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Development of novel, simple and low–cost microfluidic platform for supporting 3D dynamic cell culture

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Abstract. Drug testing *in vitro* cell culture models more accurately would be of significant value to the medical field and pharmaceutical industry. To achieve this goal, microfluidic cell culture platforms are created and improved for modeling the native cell microenvironment because they can precisely reconstruct *in vivo* cellular behavior. In this study, a 3D low-cost microfluidic device is used to compare the difference between the static and dynamic environment in 3D cell culture. Cells were seeded in the microfluidic device, and to produce the fluidic flow, the pump was used with the set speed of 0.045 ml/min. In 3D cell culture, the viability of cells was monitored by size growth of the spheroids for 7 days. All systems were designed and optimized without leakage of the medium. In the results, the 3D dynamic condition showed a faster increase in size than in the static condition. Overall, the study was prepared for microfluidic platforms with low-cost and simple settings. Moreover, the usage of 3D microfluidic for cell culture *in vitro* that can mimic *in vivo* returned favorable results that were expected for drug testing in the future.

Keywords: 3D cancer spheroid, microfluidics, laser engraving, dynamic cell culture.

Classification numbers: 2.4.3, 2.7.1.

1. INTRODUCTION

Drug development is essential in numerous fields such as improving disease treatment, promoting the progress of fundamental and applied sciences [1]. However, the process of drug development is a long complex and highly-cost series through sequential steps [2]. Initially, researchers need to identify pharmacological agents by screening candidates. Then in the preclinical stage, the candidate was put into an *in vitro* cell culture model to calculate the therapeutic dosages and ranges of safety. To further evaluate these indices as well as consider the effects on cells, drug compounds were tested on the animal body before starting human

clinical trials steps. However, more than half of the drugs entered into clinical trials fail largely because of the different environment between *in vitro* cell culture and human body, that lead the leakage sufficient safety and effect of therapy indicators [1]. And the failure is a major cause of the cost and time-consuming of the drug testing process. Therefore, to solve this problem, one of the technologies expected to increase the success rates with accommodating better precision is to improve the cell testing condition or cell culture environment.

In the early 19th century, cell culture was introduced with the collecting of cells from several animal organs and the tissues were maintained in lymph fluid or plasma medium. The benefits of the 2D culture method are easy to control a single well-defined cell type and simplify the manipulation of large quantities of cells, so it became a highly common method for maintaining and examining various types of cells over the 20th century [3]. However, while 2D culture is beneficial, it cannot always exactly represent the conditions that cells contact and react to other molecules in an organism. The difficulty of 2D cell culture *in vitro* is that it cannot capture correctly interactions between different cell types, extrapolating to *in vivo* concentrations. Furthermore, it also is hard to extrapolate from perturbed pathways or biomarkers *in vitro* to adverse effects *in vivo* [4].

Adequate cell growth and cell differentiation depend on basal culture conditions. Currently, there are various techniques for 3D cell culture. It can be generally split into four kinds: suspension, hydrogels, paper-based culture, and fiber scaffolds. Several previous studies on 3D culture have shown different results of cells in morphology, viability, differentiation, and response to stimuli [5, 6]. In an attempt to improve the success rates, developing systems that better imitate *in vivo* circumstances was looked for, researchers are increasingly studying a 3D cell culture platform that allows cells to grow three-directionally [7]. The increase in morphology, stimuli reaction, and proliferation rates were demonstrated under 3D static and dynamic culture conditions [8]. In both static and dynamic 3D environments, the cell is allowed to select an orientation to optimize its polarity and change in reaction to a stimulus [9].

Currently, there are various techniques for 3D cell culture. To mimic *in vivo* environment, cancer spheroids developed in the dynamic platform. The dynamic cell culture was approached in various methods, such as spinner flasks [10], stirred systems [11], perfusion bioreactors [12], and also microfluidic systems [6]. Although spinner flasks and stirred systems supply for a homogeneous delivery of nutrients in the bioreactor chamber, the limitation of mass transport into a 3D cell-scaffold construct was shown. In contrast, perfusion bioreactors overcome this drawback by forcing the fluid to actively pass through the scaffold. Moreover, they can be used to control the mechanical environment by fluid shear forces or hydrostatic pressure [13]. However, these systems often have limitations and drawbacks. Most of the systems lack the flexibility to scaffold size and stiffness, in addition, cell culture conditions are time-consuming, expensive, and the results may not be reproducible [14].

Another approach to entering flow to the cell culture system that is expected to improve the disadvantages of the above methods is microfluidic devices. One of the first studies of microfluidic channels application in adherent cell culture was performed by Tilles *et al.* [15]. The system had also been applied to integrate with label-free biosensors [16], label-Free DNA detection [17]. Besides, the microfluidic devices were improved to the optimization of pluripotent stem cell growth and differentiation [18]. It also applied to study the formation of neutrophil extracellular traps [2] or to follow progress in tumor cells [19]. In drug development, Weltin *et al.* used a microfluidic system for identifying change and rehabilitation impacts of cellular metabolism produced by the addition of substances to the medium [20].

This study will fabricate a cheap and simple microfluidic device and examine the usage of the simple microfluidic system in cell culture and compare the cell growth. The produce will contribute to the drug development process with a novel, simple and low–cost method for highly accurate drug testing.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Materials

A polymethyl methacrylate (PMMA) FS sheet with a thickness of 2 mm, 3 mm, and 5 mm from Fusheng (Taiwan). 99.5 % of ethanol is absolute from Cemaco Company (Viet Nam). Isopropanol (IPA) solution of Scharlau (Spain). Silicon patches were bought from HuancTeng Rubber Company in China, polyvinyl chloride (PVC) decal were from PhucNamKhanh Company in Viet Nam. Polyethylene glycol diacrylate (PEGDA), propidium iodide (PI), polydimethylsiloxane (PDMS) were bought from Sigma-Aldrich in the USA.

2.1.2. Devices

Cutting and engraving laser fiber machine of Laser Top (Viet Nam) that has a capacity of 50 W. CO_2 laser cutting machine 4060 50 W (China). Hydraulic heat press machine with a capacity of 15 tons (Viet Nam). Malvern Zetasizer Nano ZS90 (Canada) particle size analyzer. The cover glass was purchased from Jiang Huida Medical Instruments Co., Ltd in China and 3-trimethoxysilyl propyl methacrylate (TMSPMA) was from Sigma-Aldrich in the USA.

2.1.3. Cell culture

HepG2 was gotten from ATCC, trypsin-EDTA 0.25 %, trypsin neutralizer solution, Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco in the USA; phosphate buffered saline (PBS), fetal bovine serum (FBS), sodium hydroxide (NaOH), polyethylene glycol diacrylate (PEGDA) were bought from Sigma-Aldrich in the USA.

2.2. Methods

2.2.1. Creating a microfluidic device

The low-cost microfluidic device is made of polymethyl methacrylate (PMMA) and manufactured using a laser etching process. All components of the device are designed by CorelDraw 2019 software (Corel Corporation, Canada) and manufactured by laser cutting. Equipment has been tested and optimized to produce the most convenient and low-cost model in terms of quality, aesthetics, and functionality. The process manufacture of this device for 3D cell culture was demonstrated in Figure 1. Except the 2^{nd} and 4^{th} layers are silicon and PVC decal, all layers were made from PMMA 2 mm sheet by cutting and engraving a laser fiber machine of Laser Top. The silicon layer has 1 mm wide grooves that functioned as fluidic channels. The fluidic channels on the left and right of the silicon piece pass through the cell culture chamber. The cell culture chamber is a circular well with a size of 13 mm x 2 mm. The channel was connected to a set of inlet and outlet chambers (Figure 2).

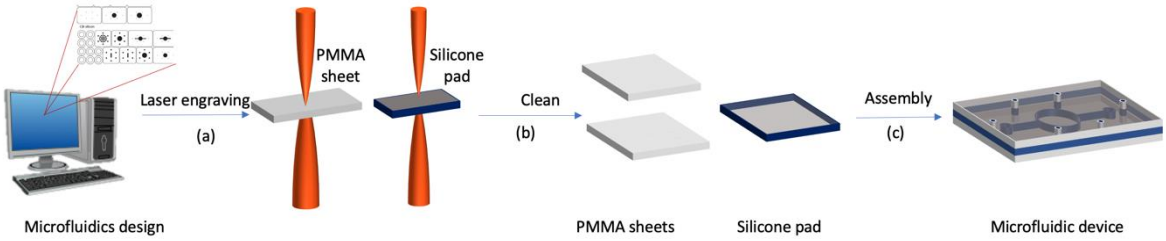

Figure 1. Process manufacture of microfluidic devices in 4 steps: Design, laser engraving, clean, and assembly. The CorelDraw 2019 software was used to create a model of the chip. The design was taken to Laser Shop to engrave by using a laser fiber machine. Microfluidic device's parts were then clean and assemble to form a device.

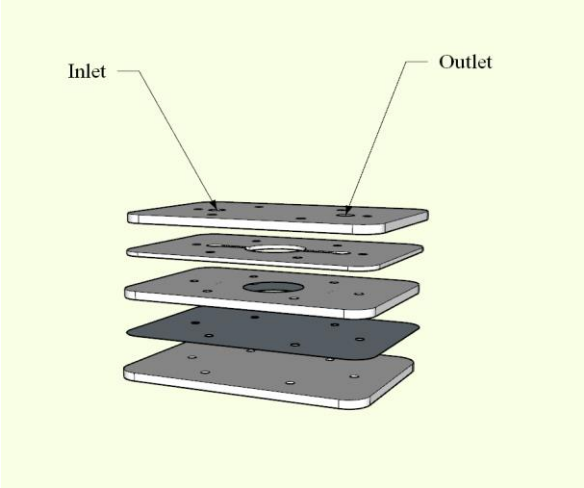

Figure 2. Schematic of platforms images with layer by layer. PMMA pads are thicker because they are 2 mm in height. The 2nd layer is a 1 mm piece of silicone and the thinnest layer is a PVC decal in the 4th layer.

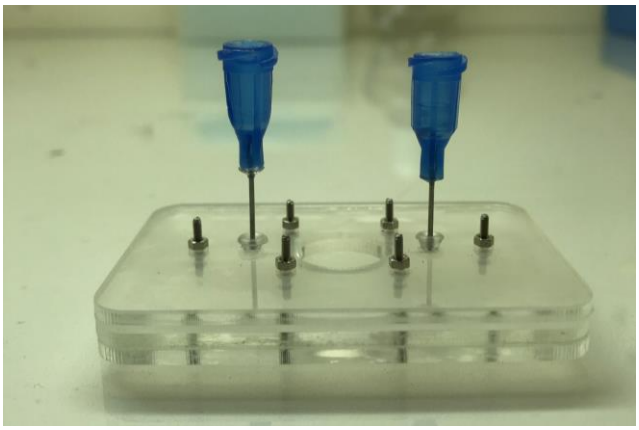

Figure 3. Microfluidic platform attached with inlet and outlet.

Before the two layers are stacked, PMMA sheets and silicone patches were sonicated for 10 minutes in ethanol as a sterilizing and washing process. After the microwell process is

completed, the microwell system is placed in the culture chamber in a microfluidic device. Two flow pipes are attached to the inlet and outlet of the unit. Finally, the device was assembled as shown in Figure 3.

Cover glass was placed in 10 % NaOH overnight and before being dried at 70 ^{o}C ambient temperature, glass slides were washed with distilled water, ethanol 70 %, and ethanol 100 %. Glass slides were stacked on a beaker and wetted by 2 ml of TMSPMA overnight. Distilled water, ethanol 70 %, and ethanol 100 % were used to rinse coated glass slides. The glass slides were stored at 37 ^{o}C. Hydrogel mixture liquid was repaired for polymerization that includes 89 % PBS, 10 % PEGDA, and 1 % PI. Then 10 µl of the solution was dropped on the PDMS stamp and put the treated cover glass on this. The polymerization is initiated thermally by UV-irradiation for 2 minutes.

2.2.2. Investigation of leaking

To test the leaking of the system, a colored aqueous solution is allowed to flow through the system. The flow depends on the pressure difference at the inlet and the outlet like Figure 4(A). To enhance the leaking inspection, a pump system has been fitted to the inlet of the unit as shown in Figure 4(B). The flow rate was adjusted from 0.02 ml/s to 0.1 ml/s in Figure 4(B).

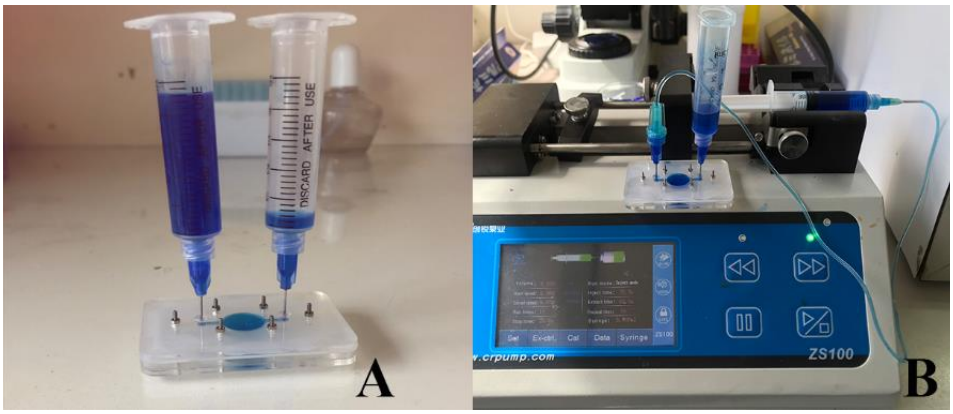

Figure 4. Leaking test of the device. The solution flow depends on pressure difference at the inlet and the outlet (A) and electric pump (B). The maximum speed was 0.1 ml/s, set up in the machine.

2.2.3. Cell seeding and 3D cell culture

The HepG2 were cultured and maintained in a DMEM medium supplemented with 10 % FBS, 1 % PI at 37 °C in a 5 % CO_2 humidified incubator. After gentle washing with PBS, trypsin-EDTA 0.25 % was added to separate cells from the flask. After the remnant substances were pipetted off, the new medium was added.

For microfluidic cultures, the device was placed at UV light at 37 ^{o}C overnight before injecting the medium. Before cell seeding, the microenvironment was clean by PBS. Then, the cell solution was prepared with a concentration of 105 cells/ml and pumped into the inlet of the device. HepG2 cells followed the flow and went through the culture chamber to fill up the bottom of the microwells. After seeding, the microfluidic device was kept stable for 5 minutes for cells deposited inside the microwell, and excess cells in devices were removed by cleaning with DMEM. Cells were cultured in microfluidic at 37 ^{o}C in a 5 % CO_2 incubator for 7 days for spheroid growth, and viability. The setting flow speech of the media was 0.045 mL/min during

cultivation. For static 3D cell culture, instead of waiting 5 minutes, the medium is added to the inlet and outlet sides with equal solution levels after cell seeding. For 2D cell culture, cells were seeded directly into the culture chamber, and growth was observed for 7 days. The medium of all was changed after 24 hours.

Cell spheroids were imaged under a microscope. The measuring of the spheroid's size was started from the 3rd day after cell seeding by using ImageJ software.

3. RESULTS AND DISCUSSION

3.1. Microfluidic platform characterization

The objective of this study was to fabricate suitable microfluidic devices for the cultivation of cell spheroids. In the microfluidic devices, cells were immobilized and cultivated on a cover glass for 2D cell culture and microwell for 3D cell culture. All of them were placed in an incubator for 7 days with a temperature of 37 ^{o}C. In the 3D cell culture model, the inlet of the dynamic medium is connected to a syringe of medium solution and an electric pump. The flow rate is set on the pump at 0.045 ml/min. Medium from the inlet will follow the channel to flow through the cell culture chamber and go to the outlet of the device which is fitted with a tube containing 15 ml capacity. In a stationary environment, according to the pressure balance in the liquid, two syringes containing the medium are attached to the two inlets of the device. Both tubes contain 1 ml of medium to equilibrate and ensure that the medium in the microfluidic culture device is not completely evaporated after 24 hours. In dynamic and static 2D cell cultures, cells are cultured on a circular glass surface placed in the instrument. The culture system is designed to be similar to 3D cultures. All systems were designed and optimized without leakage of the medium to ensure cell growth as shown in Figure 5.

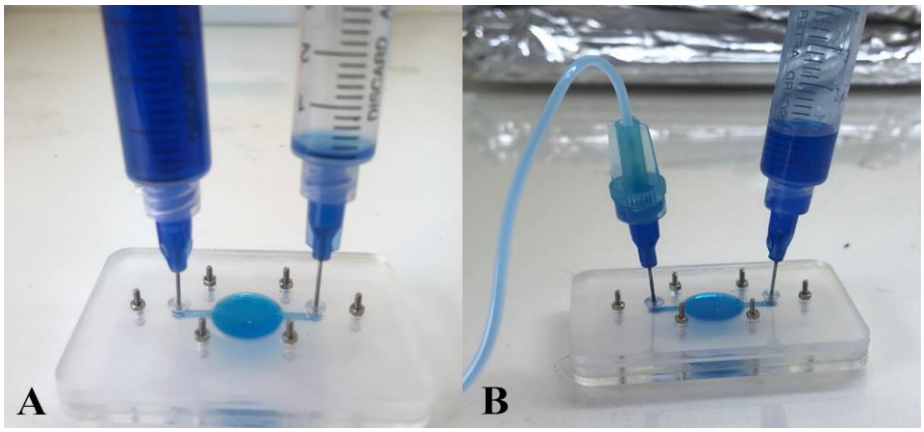

Figure 5. Leaking examination of microfluidic devices. (A) Platform leaking test with DAPI dye, (B) the system runs with syringe pump at maximum velocity of 0.1 ml/s.

3.2. 3D cell culture

After seeding for 5 minutes, cells were deposited inside and outside the microwell. The process of monitoring the cell growth in the 3D model kept only cells in the microwell, cells outside this one was taken out by washing the device with DMEM.

To compare the morphology and growth properties of cells in 3D static and dynamic conditions, cells were dissociated into single cells and seeded at the density of 10^5 cells/ml per cell culture chamber. During 48 h, the single cells formed to aggregation. And then, the size of the aggregated cell progressively increased which was observed in 5 days after (Figure 6). There was a different growth rate between the two conditions of cell culture. On the 3rd day, cells aggregated to spheroids with the same diameter of 40 μm ± 2 μm. The differences in the size of spheroids started appearing on the day 4 and this distinction increased. As shown on the chart (Figure 6) after the 5th day, the cell spheroid's size in a dynamic culture grew to 250 μm ± 10 μm, 6.25 times more than the 3rd day. While in a static system, the diameter of the spheroid increased to 180 μm ± 9 μm, only 4.5 times. On the 7th day, diameters were 230 μm ± 10 μm and 340 μm ± 15 μm in static and dynamic culture, respectively.

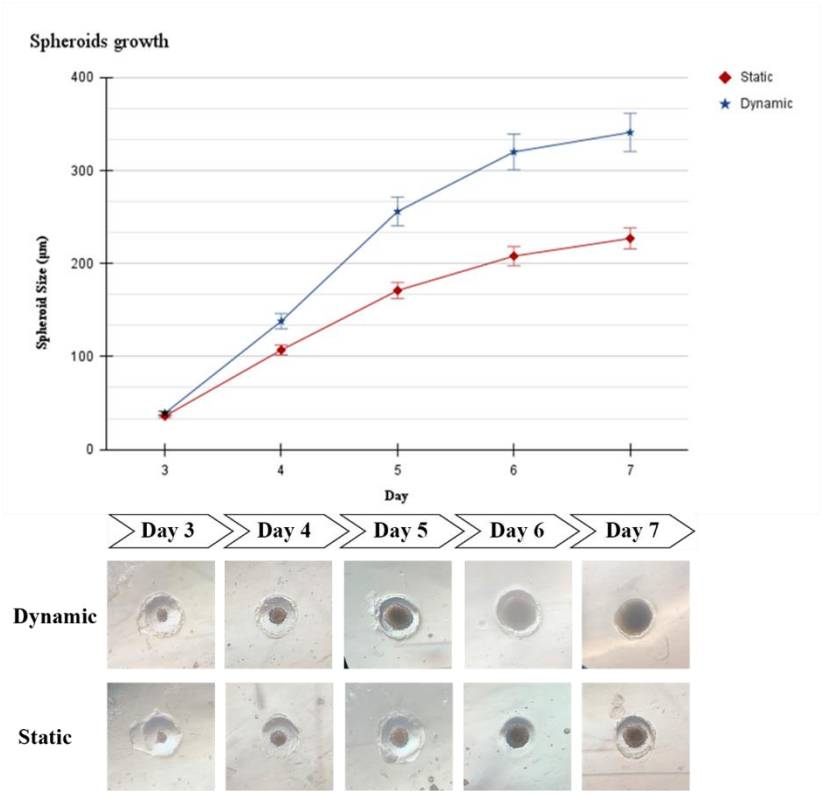

Figure 6. Timeline of cancer spheroids development in size in static 3D cell culture and dynamic 3D cell culture.

This microfluidic device has been shown to have cell growth and vitality. The distinct differences were not observed during 2D culture because cells grew as a monolayer. However, in 3D culture, all cells aggregated into spheroids and were larger than 150 μm in size after day 4. *In vitro* tumors with a diameter of over 150 μm had been verified to mimic similarly to *in vivo* tumors with diffusion limitation to various particulars [6]. The aggregates formed in the 3D dynamic culture were demonstrated, showing extensive agglomeration, with a much faster degree than in 3D static cultures. The disruption of aggregation depends on flow velocity and exposure time [21].

Furthermore, during 7 days, the size of spheroids was observed with the bigger diameter, the slower growth rate in both types of 3D cell cultures. During 7 days, the size of spheroids was observed with the bigger diameter, the slower the growth rate. From day 3 to day 5, diameter increased by more than 200 μm in a dynamic culture. However, on day 7, the measured size of the sphere only increased by approximately 100 μm. The growth rate in stationary culture was slower but from day 3 to day 5 there was a sharp increase in the size of about 140 μm from 40 μm to 180 μm. And on the 7^{th} day, the growth rate started to slow down, rising only approximately 60 μm. The explanation for this phenomenon is that high cell activities like metabolism and proliferation mainly occurred in the outer curved surface of the spheroids [22].

4. CONCLUSIONS

As mentioned earlier, the main advantage of microfluidic culture is minimizing the number of chemicals and cells required to form spheroids to perform drug testing. This experiment was focused on fabricating and optimizing device geometry for cell culture protocols in cheap, simple, and single chamber microfluidic devices. The device consists of one cell culture chamber, a pair of inlet-outlet.

In conclusion, this study was successful in the preparation of microfluidic platforms with low-cost and simple settings. In addition, the usage of 3D microfluidic to mimic *in vivo* returned favorable results. The cancer spheroids grow in 3D dynamic cell culture faster than those in 3D static cell culture. In the future, this system will be suitable for drug testing. It is hoped that it can be further optimized to improve the drug's effects on cancerous tumors.

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Credit authorship contribution statement. Pham Thu Uyen: Methodology, Experimental Process, Formal analysis. Tran Dinh Thiet: Experimental Process, Formal analysis. Nguyen Thanh Duong: Supervision, Experimental Process, Formal analysis, Investigation.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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