip technique aims at mimicking in vivo physiological activities, functions, and understanding the operation mechanism of organs on a 3D cell culture microfluidic platform. 3D cell culture of one specific cell type can hardly inspire its functions. The intercellular communication is the key factor of maintaining normal cell activities [70, 71]. Therefore, multicellular culture systems with controllable cell distribution are significant in the construction of organ-on-chip devices. Dongeun Huh developed a very typical lung-on-a-chip microsystem [72], as illustrated by Fig. 6.15. They succeeded in detecting organ-level responses to bacteria and inflammatory cytokine carried on alveolar-capillary interfaces using this organ-on-chip device. As another example, a high throughput "heart on a chip" system was successfully prepared using submillimeter-sized thin-film cantilevers made of soft elastomers. Subsequently, muscular thin films (MTFs) were obtained through building anisotropic cardiac microtissues on the cantilevers [73], as shown in Fig. 6.16.

According to the preparation characteristic of microfluidic chips in constructing organ structures, organ-on-chip systems can mainly be divided into two categories: bottom-up stacking and 3D hydrodynamic flow focusing.

The bottom-up stacking can simulate tissue interfaces in the similar way as "sandwich" structures, that is, from the bottom to the top, accumulating layer by layer. In bottom-up microfluidic devices, two or more types of cells are incubated in different chambers that are separated by porous membranes. This kind of

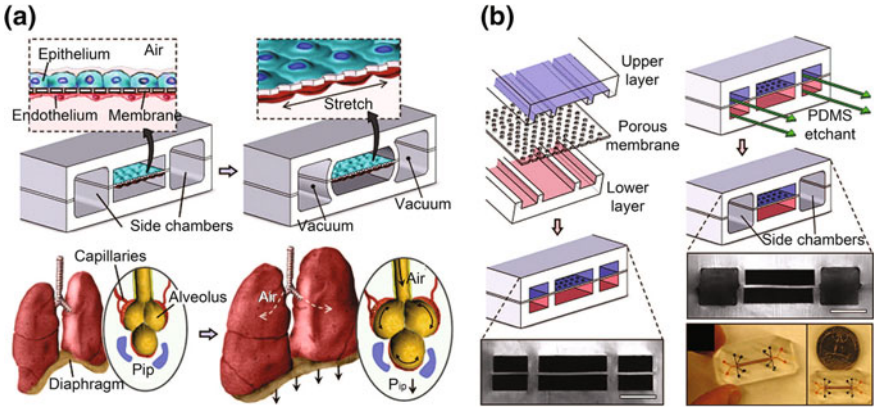

Fig. 6.15 Biomimic design of a human breathing lung on microchip. **a** Simulation of an alveolar-capillary barrier on microdevice; **b** Construction and photograph of the microchip. (Reprinted with permission from Ref. [72])

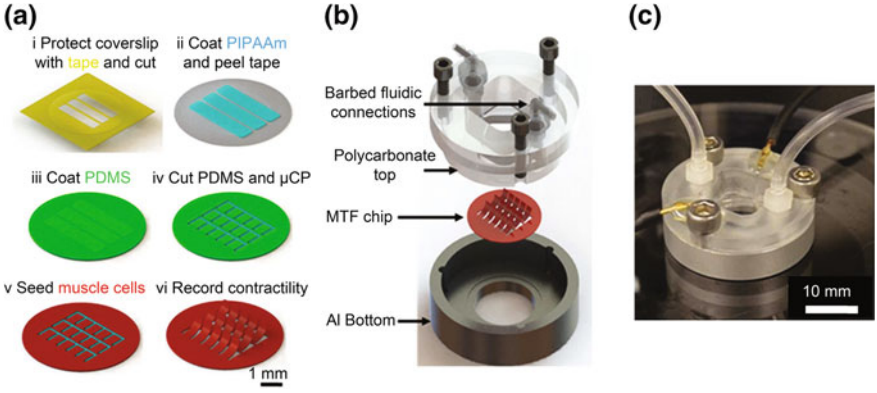

Fig. 6.16 Heart on a chip microdevice. **a** Schematic of fabrication process of MTF chip; **b** Assembly of the artificial heart fluidic device; **c** Photograph of the actual device in action. (Reprinted with permission from Ref. [73])

microfluidic chip is capable of providing fluidic shear stress, through which the lung alveolar-capillary interface and some unique structures in vivo, such as blood-brain carrier (BBB) [74], can be better simulated. The BBB is a unique feature of the central nervous system. Tight junctions of endothelial cells in BBB can prevent exogenetic substances from entering brain tissues. Figure 6.17 exhibits a microfluidic-built BBB structure. Two exogenetic flow channels were designed in one microchip, and EC cells and astrocytes were cultured on two sides of the membrane respectively. EC cells showed good viability of long time incubation, with high expression of tight junctions. C8-D1A (astrocyte) cells on the porous PC

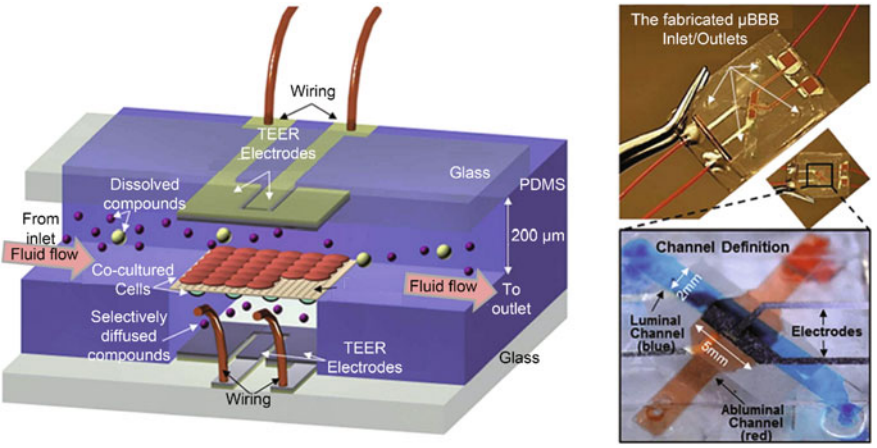

Fig. 6.17 Structure and design of the developed μBBB platform. (Reprinted with permission from Ref. [74])

membrane exhibited typical astrocytic morphology. Detection of the trans-endothelial electrical resistance (TEER) of the artificial microchip and transwells showed TEER value of the dynamic system (exceeded 250 Ω cm^2) was significantly higher than that of the static system (merely 25 Ω cm^2), which could due to the fluidic shear stress generated mecho-transductive effect on endothelial molecular pathways.

In 3D hydrodynamic flow focusing, specific cell arrangements such as spherical [75] and fibrous cell aggregations [76] can be achieved. Spherical and fibrous structures have particular importance in organ construction, because these configurations can be commonly observed in tissues and organs. Spherical structures find significant applications in mimicking tumor tissues and investigating intercellular communications between tumor cells and their neighboring cells. As shown in Fig. 6.18a, Alessanbri et al. [75] fabricated a coextrusion microfluidic device that can produce cell-based assays. They used 3D hydrodynamic flow focusing technique in preparing alginate microcapsules, and CT26 cell spheroids were successfully generated by gel-free and gel-encapsulating ways. To form spheroids, a side flow channel perpendicular to the fluidic direction is always needed to provide shear force in cutting the flow into droplets. Fibrous structures are fabricated in the same way as spheroids in terms of the fabricating mechanism. Both of them applied the coextrusion technique to form a coaxial geometry, but comparing with spheroid fabrication, synthesis of continues fibrous structures do not need the shear force. Cell-embedded hydrogel fibers embedded with cells have potential applications in mimicking fibrous structures in vivo, such as vessels, nerve fibers, and muscle fibers. As shown in Fig. 6.18b, Takeuchi's research group fabricated several meter-long core–shell structure biological fibers with using the hydrodynamic

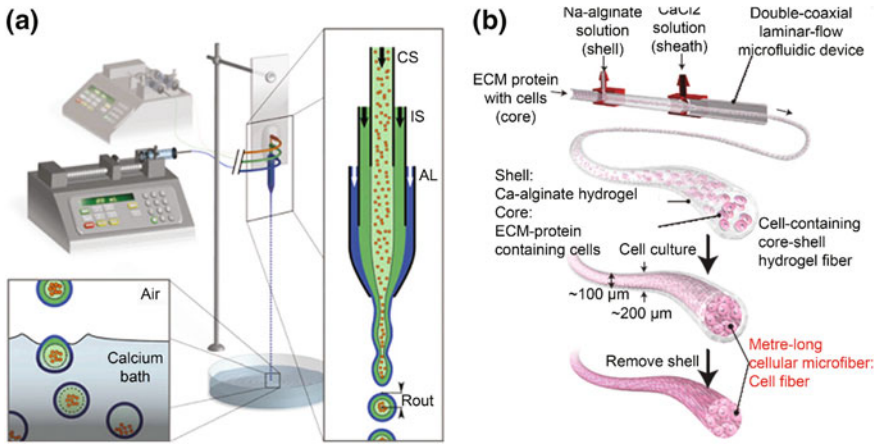

Fig. 6.18 Hydrodynamic flow focusing in forming 3D cell structures. **a** Spherical preparation system (Reprinted with permission from Ref. [75]) and **b** fibrous cell aggregations fabrication with coextrusion microdevices. (Reprinted with permission from Ref. [76])

focusing technique [76]. The coaxial fibers were continuously extruded out from a double-coaxial microfluidic chip. Cells in ECM proteins served as the core and the Ca-alginate hydrogel acted as the shell of the fibers. Using this platform, Cardiomyocyte-Fib, HUVEC-ACol, and Cortical cell-PCol fibers were successfully fabricated. Furthermore, these functional biological microfibers can be collected by waving or reeling, which can be used to prepare fiber-shaped tissues and organs.

On-chip tissues or organs with multi-cellular structures also can be realized through optimizing the chip design. For example, Lee et al. developed a 3D liver-on-chip system [77] in studying paracrine effect of hepatic stellate cells (HECs) on hepatocytes. Hepatocyte spheroids were generated in concave microwells on chip. Although no direct contact existed between the two types of cells, intercellular communications happened as a result of culture medium flow. Through comparing the spheroid structures and the level of albumin and cytochrome P450 reductase of mono-cultured hepatocyte spheroids or co-cultured spheroids, paracrine effect of HSCs was found to have positive effects on maintaining the functions and structures of hepatocyte spheroids.

6.5 Simulation and Manipulation of Cell Microenvironment on Chips

In vivo microenvironment contains many complex chemical and mechanical factors, which leads to various specific physical and chemical properties (e.g., temperature, osmotic pressure, oxygen content, and pH), soluble factors, and intercellular or cell-matrix interactions [78]. Besides, the in vivo microenvironment

is a dynamic system, in which every factor is dynamically balanced to maintain the state stable for lives. One unique advantage of the on-chip cell culture is that microchips can mimic the cell environment with manipulation in continuous flow.

In drug screening research, the microfluidic technology can meet demands such as achieving parallel tests with concentration gradients through structure design. A typical microchip design for producing concentration gradients is called the "Xmas-tree" structure. In this structure, laminar flowing effect is utilized to make better mixing of two different solutions and produce concentration gradients along the direction perpendicular to parallel channels [79, 80]. Gao et al. [81] integrated the "Xmas-tree" structure in their microfluidic device to generate drug solutions with gradually changed concentrations. They analyzed cytotoxicity, drug absorption and metabolites with high throughput. Chen et al. [82] fabricated a network structure on chip to generate concentration gradients for cell culture and drug screening. Chen used an isotope-labeled internal standard method in detection, because the mass spectrometry can only obtain semi-quantitative results. This method improved the linear exponential and expanded the linearity range, which achieved real-time qualitative and quantitative detection of metabolites. Scherber et al. [83] found endothelial cells can "perceive" their way along the concentration gradient of endothelial growth factors (EGF) by using a microfluidic chip.

Hypoxia environment proved to be able to facilitate the tumor growth, invasion and metastasis. Accordingly, the simulation of in vivo hypoxia microenvironment and the investigation of hypoxia microenvironment's impact on tumor development are hot topics in tumor research [84]. Wang et al. [85] investigated the cell response to drug stimulation in hypoxia microenvironment fabricated on an oxygen concentration gradient producing microfluidic device. Lin et al. [86] fabricated a double-layer microfluidic chip to mimic the hypoxia environment in tumor. In their work, aptamers were fixed on both the two layers, through this, real-time detection of vascular endothelial growth factor (VEGF) 165 was completed (Fig. 6.19). Besides, the distance influence on endothelial-tumor intercellular interactions and oxygen concentration gradient influence on cell migration were investigated. This investigation of signaling pathways and cell migration behavior in tumor angiogenesis has significant reference values for investigations of the formation and development of cervical cancer.

Cell density can affect cell behavior through the way of leading to intercellular matrix differences and local concentrations of different autocrine or paracrine cytokines. This series of changes caused by cell density lead to diverse in vivo cell reactions, such as intercellular interactions and tissues functions [87]. Wu et al. [88] designed a microfluidic device with a microvoid-embedded channel to generate cell density with high accuracy and good repeatability. Through regulating the microvoid number in channel, cell density can be controlled. Moreover, the cell density had linear proportional relationship with the number of designed microvoids. The obtained cell density showed good consistency with the theoretical calculation. Subsequently, an investigation on the cytotoxicity of carboxyl group coated CdTe quantum dots (QDs) under cell density gradients was performed. As a result,

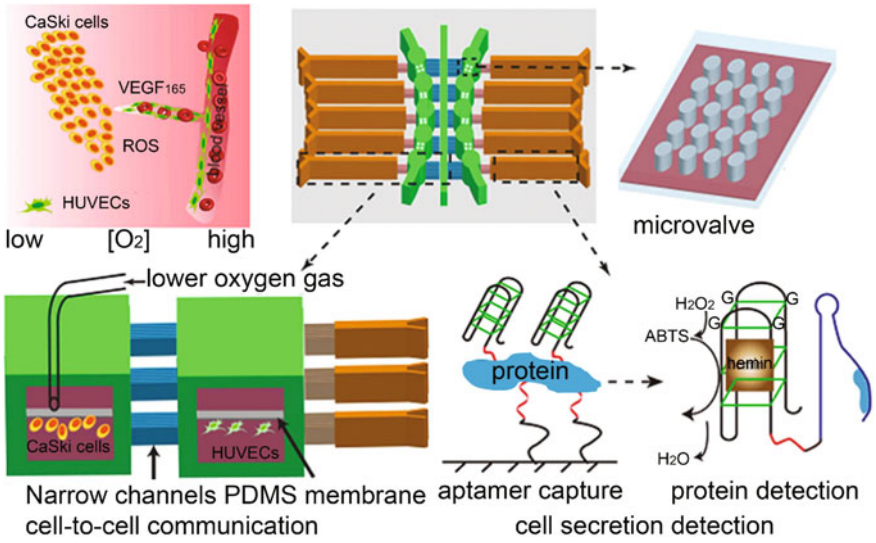

Fig. 6.19 Investigation of hypoxia microenvironment's impact on tumor development. An oxygen concentration gradient producing microfluidic cell co-culture device was constructed for on-line determination of protein $VEGF_{165}$. The $VEGF_{165}$ was qualitatively and semi-quantitatively analyzed by the functional nucleic acid, hemin, ABTS and peroxide system. (Reprinted with permission from Ref. [86])

quantum dots produced lower cytotoxicity under high cell density than the condition of low cell density.

Microfluidic chips made great contributions to investigations of some important cell behaviors (e.g. chemotaxis [89] and electrotaxis [90] of fibroblasts) and understanding of the roles of some proteins involved in cellular activities, for example, the function of selectin in neutrophil adhesion during inflammatory response [91]. Microfluidic platforms are also capable of regulating the cell microenvironment, which can be utilized in investigating paracrine and autocrine signals. For instance, mES-secreted factor regulation has been successfully performed on microfluidic platforms, which has significant importance for the development of dynamic investigation of signaling molecules [92, 93].

6.6 On-Chip Cell Observation

Main applications of microfluidic devices are in cell research, because microchannels have size accordance with cells and possess superiorities of low regent consumption and high detection sensitivity meanwhile. Different form static cell culture on culture dishes or bottles, the dynamic on-chip cell experiments can carry on real-time monitoring under specific flowing environment. The flow culture

creates more realistic simulations for cell experiments, but gives several challenges for cell observation at the same time, for example, cells have difficulties in adhering on chip surfaces after cell suspensions were injected into microchannels. Therefore, cell immobilization is a key step in on-chip cell observation and analysis. After successful cell immobilization and incubation in microchannels, the cell morphology and behavior can be observed through various cell imaging methods, in which the fluorescent imaging is the most commonly used method.

6.6.1 Cell Immobilization

In general, the inefficiency and poor regional location capability of cell immobilization lead to great challenges to the on-chip cell observation. At present, cells isolation methods have three main categories: cell adhesion enhancement, porous membranes-assisted isolation, and cell encapsulation using hydrogels. It is important to choose an appropriate way of cell immobilization based on specific features and applicability of different methods.

Cell adhesion enhancement

Microchannels with good cell affinity can be achieved by altering the chemical and physical properties of channel surfaces. Surface modifications can efficiently improve the cell adhesion through adjusting the surface charge property, wettability, and functional groups of microfluidic channels. Most cells have negatively charged cell membranes. Therefore, cell adhesion enhancement can be achieved in the way of coating cationic species on channel surfaces, such as ECM proteins, poly-L-lysine, and APTES. As we have mentioned in the previous parts, the hydrophobicity of PDMS-based microfluidic chips leads to poor affinity between cells and the matrix surfaces. Proper wettability of the matrix surfaces can be prepared by physical protein adsorption and covalent surface chemical modification. In addition, changes of surface morphology of microchannels also work effectively in cell isolation. For example, Wu subtly applied microwell arrays as cell density generators to controllably store cell suspensions by altering the number of microwells [88] (Fig. 6.20).

Porous membranes-assisted isolation

Porous membranes-assisted isolation is developed based on size distinctions between different cells. Cell filtration with a porous membrane can easily achieve cell isolation in a short time. For example, circulating tumor cells (CTCs) enrichment can be realized by using porous membranes. Lin et al. [94] used a parylene membrane-based microdevice in isolating CTCs from human peripheral blood, as shown in Fig. 6.21. More than 90% recovery was achieved when only five tumor cells were seeded in 7.5 mL of blood. The porous membranes-assisted isolation is also often applied in 2D/3D hybrid systems, that is, the before mentioned

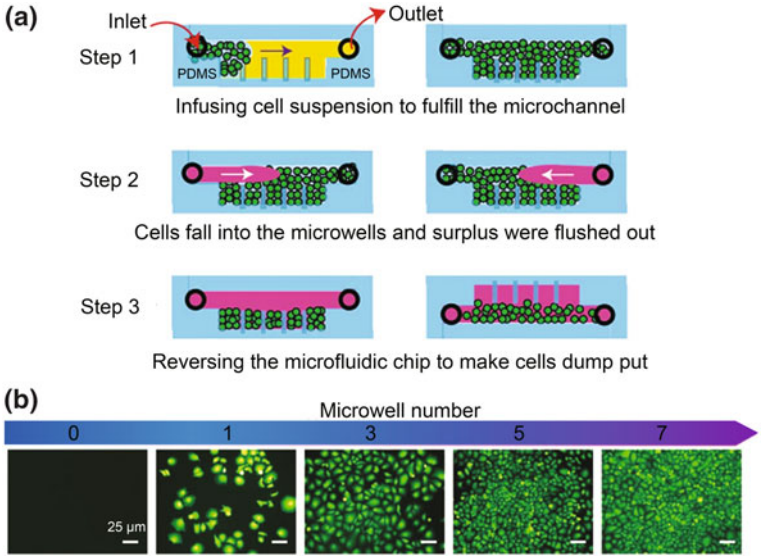

Fig. 6.20 Microwell-based cell density gradient generation microdevice. **a** Schematic of cell density gradient generation on the microfluidic chip; **b** Live/Dead kit staining fluorescent images of cells on microfluidic chips with different microwell numbers. (Reprinted with permission from Ref. [88])

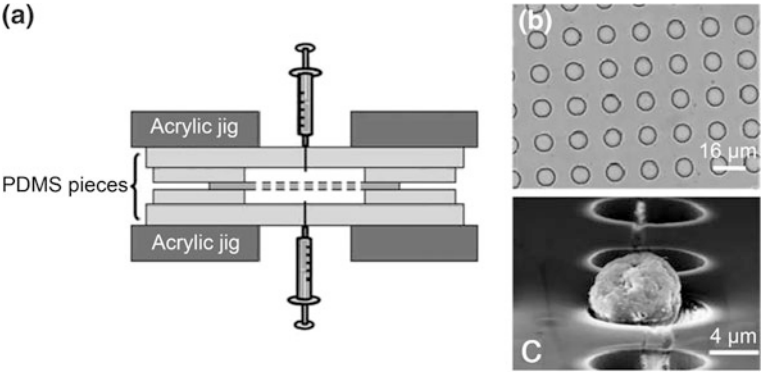

Fig. 6.21 Porous membrane-based cell isolation microdevice. **a** Device assembly; **b** Bright field image of the optically transparent parylene filter; **c** SEM image of the captured tumor cell on the membrane. (Reprinted with permission from Ref. [94])

"sandwich" structures. In the hybrid system, porous membranes are used as filter membranes for target cell isolation in 2D systems, but the whole microchip is a 3D system with two or more functional zones distributed on the two opposite sides of the membrane.

Cell encapsulation in hydrogels

Hydrogels, which have porous structures and are widely employed in 3D cell culture, are generally used in the way of cell encapsulation in cell isolation. Hydrogel-based cell-trapping technique can immobilize target cells in flow and provide a 3D microenvironment for cell proliferation and analysis. Gao et al. [95] obtained cell encapsulation of HepG2 cells and A549 cells simultaneously in PEG hydrogels using a photolithography approach (Fig. 6.22). The immobilized cell arrays can further be utilized to evaluate the anticancer drugs influence on cell viability and intracellular redox parameters.

6.6.2 Cell Imaging

Most cells are transparent and colorless under lamps, which makes direct observations of cytomembrane behaviors and intracellular components very difficult. For this reason, proper cell imaging techniques are necessary in real-time monitoring or detection

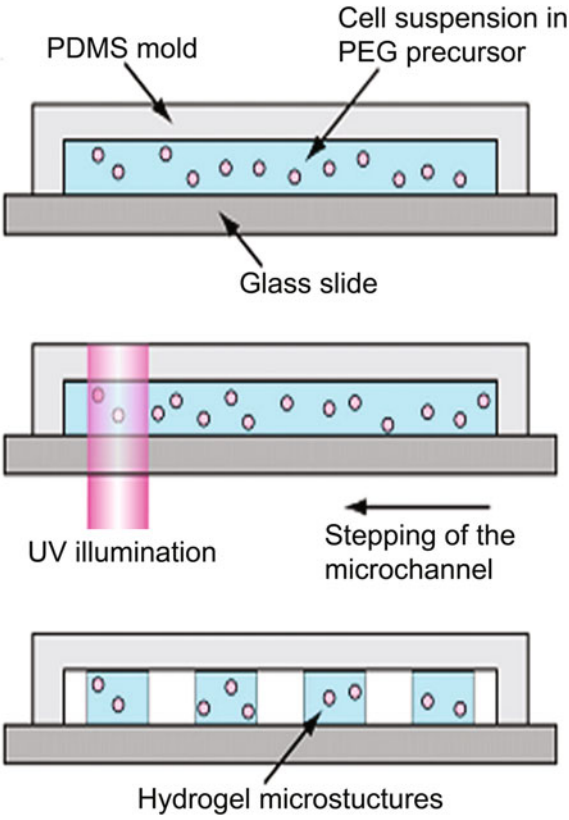

Fig. 6.22 Schematic diagram of the cell immobilization through cell encapsulation in hydrogel by photopolymerization. (Reprinted with permission from Ref. [95])

[96]. Cell imaging techniques are generally noninvasive and highly sensitive, such as photoluminescence (PL), magnetic resonance imaging (MRI), and surface-enhanced Raman scattering (SERS). Here, the fluorescent imaging is selected to elaborate how biomaterials are applied in cell imaging because the fluorescent imaging which contains fluorescent probe dyeing and subsequently appropriate fluorescence detections have been extensively studied and widely used in cell imaging.

Although some kinds of cells have green fluorescent protein (GFP), which makes them visible through emitting fluorescence under the excitation of certain wavelength, biomaterials are still necessary to assist cells in generating fluorescence in some cases, because only small parts of cells have GFP. Metals, polymers, and composite biomaterials, nano-biomaterials in particular, attracted great interests in cell imaging to label cell membrane, cytoplasm, or nucleus.

Inorganic nanomaterials, especially QDs, have been widely studied and applied because of their excellent fluorescence emission performance and tunable emission wavelength through diameter adjustment. Metal and transition metal QDs, graphenes and carbon dots, are some frequently used fluorescent-emitting materials, which has been reviewed in detail by Li et al. [97]. However, these inorganic nanomaterials have drawbacks of quenching and poor hydrophilicity. These problems can be solved in ways of polymer coating [98] or aptamer modification [99]. Recently, a kind of inorganic nanomaterial with excellent energy-converting properties, rare earth (RE) based upconversion nanoparticle (UCNP), was synthesized and applied in biological imaging (Fig. 6.23) with low cytotoxicity [100]. The hydrophilic hollow NaREF4 (RE = Y, Yb, and Lu) nanoparticle (NP) was synthesized through a facile liquid–liquid two-phase method. In vitro tests proved the UCNPs have bright-red emission without apparent noise background, which confirmed the suitability of using UCNPs in cell imaging.

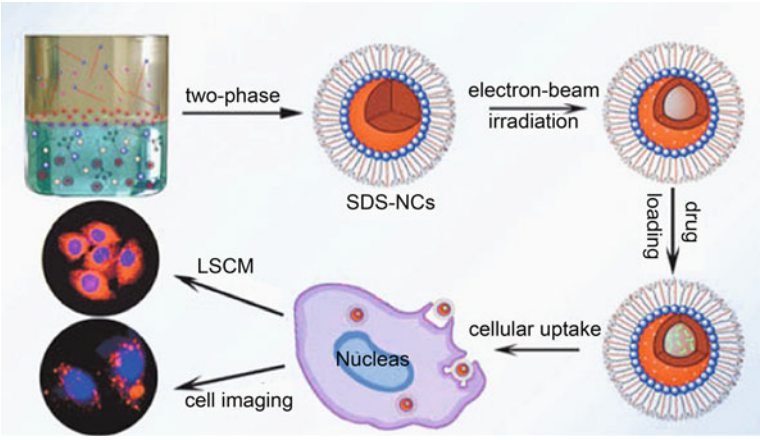

Fig. 6.23 An inorganic cell imaging biomaterial nanoparticle. The synthesis of small NaREF4 (RE = Nd, Sm–Lu, Y) and hydrophilic hollow NaREF4 (RE = Y, Yb, and Lu) NPs for application in cell imaging. (Reprinted with permission from Ref. [100])

Polymers and their nanocomposites are splendid imaging regents for optical detection, because polymeric materials have good transparency, biocompatibility, and easy bio-functionalization property. Fluorescent-emitting NPs, fluorescent-conjugated NPs, and degradable polymers encapsulating fluorophores are commonly used for cell imaging. For example, Zheng et al. [101] employed a single-step assembly and nanoprecipitation approach in preparing folate receptor-targeted (FA) indocyanine green (ICG) dye-doped poly(D,L-lactide-co-glycolide) (PLGA) liquid NPs for cell imaging with good biocompatibility and excellent stability of photobleaching resistance. Some commonly existing defects of routine fluorescent materials, such as the poor aqueous stability, target specificity and rapid elimination of ICG were overcome with using the functional FA-ICG-PLGA-liquid NPs. Comparing with in folate receptor-negative A549 cells, the endocytosis of biomaterial shells in folate receptor-overexpressed MCF-7 cells was found to be more efficient.

6.7 On-Chip Cell Analysis

Microfluidic chip is a multifunctional platform that can integrate cell isolation, cell culture, metabolite enrichment and analysis in a very small area. In biological research, the aim of lab-on-chip experiments is cell analysis, which provides theoretical information to research on cytotoxicity, metabolic pathways, drug evaluation, and intercellular interactions.

6.7.1 Population Cell Analysis

Microfluidic chips have advantages in cell analysis due to their low demand of reagents and cell quantity. However, this property also brings difficulties for analysis due to the limited chamber capacity and the continuous flow lead to very low metabolite concentration. Also, the metabolites always have very complex compose and structures for further analysis. Therefore, accumulating and purifying target metabolites with high efficiency is very important for on-chip cell analysis. The solid-phase extraction (SPE) is frequently adopted in liquid chromatogram (LC). Bacause microfluidic chips are potable LC devices in micro-scale with high throughput, the SPE is also feasible for on-chip analysis. Gao et al. [81] built an online electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-Q-TOF MS) to determine drug absorption and cytotoxicity. Two functional parts, cell culture chambers combined with a drug solution flow and an on-chip SPE column, were connected by PE tubes. In the cell culture part, surface modification of 0.1% poly-L-lysine (PLL) was carried out to improve the cell adhesion. After providing drug stimulation to the cells, the SPE purification was used to determine the drug absorption. Also, SPE columns participated in the investigation of drug permeability [102]. As shown in Fig. 6.24, a “sandwich” microchip was fabricated

with using a semi-permeable PC membrane. The model drug releasing was performed at one side of the membrane to observe the drug permeation to the other side of the channel. ESI-Q-TOF MS detection was used to determine the permeated curcumin concentration. The results showed that only 30 min is necessary to accomplish the analysis on this platform, and only 6 μL of the drug solution is required for one permeation test, which proved the integrated microfluidic device had high sensitivity and efficiency for drug discovery and development. Moreover,

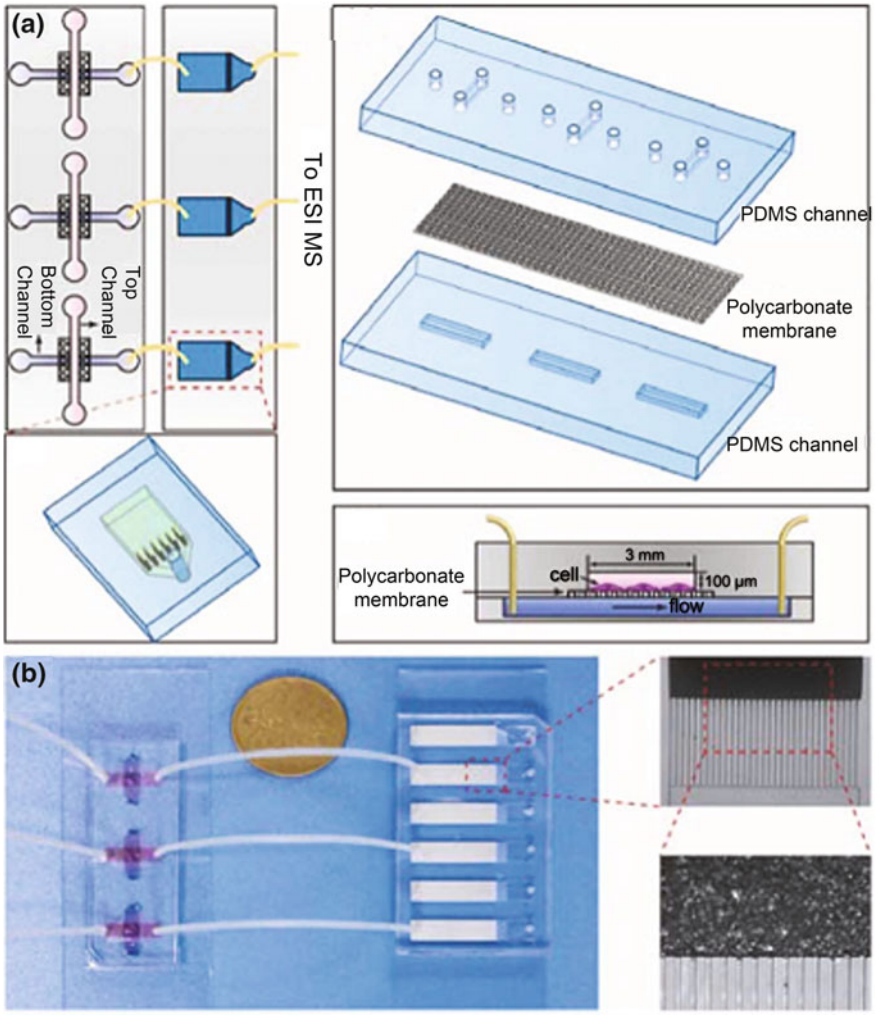

Fig. 6.24 A real-time online drug permeability assay with direct coupling to MS. **a** The integrated microdevice contained a "sandwich" cell culture section, and a sample pretreatment section consisted by μSPE columns prior to MS detection; **b** Photograph of the microfluidic device. (Reprinted with permission from Ref. [102])

Lin et al. adopted porous polymeric monolithic columns in SPE and chemiluminescence (CL) detection [103]. A porous polymeric column made of ethylenediamine modified poly(glycidyl methacrylate-co-ethylene dimethacrylate) was used in catechin enrichment. Then, the CL was generated through the reaction of catechin and potassium permanganate. This method has advantages of elution skipping, low limit of detection (LOD, 1.0 - 10^{9} M), and high recovery ranging from 90 to 110%. Lin also synthesized poly(ethyleneglycol)diacrylate (PEG-DA) microcolumn arrays in microchip channels as the probe for proteins and glucose detection [104].

6.7.2 Single Cell Analysis

In general cell analysis, analytic results are collected from a large amount of cells exposed to specific culture conditions, thus the results represent overall cell states in testing system. However, each cell is unique and not identical to the overall cell groups. Besides, recent research proved that there are obvious differences existing between individual cells. Therefore, developing single cell manipulation, screening, analysis, and cell heterogeneity investigation methods are meaningful for biological medicine. Microfluidic devices have superiority in low volume control with high accuracy, which is suitable for single-cell manipulation, such as cell sorting, cytomixis, transfection, gene sequencing and labeling [105].

At present, cell separation and capture techniques are commonly used to isolate intact single cell from the cell population and further intracellular substance analysis is performed after the cell lysis treatment. As one of the most direct physical method, some special microstructure design of microchips can realize cell separation and capture [106–109]. Microwell arrays-assisted cell trapping is one efficient way of single cell isolation and analysis. Chen et al. [106] achieved specific capture of single cell on chip through combining microvoid arrays with aptamer immobilization (Fig. 6.25). In this method, 3D microvoid structures assisted in enhancing the interaction between the aptamer-immobilized microvoid surfaces and cells. As a result, high single-cell occupancy of 88.2% was obtained. Using this highly specific cell capture microchip, target cells were isolated from the complex cell suspensions and formed cell arrays. Further single-cell level enzyme reaction dynamics analysis revealed that metabolic differences existing between different individual cells. Liu et al. [107] fabricated PDMS microvoid array on glass surface by employing the self-assembly of polystyrene microspheres (Fig. 6.26) for single-cell trapping. Subsequently, the real-time single-cell enzyme activity analysis confirmed the dissimilarity of viability and conditions among the trapped cells by analyzing the fluorescence intensity change of calcein AM-stained HeLa cells.

Aside from the microwell trapping ways, encapsulation, current separation, and some other methods are also effective in single-cell capture and analysis. For instance, Liu developed a modified microscope projection photolithography system that can activate the photopolymerization of PEGDA under control and finally

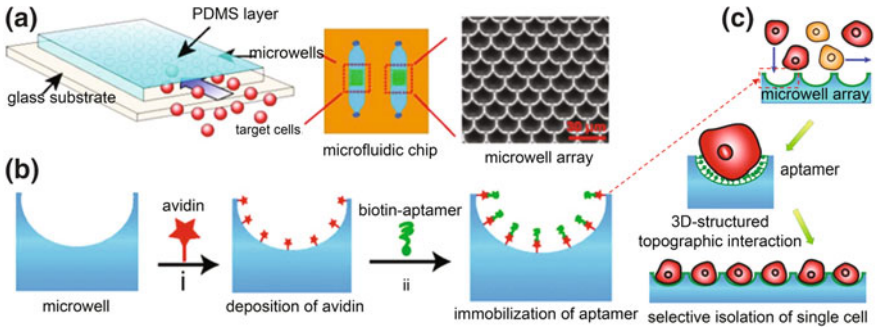

Fig. 6.25 A single-cell specific trapping microdevice. **a** Schematic illustration of the microwell arrays-assisted single target cells isolation microfluidic platform; **b** Functionalization of glass surface was conducted by the deposition of avidin and the immobilization of biotin–aptamer in turn; **c** Single-cell specific isolation was accomplished through the combination of microwell trapping and biotin–aptamer immobilization on glass substrates. (Reprinted with permission from Ref. [106])

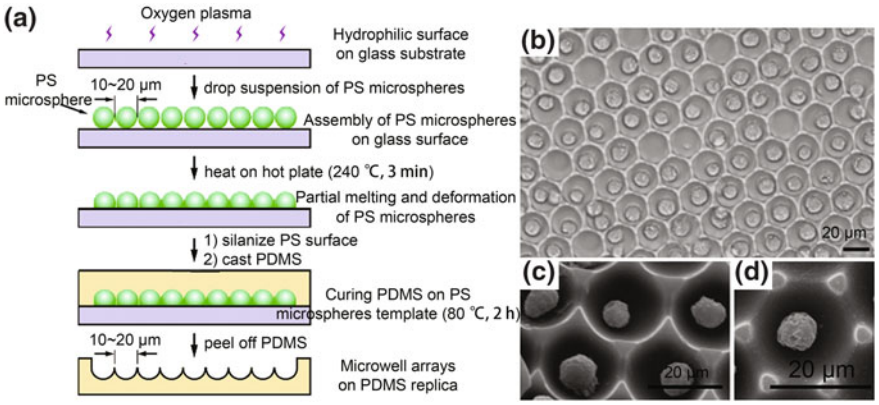

Fig. 6.26 Cell arrays generated on microwells for single cell enzyme activity analysis. **a** Schematic illustration for the fabrication of PDMS microwell arrays; **b** Dense trapping of Ramos cells; (**c**, **d**) SEM images of Ramos cells isolated in microwells. (Reprinted with permission from Ref. [107])

achieved single-cell encapsulation with an efficiency of 80%. Mach et al. [110] developed a microfluidic centrifuge for cell isolation based on eddy current separation. Wang completed single cell distribution in aqueous droplets surrounded by oil phase [111]. They further carried out cell incubation on a microfluidics platform with high throughput and chemiluminescence was employed for cell analysis. By this mean, only saccharomyces cerevisiae cells which excessively consumed xylose was successfully isolated from 10^4 cells, and the genomic variation resulting in excessive consumption of xylose was also analyzed.

With the development of microfluidic technology, single-cell analysis will develop quickly, and cell heterogeneity which covered by massive cells will be disclosed in the future.

6.7.3 Chip-MS Technology

Microfluidics and capillary electrophoresis combining with chemiluminescence, laser-induced fluorescence and electrochemical detection are some mature and commonly used cell analysis methods, but their testing targets in separation or detection still limited in molecules with simple or known structures so far [112, 113], which only contains less species. Lin et al. [114] realized rapid determination of mini-short tandem repeat (miniSTR) loci by means of combining polymerase chain reaction (PCR) with microchip capillary electrophoresis (MCE). Under the optimized conditions, merely 0.001 ng of DNA templates was necessary for miniSTRs generation, and the produced 15 miniSTRs had good narrow distribution with the relative standard deviations in the scope of 0.49–4.41%. Also, the miniSTR detection method was workable in practical samples, such as human hair, which has great potentials in criminal identification and paternity tests. Yi et al. [115] used a similar objective amplification detection to perform genotyping test on 11 human papilloma virus (HPV) with high sensitivity (detection limit was 200 copies) and accuracy. Validity of the method was further confirmed with using clinical samples. All HPV types were recognized with a good compatibility degree of more than 90%, which proved the high feasibility and reliability of the established separation and detection method in the screening and prognosis of cervical cancer.

MS detection can rapidly analyze multiple components at the same time with high sensitivity. Moreover, the detectable biological species of MS cover small molecules, peptides, amino acid and protein, which have a broad size distribution. More importantly, unknown substances can be analyzed and speculated based on the mass-to-charge ratio obtained from MS results. Consequently, MS plays an increasingly important role in biological medicine research, such as investigations of signaling pathways and protein structures. Though MS has lots of merits, the direct employment of MS in cell analysis is of great challenge because cell products always have complex compositions and strong matrix interference, which leads to fail results. As a solution, effective separation units are frequently empolyed to purify or separate the test solution before MS detection.

As exhibited in Fig. 6.27, since the 1950s, the developed separation units have experienced gas chromatography, liquid chromatography, capillary electrophoresis and microfluidic chip in turn [116]. Among those, the microfluidic chip is much more particular than other column-based separation techniques, because microfluidic chips have superiorities in connecting effective separation units with cell experiments with high integration, online processability and low reagent consumption [117]. In chip-MS systems, the electrospray ionization mass spectrometry

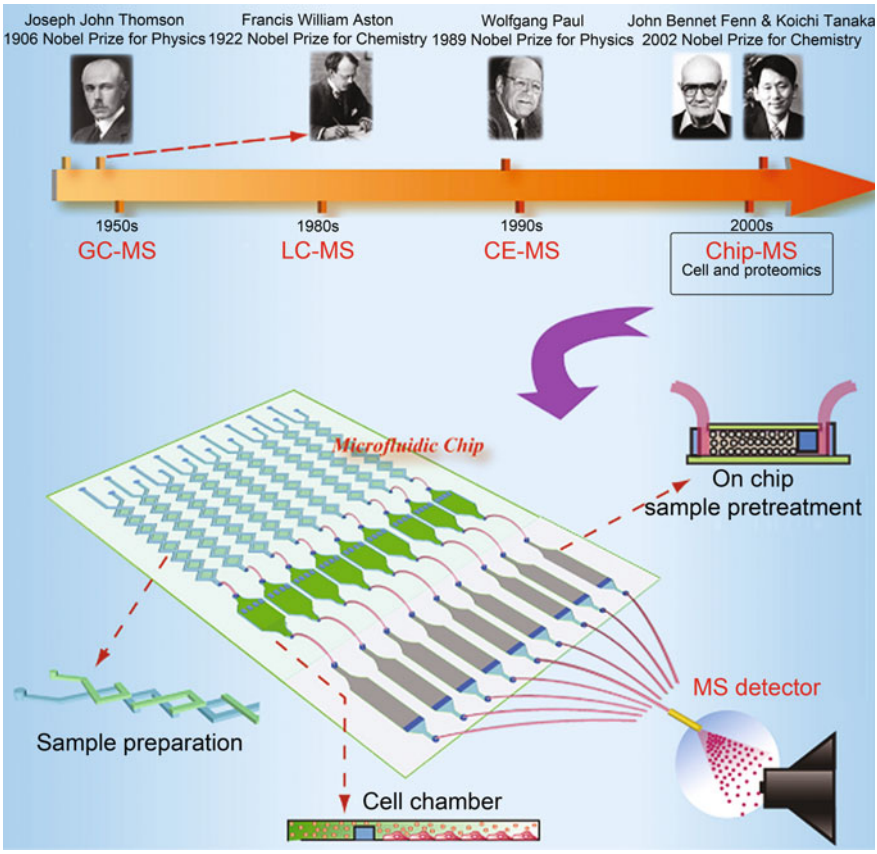

Fig. 6.27 The development of MS with coupling to different separation technology. The chip-MS system integrated sample preparation, cell chambers, on-chip sample pretreatment, and direct MS detection. (Reprinted with permission from Ref. [116])

(ESI-MS) is the most frequently used MS technique. On-chip sample pretreatments and the interface design of sample injection to MS are very important to obtain good detection results. Integrating monolithic columns or extracted materials are intensively utilized in sample pretreatment on chips [81, 82, 118] to accomplish desalination and target molecule enrichment of solutions with complex compositions, such as serum. In Gao's microfluidic device [81], SPE micro-column chambers were integrated on-chip, through which the rinsing and enrichment of target molecules were completed in fluids. They selected the methotrexate, an inhibitor of folate metabolism enzyme systems, as the drug model. HepG2 and Caco-2 cells were used as target cells to evaluate the effectiveness of the fabricated microfluidic chip on drug absorption. The results confirmed the feasibility of the fabricated platform in investigation of real-time pharmacokinetics of living cells. Moreover, to make the design and operation of sample pretreatment on chip

simpler, droplet paper-spray ion source interfaces and inkjet toothpick-spray ion source techniques were developed [119]. In the droplet paper-spray, non-easily ionized matters were maintained on paper fibers in advance, because paper fibers are capable of improving the separation and purification of complex solutions simultaneously. Liu et al. [120] fabricated a multi-channel microfluidic platform and determined the association constant of a noncovalent protein-protein complex using the paper-based electrospray MS (Fig. 6.28a). The results proved that the platform is effective in protein analysis. In the same way, the inkjet toothpick-spray ion source technique also realized separating and purifying complex matrixes on toothpick wood fibers. Moreover, the ink-jet system can be applied in accurately generating picoliter droplets on toothpick tips to form sprays for MS analysis. Luo et al. [119] set up a testing platform consisting of the inkjet droplet generation and a toothpick electrospray ion source (Fig. 6.28b). In MS detection, theobromine was used as the internal standard in linear measurement of caffeine. Good linearity exhibited in range of 1–200 ppm. More importantly, due to the shorter duration of electrospray, the obtained peak intensity ratio of different peaks almost equaled to the peak area ratio, which makes the quantitative examination possible on this platform.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has good salinity tolerance, which is very suitable for biological macromolecules detection, such as proteins and peptides. MALDI-MS is particularly suitable for single-cell and rasterization analysis due to its characteristic of rasterization detection. However, in MALDI-MS analysis, providing direct laser illumination eutectic laminas is necessary. Therefore, an open test area is required, which brings difficulties in connecting microfluidic chips to MALDI-MS. Zhang et al. [121] achieved direct, in situ MALDI-MS lipid analysis of mammalian cells on the platform shown in Fig. 6.29. First, they had mammalian cells directly cultured on indium tin oxide (ITO) coated glass. Then, a homogeneous eutectic lamina was prepared through electrospray ionization. The in situ determination of cell membrane lipid was carried out using a MALDI-MS. As a result, abundant information

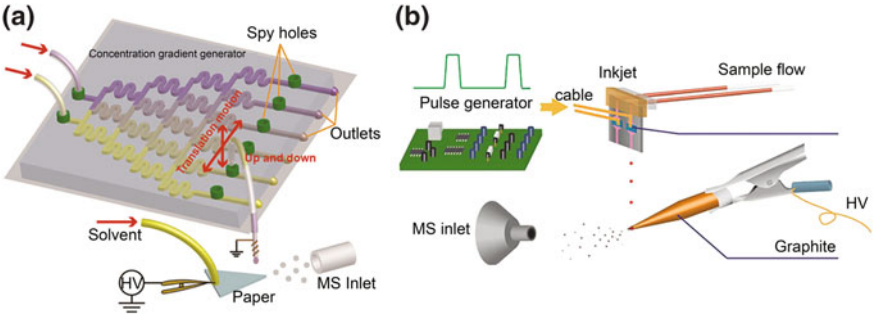

Fig. 6.28 Droplet spray ion source interfaces. **a** A droplet paper-spray ion source interface (Reprinted with permission from Ref. [119]) and **b** a inkjet toothpick-spray ion source technique in real-time monitoring of cells. (Reprinted with permission from Ref. [120])

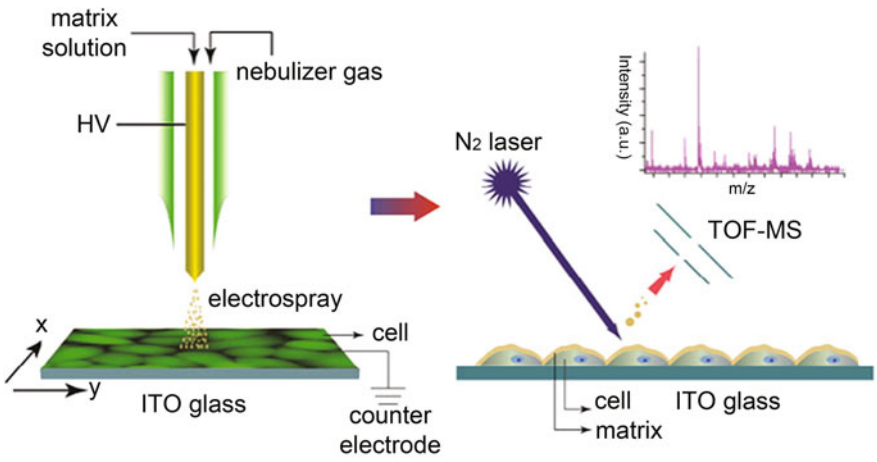

Fig. 6.29 Direct and in situ lipid analysis of mammalian cells on ITO glass by MALDI-MS. (Reprinted with permission from Ref. [121])

of the membrane lipid was obtained, which can be used as a relatively rough molecular mapping in cell identification.

6.8 Summary

The exploitation of microfluidic chips originally aims at developing portable chemical analysis equipments. So far, microfluidic chips are no longer just miniaturized analytical instruments, they are also being expected to realize biostructures or tissues constructions in vitro, and investigate biological targets, such as cells, proteins, and metabolites. Now, microfluidic chips are the most powerful tool for drug evaluation, diagnosis and therapy, biological research and fields based on cells, because they have tremendous advantages such as low reagent consumption, high throughput, easy integration, good designability, and the ability of mimicking complex microenvironment.

The principle materials of constructing microfluidics experienced the age of inorganic materials now have entered the organic era dominated by synthetic polymers. Performances of microfluidic devices in cell culture, cell observations and cell analysis can be improved by using adequate biomaterials. The hydrogels and smart materials have great potential to improve the biocompatibility and biological functionality of microfluidic devices. Meanwhile, making use of biomaterials and optimized chip design, 3D cell culture and single-cell analysis that can hardly be performed on traditional in vitro devices are realized on microfluidic chips, which have great potential in the future. In addition, the combination of microfluidic chips with MS detection efficiently extended the scope of testing

targets and is capable of analyzing complex compositions, which is the future direction of on-chip cell analysis.

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