

Droplet-Based Microfluidic System for Tumor Cell Encapsulation in Alginate Beads and Anticancer Drug Testing

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I. Introduction

Multicellular tumor spheroids (MCTS) have recently received a great deal of attention in cancer research and have been applied to the evaluation of anticancer drugs [1]. This is because the 3D multicellular aggregates more accurately simulate the tumor microenvironment in vivo [2]. The intercellular adhesion in tumor spheroids is thought to give multicellular resistance, reducing the cytotoxic effects of anticancer drug in MCTS [3]. and this information is valuable for identifying correct clinical dosing [4]. There are several techniques to form tumor spheroids but most of them lack the ability to precisely control the number of cells in each spheroid and do not allow testing on the growth platform. Here we present a droplet-based microfluidic system for formation of uniform alginate gel beads, trapping alginate beads in microsieve structures, and characterization of the interaction between tumor spheroids and anticancer drug, all in one integrated platform. In our microfluidic device, breast tumor cells (LCC6/Her2) were used as a model system to validate the platform. After tumor spheroid formation, serial concentrations of doxorubicin (Dox) were used to investigate the dose-effect relationship between Dox, cell proliferation, and cell death. Compared with that of the monolayer culture cells, small tumor spheroids have higher resistance to anticancer drugs than monolayer culture cells which is valuable for identifying correct clinical dosing. This microfluidic platform requires minimal

culture reagents and mechanical labor which will significantly reduce the cost and time and realize the high throughput cell cytotoxicity assessment. In future it may replace labor intensive, microtiter-plate based screening platforms currently implemented in the laboratory.

II. RESEARCH DESIGNS AND METHODS

2.1 Microfluidic Device Design

The microfluidic chips used for droplet based alginate beads formation and self-assembly tumor spheroids culture are shown in Figure 1. The microchannels in the droplet formation chip were 113 μm in depth and 400, 100 μm in width respectively at the main section and the nozzle. In the cell culture chip there are two chambers. Each chamber contains fourteen micro-sieves for alginate droplet trapping. Each sieve is semicircular with an inner diameter of 300 μm , with two apertures (48 μm width). The channels are fabricated using soft lithography [5]. The bonded PDMS chambers maintain a sterile culture environment. We generated a non-adherent trapping array by alginate beads. Approximately one hundred LCC6/Her2 cells were encapsulated in each alginate bead and cultured to form tumor spheroids in the

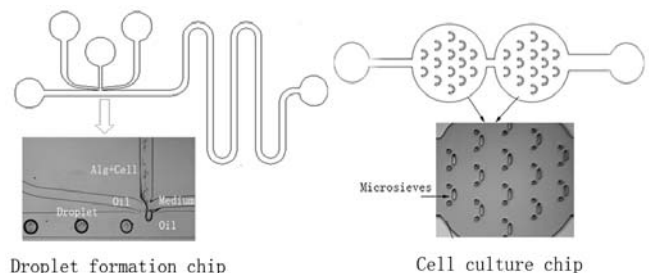


Fig.1 . Schematic of the droplet formation and cell culture chips

device. Continuous media perfusion was controlled by syringe pump after cell trapping. Drug injection can then be conducted using the same channels.

2.2 Gel Droplet formation and trapping

Gel-based techniques for approximating 3-D cell culture display enhanced cell biological activities and more biologically relevant to living organisms. Polymers such as calcium alginate have shown promising results for cell trapping and culture. In this study, hydrogel bead formation was generated using a T-junction in a microfluidic channel. Figure 1 shows the formation of hydrogel-droplets one by one. First, the dispersed phase (alginate solution containing cells and culture medium) and the continuous phase (n-hexadecane with 2% span-80) were injected from two branches of the “T”. Alginate droplets were produced as a result of the shear force and interfacial tension at the fluid-fluid interface. Second, the formed alginate droplets flow downstream of the main channel and are collected in a vial through tygon tubing. Then the alginate droplets were rapidly reacted with calcium in the vial, thus cross-linking the polymer, and gelled beads containing the cells were formed. Third, the hydrogel beads were loaded to the cell culture microfluidic chip and trapped in the microsieve structures. All flow rates in this work are controlled using an 8-channel mass flow controller (Fluigent, France). The pressure above each individual external reservoir is controlled using the software interface, permitting control of the flow rate in each channel.

2.3 cell culture and drug exposure

LCC6/Her2 cells (kindly provided by M. Bally at the BC Cancer Research Centre) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cell viability was subsequently determined by trypan blue exclusion.

The cell density was controlled in the range of $\sim 1 \times 10^7$ /mL to make sure that ~ 100 cells could be encapsulated in each hydrogel bead. After loading the alginate beads onto microfluidic chip, the chips were put in 6-well plates and immersed in culture medium. The medium was continuously perfused through the microchannels using a syringe pump, and the microchip was put back into the incubator for long-term culture. The cells in alginate beads were cultured for 4 days to permit MCTS formation. After tumor spheroid formation, drug assays of the spheroids were performed immediately by loading 0, 800 and 1600 nM Dox via the same perfusion system for 2 days. In this experiment, three concentrations of drugs were treated with tumor spheroids to investigate the significant dose-effect relationship between drug and cell death. These results were also compared with that of the monolayer culture cells.

2.4 Assessment of cell viability

After toxin exposure, cell cytotoxicity was assessed using fluorescent live/dead stains, Calcein AM and ethidium homodimer-1 (EthD-1, Molecular Probes). Calcein AM is a fluorogenic esterase substrate that is hydrolyzed intracellularly to a green fluorescent product; thus green fluorescence is an indicator of live cells. EthD-1 is a high-affinity, red fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. The toxin-treated cells will be stained with the fluorescent viability dyes by syringe and incubated at room temperature for ~ 30 min. After viability staining, evaluation of the viability of the seeded cell lines after toxin exposure will be achieved by confocal microscopy (Nikon).

III. RESULTS

3.1 Droplet bead uniformity

For drug assays, homogenous droplet size is necessary for statistical analysis. Homogenous droplet size and same cell concentration is better to generate spheroids with a narrow size distribution for tumor formation. The results of trapped droplet uniformity are shown in Figure 2. The average of droplet size and standard deviation are 247 μm and 32.4.

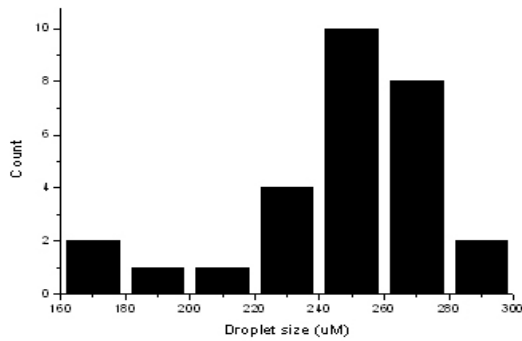


Fig.2. the distribution of droplet size in microchip

3.2 Morphology of Tumor spheroids

Fig.3. shows the morphology of LCC6/Her2 cells cultured for 1, 4, 6 days and the fluorescent images of tumor spheroids treated with 0, 800 nM, 1600 nM Dox for 2 days. It observed that tumor cells gradually formed aggregates after 4 days culture in alginate beads. Morphology observation also showed the damage of cell membrane increased with the increase of drug treatment for 2 days. The fluorescent images revealed the proportion and distribution of live and dead cells inside the alginate beads. The live cells were mainly localized to the periphery of the cell spheroid and the most of the dead cells were in the center (Figure 3c). With the increase of drug concentration, the viability of cells decreased.

3.3 Cytotoxicity in Drug-Treated Tumor Spheroids

The cytotoxicity was assessed by the cell proliferation rate and survival rate. The cell proliferation rate was calculated using the following formula:

$$\text{Proliferation rate} = (N_4 - N_1) / N_1 \text{ or } (N_6 - N_4) / N_4$$

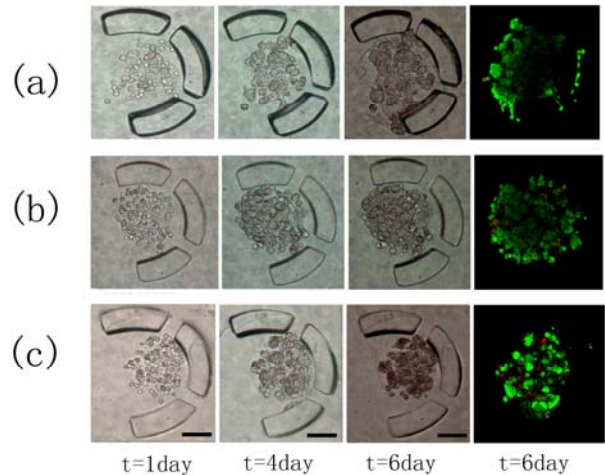


Fig.3. The morphology of LCC6/Her-2 Tumor Spheroid cultured for 1, 4, 6 days and the fluorescent images of tumor spheroids treated with different concentrations of drug for 2 days. (a-c) Control group cells and Cells treated with 800, 1600 nM drug respectively. Scale bar: 100 μm .

Where N_1 , N_4 and N_6 are the number of cells cultured for one, four and six days respectively. Figure 4 showed that the cell proliferation of three groups were almost 0.66 before drug treatment. After two days Dox incubation, the cell proliferation decreased markedly. With the increase of drug concentration from 800nm to 1600 nm, the cell proliferation rate was decreased from 0.133 to 0.027. It indicated that at high concentration of drug almost no cancer cell proliferated which means that anticancer drugs showed the strongest cytotoxicity in the tumor spheroids. The cell survival rate was calculated using the following formula:

$$\text{Survival Rate} = N_G / (N_G + N_R)$$

Where N_G , N_R are the number of cells showing green and red fluorescence respectively after staining with calcein AM and ethidium homodimer-1. Figure 5 shows that in both monolayer culture cell and small tumor spheroids, the cell survival rate decreased with the increase of drug concentration. But the cell survival rate in monolayer culture was much lower than that in small tumor spheroids at high concentration of doxorubicin. The cell survival rates

were 80.6%, 65.8% in small tumor spheroids, and 87.3, 26.2% in monolayer culture cells treated with Dox at 800 and 1600 nM for 48 h, respectively. This has demonstrated that tumor spheroids were more

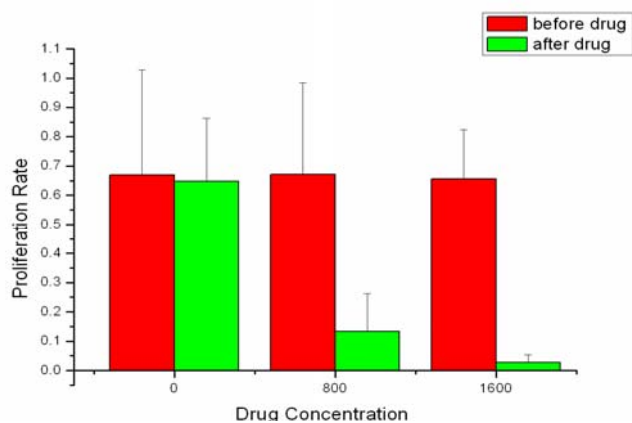


Fig.4. Effects of drug concentration on the cell proliferation rate of three groups of tumor spheroids. A group of control and two groups of tumor spheroids treated with 800, 1600 nM Dox respectively were investigated.

drug-resistant than cells in monolayer culture.

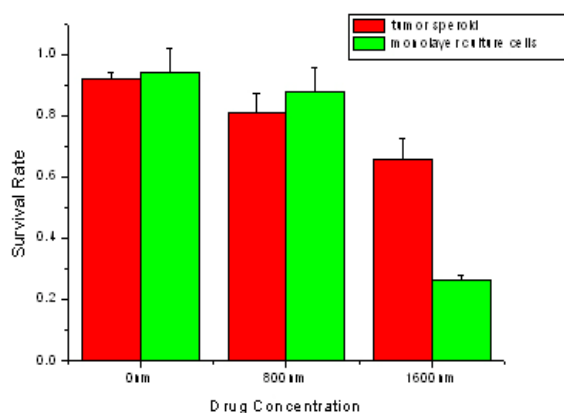


Fig.5. Effects of Dox concentration on the cell survival rate of small tumor spheroids and monolayer culture cells. A group of control and two groups of cells, treated with 800, 1600 nM Dox respectively, were investigated.

IV. Conclusion

In this work, a new method to self-assemble arrays of 3D tumor spheroids in alginate beads on droplet based microfluidic system was demonstrated. The microfluidic chip was capable of forming uniform

alginate beads for cell encapsulation. We also demonstrated 3D tumor spheroid formation after long-term cell culture under continuous media perfusion and a sterile microfluidic culture environment. With this platform, anti-tumor assays can be done immediately after the spheroid formation right in the growth platform. Unlike high density microplate systems, microfluidic networks have the capability to reduce individual assay volumes to picoliters and create highly integrated channel with multifunctional possibilities. In addition, due to microfluidic systems rapid device prototyping, disposability, it may be very useful in clinical and pharmaceutical applications.

Acknowledgments

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V. References

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