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A microfluidic chip with a U-shaped microstructure array for multicellular spheroid formation, culturing and analysis

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Abstract

Multicellular spheroids (MCS), formed by self-assembly of single cells, are commonly used as a three-dimensional cell culture model to bridge the gap between *in vitro* monolayer culture and *in vivo* tissues. However, current methods for MCS generation and analysis still suffer drawbacks such as being labor-intensive and of poor controllability, and are not suitable for high-throughput applications. This study demonstrates a novel microfluidic chip to facilitate MCS formation, culturing and analysis. The chip contains an array of U-shaped microstructures fabricated by photopolymerizing the poly(ethylene glycol) diacrylate hydrogel through defining the ultraviolet light exposure pattern with a photomask. The geometry of the U-shaped microstructures allowed trapping cells into the pocket through the actions of fluid flow and the force of gravity. The hydrogel is non-adherent for cells, promoting the formation of MCS. Its permselective property also facilitates exchange of nutrients and waste for MCS, while providing protection of MCS from shearing stress during the medium perfusion. Heterotypic MCS can be formed easily by manipulating the cell trapping steps. Subsequent drug susceptibility analysis and long-term culture could also be achieved within the same chip. This MCS formation and culture platform can be used as a micro-scale bioreactor and applied in many cell biology and drug testing studies.

Keywords: drug screening, microfluidic systems, micro-scale bioreactor, multicellular spheroids, photoresponsive hydrogel

(Some figures may appear in colour only in the online journal)

1. Introduction

Three-dimensional (3D) cell culture produces tissues resembling those in animals in many aspects including cell–cell and cell–microenvironment interactions and is therefore more suitable than the conventional monolayer culture for both basic and applied cell-based studies [1, 2]. A large number of studies describing the development of 3D culture models has been reported over the past few decades to meet the increasing demands for such research systems [3–6]. Multicellular spheroids (MCS), with advantageous properties such as simplicity in production, functional and structural

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similarity with tissue *in vivo*, and potential for automation and high-throughput screening, is one of the well characterized and widely used 3D culture models [7–11]. MCS are typically several hundred micrometers in diameter and possess unique chemical gradients and cell–cell interactions found in tumors [12–14]. These properties make MCS especially suitable to be used as an avascular tumor model [15–18].

Traditionally, MCS can be generated by the hanging-drop method using single cell suspension, or by growing cells in spinner flasks and non-adhesive culture wares. These methods prevent cells from attaching on substrates to enhance cell–cell interaction forcing cells to assemble into aggregates [19]. However, each of these methods suffers several of the following shortcomings: labor-intensive, low production efficiency, poor MCS size uniformity, insufficient mass exchange for long-term MCS culture, difficult to use directly in subsequent applications. These limitations prohibit the extensive utilization of MCS in biomedical research.

The recent advancement of microfluidic technology has provided scientists many new capabilities in manipulating, culturing and analyzing cells [20]. Microfluidic systems can provide a continuous perfusion platform for long-term culture, drug treatment and assay readout acquisitions of cells [21–24]. A number of studies concerning the use of microfluidic systems in 3D cell culture have been reported. For example, a tumor cell intravasation model for endothelial barrier function analysis has been established in a microfluidic chip [25]. A model mimicking angiogenic sprouting morphogenesis has also been developed using a microfluidic system [26]. These findings indicate that a microfluidic system has good potential to be used in 3D cell culture.

Several microfluidic chips have been designed to overcome the problems of conventional MCS production methods. For example, the size of MCS can be accurately controlled through non-adhesion multi-microwells [27–29], ultrasound trapping [30], dielectrophoresis [31] and hydrodynamic force manipulations [32–35]. Taking advantage of the elasticity of polydimethylsiloxane (PDMS), a chip with dynamic pneumatic microstructures allowing sequential cell capture, spheroid formation and release, and subsequent analysis has been reported [36]. Different microfluidic designs to integrate MCS for anti-cancer drug screening and stem cell research have also been reported [27, 32, 33]. Mass exchange efficiency and automation are two important future directions of improvement on microfluidic systems for MCS-based studies.

Polyethylene glycol (PEG) hydrogels are materials with good biocompatibility and permeability, and have been widely applied in many biomedical research fields, in particular tissue engineering [37–39]. Photoresponsive PEG, such as PEG-diacrylic (PEG-DA), can be cross-linked to form different shapes through the photo-polymerization procedure [40] and has already been used in the last few decades for drug delivery, tissue scaffold fabrication and cell encapsulation [41, 42]. Photolithography, a method to define the exposure area through a photomask, can generate specific PEG-DA hydrogel patterns and precise micro-scale structures with efficient diffusion ability in microfluidic systems [43–48].

This study demonstrates a microfluidic chip integrated with U-shaped PEG hydrogel microstructures that can perform cell trapping, *in situ* MCS formation and long-term culturing. The U hydrogel microstructures were fabricated using the photolithography technique in a pre-assembled microfluidic chip. Cell trapping was carried out by applying gravity against fluidic flow and the sizes of MCS can be fine tuned according to the magnitudes of the U microstructure. The U-shaped structures prevent cells from shear force damage while allowing free diffusion of nutrients and wastes. Finally, we exemplify the usefulness of this microfluidic chip with a cytotoxicity assay and heterotypic spheroid generation.

2. Materials and methods

2.1. Materials and reagents

All tissue culture media, antibiotics, fetal bovine serum and fluorescent dyes were purchased from Invitrogen Corp (Carlsbad, CA). CellTracker^{TM} dyes 5-chloromethylfluorescein diacetate (CMFDA) and 5- (and 6) (((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) were used for cell labeling. Balb/c 3T3 fibroblasts and the human hepatoma cell line HepG2 were obtained from the Bioresource Collection and Research Centre (Hsin Chu, Taiwan). The cells were routinely maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37 °C in a 5% CO_2 humidified incubator. Other chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO) unless indicated otherwise.

2.2. Fabrication of hydrogel-integrated microfluidic systems

This study utilized a PDMS growth microchamber to accommodate a U-shaped microstructure array to form and culture MCS (figure 1(*A*)). The pre-polymers of PDMS were purchased from Dow Corning (Midland, MI). The acrylic mold of the microfluidic chip was designed using the SolidWork software (Dassault Systèmes SolidWorks Corp., Waltham, MA) and fabricated with a computer numerical control professional engraving machine (Roland, EGX-400, Irvine, CA). The microchamber was fabricated using the conventional PDMS casting method, mixing A and B reagents in the ratio of 10–1 and pouring into the acrylic mold. After baking at 80 °C for 2 h, the cured PDMS was demolded, oxygen plasma treated and bonded onto a glass slide to form a growth microchamber. The 3-methacryloxypropyltrimethoxysilane was used to introduce reactive acrylic groups on the glass and PDMS surfaces to form covalent binding with PEG-DA hydrogel. The U-shaped microstructures were generated in the PDMS microchamber using *in situ* photolithography. Photomasks were designed using the L-Edit v10 software (Tanner Research, Monrovia, CA) and printed on emulsion films with a resolution of 20 000 dpi (Taiwan Kong-King, Taoyuan). The PEG-DA precursor solutions contained 70% PEG-DA prepolymer (MW = 575 Da) in distilled water and 0.1% (v/v) photoinitiator, 2,2-dimethoxy-2-phenylacetophenone.

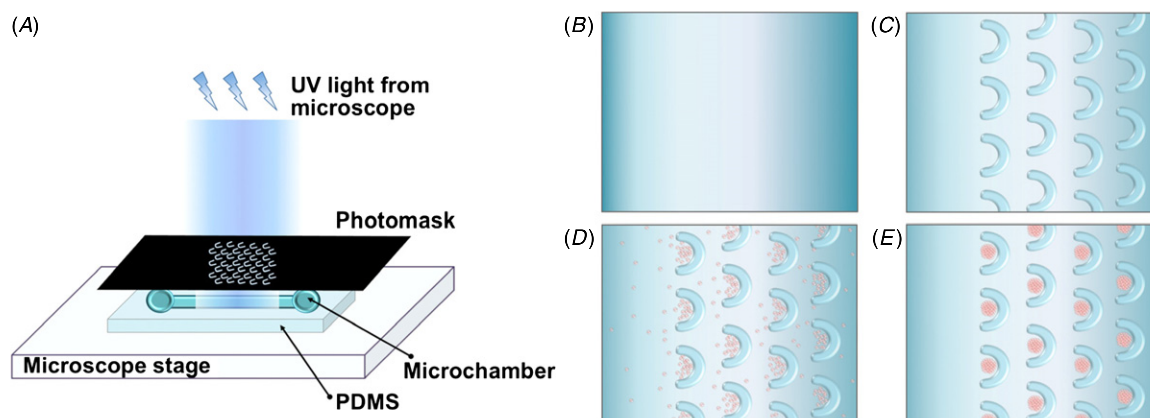

Figure 1. The flowchart of U-shaped microstructure fabrication, cell trapping and spheroid formation. (*A*) Fabrication setup of U-shaped microstructure-integrated microfluidic chip; (*B*) injection of PEG-DA pre-polymer solution; (*C*) generation of hydrogel microstructures; (*D*) cell trapping into U-shaped structures; (*E*) spheroid formation and culturing.

Figures 1(*B*)–(*E*) illustrate the processes of U-shaped structure fabrication. The photoresponsive PEG-DA precursor solution was first injected into the PDMS microchamber and then exposed to UV light through a designed photomask. After photopolymerization, non-reactive PEG-DA precursor was removed by washing the chip with distilled water. In this study, the light source was the mercury lamp on a fluorescence microscope (BX-51, Olympus) [49].

2.3. Cell seeding and trapping

Under a flow rate of 2 μl min^{-1}, cells in the PDMS microchamber primarily followed the main flow field and were hardly trapped into the U-shaped structures. To improve the trapping efficiency, we applied the force of gravity against the flow field by tilting the microfluidic chip. The chip was tilted at three angles (0°, 45° and 90°) and the degree was confirmed with a level. Single cell suspension at a density of 8.4 $\times$ 10^6 cells ml^{-1} was loaded into the microfluidic chip using a syringe pump at the withdrawal mode under the flow rate of 2 μl min^{-1} for 8 min. Then cell culture medium was injected into the microchamber from the same inlet for 5 min to elute non-trapped cells.

2.4. Cell culture and metabolic activity analysis in the microfluidic system

After trapping, cells were cultured and allowed to form MCS under continuous perfusion. At the MCS forming stage, cells were cultured under a flow rate of 8 μl min^{-1} for 14 h. After that, the chip was placed in a horizontal position and the flow rate was reduced to 1.5 μl min^{-1} for long-term culturing. For the anti-cancer drug assay, different concentrations of doxorubicin were continuously supplied at 1.5 μl min^{-1} for five days. The cell metabolic activity was determined using the Alamar Blue reagent (Invitrogen) at the end of the study. A total volume of 130 μl of the assay reagent was continuously loaded into the microchamber for a total reaction time of 2.5 h. The reacted Alamar Blue was immediately measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm with a Wallac Victor 1420 Multilabel Counter (PerkinElmer, Waltham, MA).

2.5. Heterotypic spheroid formation

HepG2 and Balb/c 3T3 fibroblast cells were trapped into the U microstructures either simultaneously or sequentially. The parameters for cell trapping in the heterotypic spheroid formation were the same as for the homotypic spheroid formation described above. To distinguish HepG2 and Balb/c 3T3 fibroblasts, these cells were labeled with red and green fluorescence with CMTMR and CMFDA, respectively. An illustration describing the process is given in supplementary figure S1 (available from stacks.iop.org/BF/6/015009/mmedia).

2.6. Image acquisitions and analysis

All images and videos in this study were captured with an epi-fluorescence inverted microscope (Axio; Carl Zeiss) or an upright microscope (BX-51; Olympus) connected with a DSLR camera (Nikon) or a CCD recorder using the SPOT Advanced Plus Imaging software (Sterling Heights, MI). This study utilized the ImageJ software to measure cell capture efficiency. The pocket area in each of the U-shaped microstructures was circled using 'Oval selection' and then the mean gray value was measured. The size of all the circled areas was controlled between 85 006 and 85 161 pixels to ensure the comparison of the mean gray values was on equal basis.

3. Results

3.1. Design and fabrication of a microfluidic chip with a U-shaped microstructure array

Figure 2(*A*) shows the accomplished PDMS microfluidic chip 75 mm in length and 25 mm in width. Each chip contains a chamber, of size 27 mm $\times$ 3 mm $\times$ 0.7 mm (l $\times$ w $\times$ h), a volume of approximately 60 μl, and an array of 56 U-shaped structures. The wall of the U microstructure has a thickness around 100 μm with an inner space of 250 μm $\times$ 200 μm $\times$ 700 μm. In this study, the mercury lamp equipped on a fluorescence microscope served as the

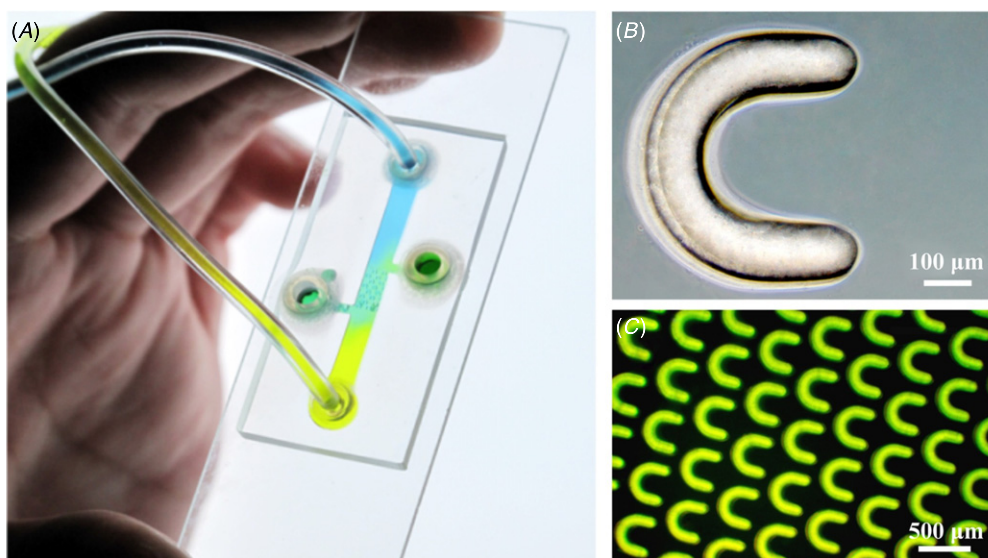

Figure 2. The hydrogel-integrated multicellular spheroid culture chip and the U-shaped hydrogel structures. (*A*) The entire chip is 75 mm long and 25 mm wide. The microchamber in the chip is 27 mm × 3 mm × 0.7 mm (l × w × h). (*B*) A U-shaped microstructure (scale bar = 100 μm); (*C*) the staggered U-shaped microstructure array. A total of 56 U-shaped microstructures are in a microchamber. The scale bar is 500 μm.

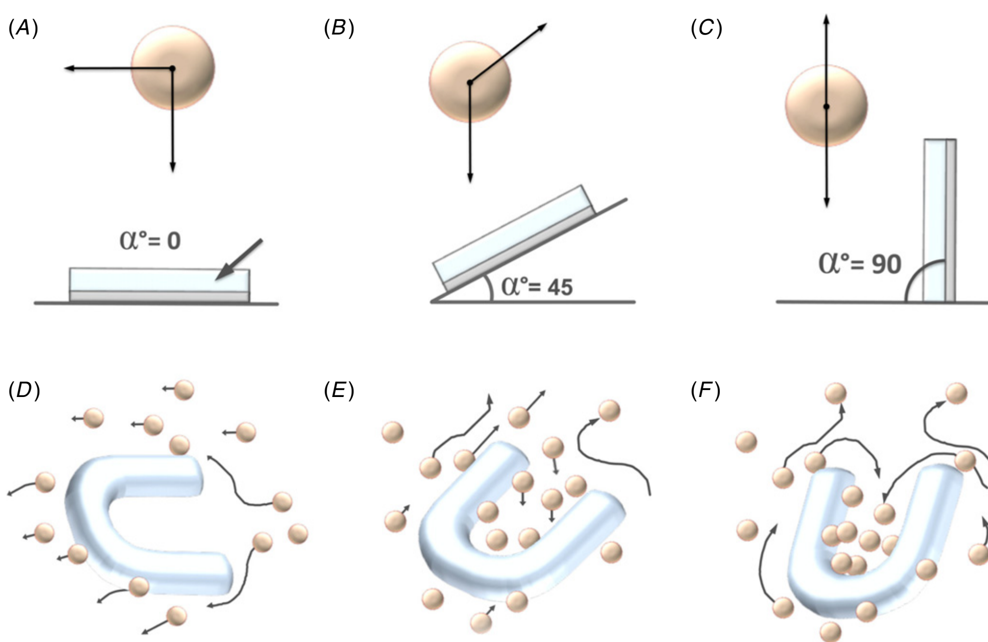

Figure 3. Cell movement in the microfluidic chip in the presence of gravity at different angles. (*A*) Horizontal chip for 0°, (*B*) tilted chip for 45°, and (*C*) vertical chip for 90°.The force exerted on cells and the resulting movement of the cells in the microfluidic chip at the angles of 0° (*D*), 45° (*E*), and 90° (*F*).

exposure light source. The optimal exposure time for PEG-DA photopolymerization was typically between 4 and 6 s. The U microstructures generated through this strategy are shown in figures 2(*B*)–(*C*). Following the optimal fabrication process, the PEG-DA microstructures could be maintained in culture medium for at least two weeks.

3.2. Cell trapping and MCS formation

This study set the chip at horizontal ($\alpha = 0°$), tilted ($\alpha = 45°$), and vertical ($\alpha = 90°$) positions, and the schematic diagrams of microfluidic chip setups are shown in figures 2(*A*)–(*C*). At $\alpha = 0°$, the dominant force is primarily the flow force. Cells were eluted out following the main stream of the flow field. Only a small percentage of the cells can get into the inner area of U-shaped microstructure (figure 3(*D*)). Because the area right above each U-shaped microstructures has relatively low flow rates, we reason that cells may be pulled down into the U microstructure by gravity if the chip is set at an inclination angle. This study tested two inclination angles for their effects on the capture efficiency of the chip. As shown in figure 4(*A*), cell accumulation increases over time at $\alpha = 90°$. On the other

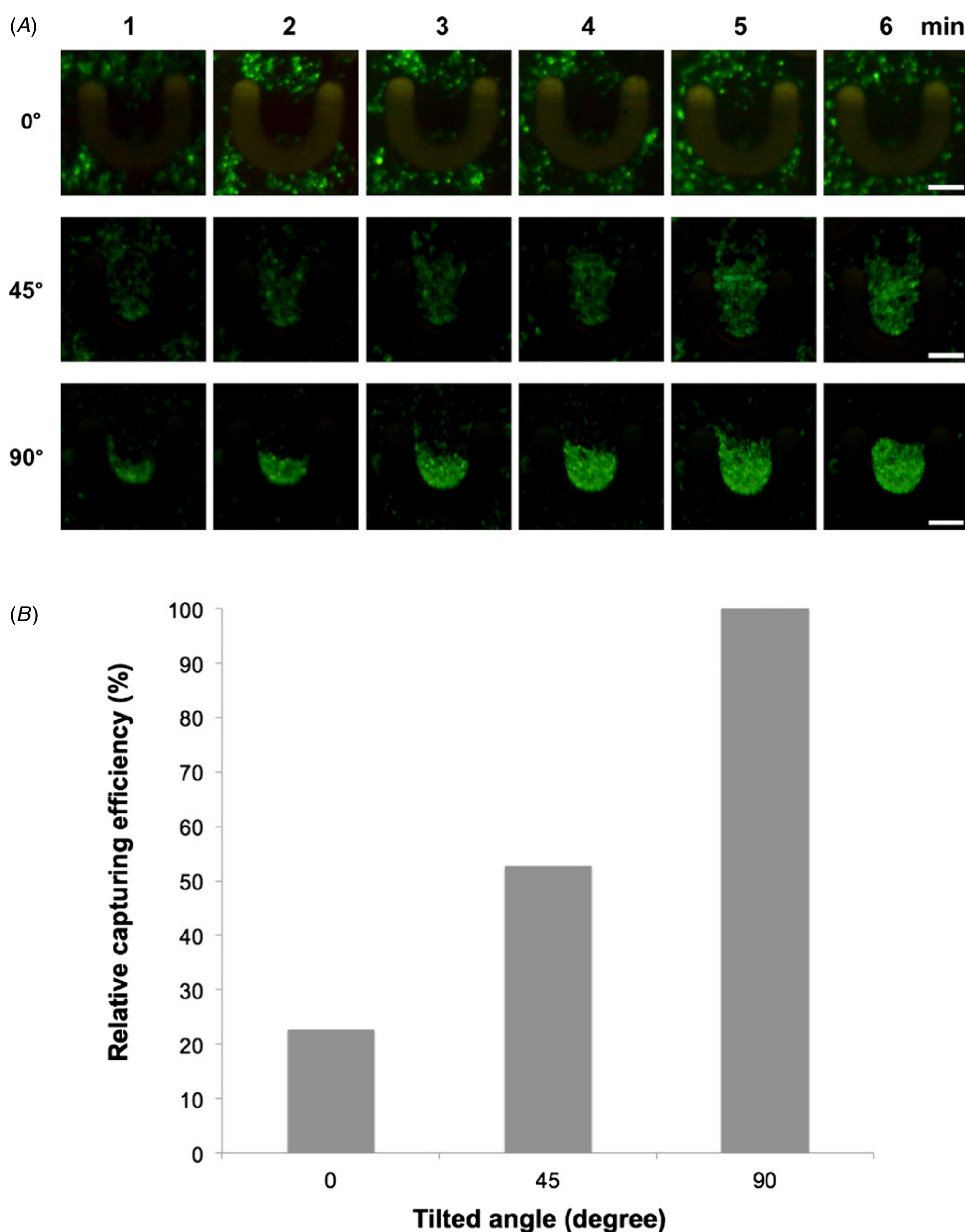

Figure 4. Cell capturing efficiency depending on different tilting angles. (*A*) Cell capturing at different angles from 1 to 6 min. (*B*) Cell capturing efficiency at the angles of 0°, 45°, and 90° after performing capturing for 6 min. HepG2 cells are labeled with CMFDA fluorescence dye. The scale bar is 100 μm.

hand, the cells scatter outside the U microstructures when the chip was maintained in the horizontal position. At $\alpha = 45°$, the trapped cells covered the entire pocket area of microstructures while at $\alpha = 90°$, cells accumulated at the bottom of U structures, presumably due to a higher gravity effect than at $\alpha = 45°$ (figures 3(*D*) and (*E*)). According to the result of capture efficiency shown in figure 4(*B*), for trapping enough cells in each U structure and improving the cell aggregation velocity and compactness, all subsequent studies were conducted with a chip in a vertical position and when the U structures were filled to nearly two-thirds full. The cell aggregates could be detected after one-day incubation and MCS with a higher degree of compactness could be observed after several days of culture. Figure 5 shows the result of the entire process from cell trapping to MCS formation. An online video recording the cell trapping process is provided as supplementary data (available from stacks.iop.org/BF/6/015009/mmedia).

3.3. Cell metabolic activity assay for doxorubicin treatment

Doxorubicin is a potent chemotherapeutic agent that functions as a DNA intercalator and topoisomerase inhibitor. This study utilized doxorubicin as a model to test whether HepG2 MCS formed on the U-structure microfluidic chip could be used in drug screening. The HepG2 MCS were formed on chip, perfused with culture medium with or without doxorubicin at a flow rate of 1.5 μl min^{-1} for 5 days, and the metabolic activity of the MCS was determined using the Alarma Blue

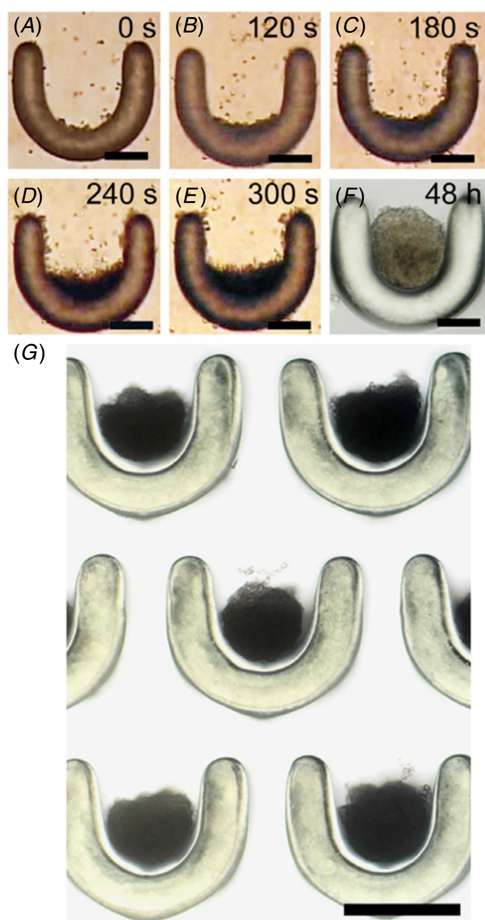

Figure 5. Cell trapping and multicellular spheroid formation at different time points. (*A*)–(*F*) Cell trapping in the U microstructure at 0, 2, 3, 4, 5 min and 48 h; the scale bar is 100 μm. (*G*) Multicellular spheroid formation cultured in the U-shaped microstructure array. The scale bar is 250 μm.

reduction assay. Unlike HepG2 monolayer cells, which are highly susceptible to 3.0 μg ml^{-1} doxorubicin and were killed in two days (data not shown), HepG2 MCS formed on the microfluidic chip were mostly compact under the same treatment. Even when increasing doxorubicin concentration to 48 and 96 μg ml^{-1} to treat MCS for five days, the metabolic activity of the MCS remains about the same as that before treatment. Although the metabolic activity of MCS treated with high concentrations of doxorubicin was somewhat lower than that without drug treatment, the results still clearly show that cells in MCS are more resistant to doxorubicin than cells in monolayer culture, consistent with previous findings [33, 50, 51] (figure 6).

3.4. Heterotypic MCS generation

This study demonstrates two strategies to form heterotypic MCS with the U-structure microfluidic chip. When HepG2 cells (red) and Balb/c 3T3 fibroblasts (green) are loaded simultaneously, they form a MCS with the two types of cell distributed randomly (figure 7(*A*)). On the other hand, if the two cell types were loaded sequentially, a MCS with a discrete two-layer morphology, exemplified as a red fluorescent MCS covered by a layer of green fluorescent cells, shown in figure 7(*B*), could be formed. Both the thickness of the fibroblast layer and the size of the HepG2 MCS could be controlled by adjusting the cell trapping time.

4. Discussion

The use of microfluidic systems to culture cells in 3D constructs has provided scientists many new capabilities to address difficult biomedical questions [18]. Several challenges to realize a convenient to use microfluidic system for 3D cell culture remain to be overcome, and how to provide efficient mass transfer for cells is one of the major issues. The use of the high permeability of hydrogels in microfluidic systems can potentially solve the problem and hydrogels were thus tested in various studies [48, 52–54]. Small molecular weight nutrients and metabolic wastes can diffuse across the 100 μm of PEG-DA structures within several minutes providing an efficient mass exchange.

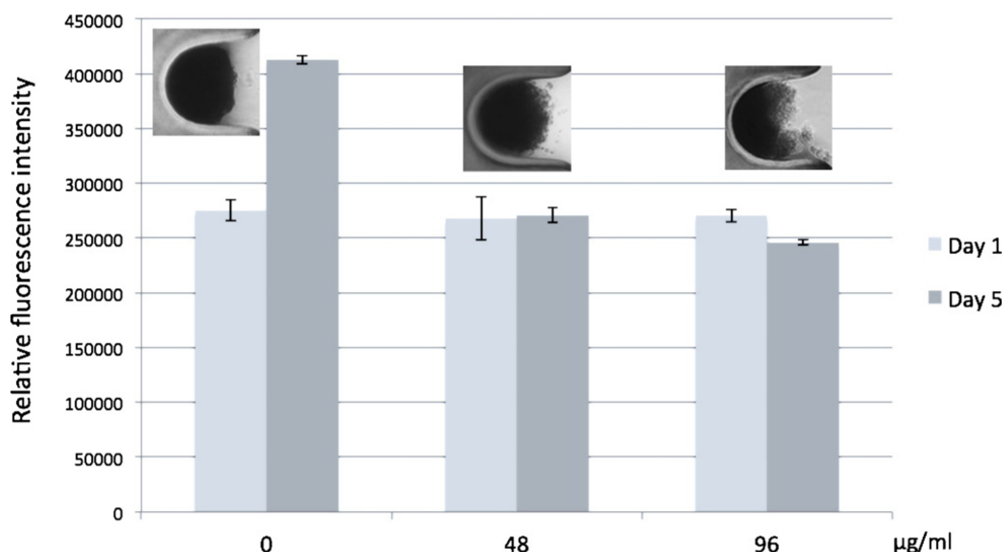

Figure 6. Doxorubicin treatment of the multicellular spheroids formed in the microfluidic chip with U-shaped microstructures.

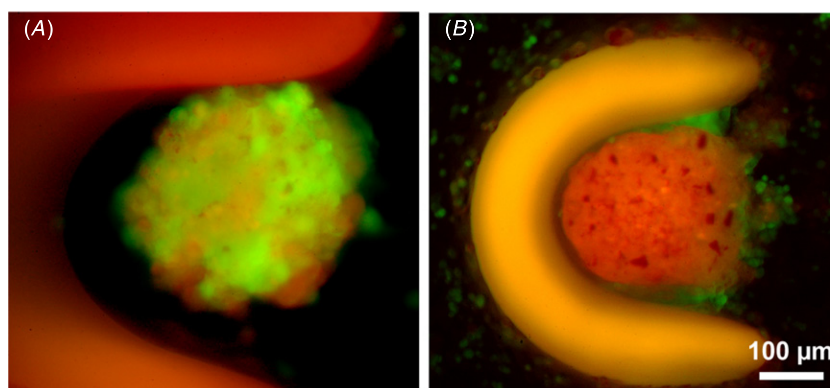

Figure 7. Formation of heterotypic spheroids in the U-shaped hydrogel-integrated microfluidic system. (*A*) Loading a mixture of HepG2 cells (red) and Balb/3T3 fibroblasts (green) to form MCS with random distribution of the two cells. (*B*) Sequential loading of HepG2 cells (red) and Balb/3T3 fibroblasts (green) to form a two-cell type co-culture system. The scale bar is 100 μm.

In addition to their high permeability, the use of hydrogels in conjunction with *in situ* photolithography technique to form microstructures on chip offers many advantages [43–49, 55]. The chip assembly process is simple after the hydrogel microstructure is fabricated and the structures can be maintained easily. Different hydrogel structures can be generated rapidly by changing photomask designs. The light source for the photopolymerization of the hydrogel can be a mercury lamp from a microscope that is easily accessible, allowing the fabrication process to be conducted in most life science laboratories.

The U-shaped microstructure developed in this study can produce a large number of homogeneous MCS and thus the device can be exploited to improve poor size control and labor-intensive drawbacks of previous MCS formation methods. Various sizes of MCS can be fabricated easily by designing U microstructures of different magnitudes or adjusting cell-trapping parameters such as cell density and loading period. In addition, cell capture efficiency also affects the size of MCS formed in the U microstructures. Factors affecting capture efficiency include size, shape and density of the cells, the viscosity of culture medium, flow rate and distance between each U structure. We have estimated that around 80% of the cells loaded into the microfluidic chip can be trapped into the U microstructure array under optimal conditions.

It has been recently reported that 3D cell culture closely resembles *in vivo* tumor and exhibits a drug resistance profile dramatically different from that of the 2D monolayer culture [56, 57]. Our results again show a dramatic difference in the response to doxorubicin treatment between monolayer cells and MCS, implying the importance of 3D culture systems in tumor drug screening. The device can be adopted for high-throughput combinatorial drug assays by integrating with other functional chips such as a gradient generator [58]. In addition, heterotypic spheroids, which show even higher resemblance to living tissues than homotypic spheroids [59–61], can be generated in this device to provide a model for studies such as cell attachment and migration, cell or spheroid fusions, angiogenesis and cell–cell interactions.

To further improve the versatility of the U-shaped structures, the hydrogel can be modified with functional entities prior to fabrication into the desired structures. For example, biodegradable hydrogels may be used to release the complex MCS formed in the chip. Cell-encapsulating or drug-containing hydrogels can provide stimuli from the structures in a temporally- and spatially-controlled manner for paracrine and cell–microenvironment studies. Enzyme-linked hydrogels can act as sensors to measure the quantity of specific substrates. Together, we believe that the integration of multi-functional hydrogels into the microfluidic chip can help this 3D culture system to construct organoids more related to complex tissues found in the human body to provide more reliable drug screening assays.

5. Conclusion

This study developed a novel PEG-based, simple-to-fabricate, U-shaped micro-scale growth chamber, which allows cell trapping, MCS forming and culturing in the presence of a continuous perfusion flow to achieve long-term 3D cell culture. The designed microstructures can effectively control the size uniformity of MCS, and provide a high diffusion rate and reduced shear stress to MCS. Conventional drug assays are permitted in this microfluidic system and heterotypic spheroid formation can be conducted easily. We believe that the chip developed in this study can provide a useful platform for many MCS-based studies and applications in the future.

Acknowledgments

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