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Abstract

Cell-based assays can be applied to evaluate the efficiency of anti-cancer drugs but the conventional approaches are mostly based on two-dimensional cell culture which is not able to recapitulate the tumour specificity. Here we developed a method to culture millimetre size tumour spheroids that is useful for anticancer drug studies. Agarose multi wells were obtained by casting on polymethylsiloxane (PDMS) mould, which were then used for culture of U87-MG human glioblastoma. As expected, large size tumour spheroids could be generated after 24 h incubation. Comparing to the multi-well systems made of PDMS or polyethylene glycol diacrylate (PEGDA), agarose multi wells are clearly advantageous due to the hydrophobic surface and the high permeability of agarose. After culture for 10 days, the tumour spheroids in agarose wells stopped to grow and the further increase of the cell seeding density had no effect on the final size of the spheroids. To study the anticancer drug effect, combretastatin A-4 (CA4) was added on day 2 or day 4, showing clear effects on the tumour spheroids and cell viability. More importantly, the live/dead cell staining images suggested that an earlier drug treatment was more efficient to prohibit the tumour spheroid growth.

Keywords: Agarose, Tumour spheroids, Drug assay

Introduction

Cancer metastasis is a leading cause of mortality in the world for which tremendous efforts are now devoted to anti-cancer drug development [1-3]. Before metastasis, a primary tumour was formed at the anatomical site where tumour progression started to yield a cancerous mass. Therefore, one strategy in drug discovery is to against the tumour progression. However, the most previously used cell based assays for cancer drug studies are based two-dimensional (2D) culture which does not take properly into account the three dimensional (3D) characteristics of tumour formation *in vivo* [4, 5]. Suspension culture is frequently used for multicellular spheroid formation but it is not easy to control the size consistency of the spheroids and to handle them for systematic studies [6, 7]. Suspension culture using spinning flask or rotating bioreactors can improve the size uniformity of the spheroids but undesired shear stress can be introduced during the spheroid formation. Culture with hanging drops can also improve the size uniformity and overcome the problem of mechanical disturbance. This technique is however time-consuming and not easy to perform drug tests during spheroid progression. More recently 2D and 3D patterned substrates are used to support spheroid formation [8, 9]. In particular, various micro-well arrays are proposed for 3D cell aggregation, depending on microfabrication techniques [10, 11]. This approach allows controlling cell population in each spheroid as well as high-throughput screening. However, the available systems are mostly appropriate for small size spheroid formation, which cannot efficiently recapture the spheroid complexity of solid tumours.

To facilitate the diffusion of nutrients, drugs and other factors into the spheroid area, it is also important to form the 3D assays with the materials of high permeability [12]. In this work, we developed a multi-well assay form by agarose moulding. We first describe our technique and then compare the formation of spheroid culture in agarose, PDMS and PEGDA wells. We then show the relevance of agarose wells for the formation of millimetre size tumour spheroids. Finally, the results of anticancer drug test performed on the same platform on the progressing tumours are reported and the potentials of the proposed assay form for other applications are also briefly discussed.

1. Experimental

2.1 Fabrication of multi-well array

Figure 1 shows the schematic process flow of multi-well array fabrication. A flat PDMS film of 2 mm thickness was prepared by casting a mixture of PDMS pre-polymer and cross-linker (GE RTV 615) at ratio of 10:1 on a silicon wafer. After curing at 80°C for 1 h, the PDMS layer was peeled-off and an array of holes was created on the PDMS layer using a computer numeric controlled (CNC) milling machine and a biopsy punch of 2 mm diameter. Afterward, the PDMS layer was exposed in trimethylchlorosilane (TMCS, Sigma, France) vapour during 1 min for surface anti-sticking treatment. This PDMS layer with holes was then used as master to cast a second layer of PDMS with posts. After curing at 80°C in oven for 2 h, the second PDMS layer was peeled off.

The agarose multi-well arrays were produced by using the second layer PDMS as mold. A solution of 5% agarose (Fisher Scientific, France) in DI water was mixed at 120°C for 5 min and then immediately casted on the PDMS mold. After cooling down to room temperature and waiting for 10 min, a solid agarose layer was obtained by gelatinization. Then, the agarose layer was gently removed from the PDMS mold, resulting in a multi-well device for spheroid culture.

For comparison, PDMS multi-well arrays were prepared similarly, while PEGDA multi-well arrays was produced by UV assisted casting with a PEGDA solution (Mw=250, Sigma) mixed with 1v/v% Irgacure 2959 (Ciba Specialty Chemicals, France) as photo-initiator.

Prior to cell seeding, all multi-well devices were sterilized by UV irradiation and then equilibrated with culture medium for at least 15 min.

2.2 Cell culture and seeding

Human glioblastoma cell line U87-MG was prepared in Dulbecco's Modified Eagle Medium (DMEM) completed with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2

supplementation for 3-4 days. After proliferated to confluence, cells were detached by Trypsin at 37 °C for 3 min and centrifuged before re-suspended in a culture medium at a density of 5×10^6 or 10^7 cells/ml. The multi-well devices were placed in a culture dish and each well of the device was then filled with 50 μl cell containing medium. After 15 min incubation for cells settling inside the wells, 2 ml more medium was added gently around the device. Finally, the culture dish with the multi-well device was transferred into incubator. After 24 h incubation, tumour spheroids can be observed in each well.

2.3 Drug test and live/dead assay

After culture for 1 day or 3 days, new culture medium contained with 10^{-7} M anti-cancer drug Combretastatin A4 (CA4) was added to the wells. After another 2 days, apoptotic cells can be observed around cancer spheroids. Cell viability was studied by live/dead assay. Briefly, PBS solution with 2 μM of Calcein AM and 2μM EthD-1 was used to change the medium for live and dead cell staining, respectively. After 30 min incubation at 37°C and 5% CO_2, fresh PBS was used to wash out the residual dyes for 2 times. Stained cell spheroids were transferred on a glass slide for fluorescence observation with an inverted optical microscope (Zeiss, Axiovert 200) equipped with a digital CCD camera (Evolution QEI). Cell viability was calculated by live cells number divided by total cells number.

2. Results and discussion

3.1 Materials comparisons of multi-wells

Multi-wells made of PDMS, PEGDA and hydrogels could be easily obtained by casting or moulding and used for the formation of either tumour spheroids or embryoid bodies [13-15]. However, their performances for the spheroid formation should be different due to difference in cell compatibility and material permeability. Figure 2 shows microphotographs of tumour spheroids

formed in multi-wells made of PDMS, PEGDA and agarose. After 2 days, spheroid shaped cell aggregates could be found in all three types of wells. As can be observed, no cell attached on the agarose well but a large number of cells attached on PDMS both inside and outside the well (indicated by red arrows), due to the different surface properties. More interestingly, the spheroid in PDMS well is likely composed by three smaller aggregates, suggesting strong attachment to the surface of each of them during the growth phase. In PEGDA well, a flatten spheroid was observed, together with some spread cells on the board of the well. And due to the worse optical transparency of PEGDA, it's difficult to observe the tumour spheroids clearly by microscope, as shown as Fig.2(c). Agarose is a typical hydrogel, low-cost, transparent and not toxic to cells [16] but cells can poorly adhere on the surface of agarose so that cell aggregation can easily occur. More interestingly, agarose is permeable to gas and small biomolecules, which facilitate diffusion of nutrients, drug and other cell factors. Therefore, agarose was chosen as an ideal material of multi-wells for tumour spheroid generation and spheroid culture under physiological conditions.

3.2 Generation and growth of tumour spheroids in agarose wells

Figure 3 shows the growth of tumour spheroid in agarose wells. As expected, tumour cells aggregated first in the well and then formed compact spheroids. Afterwards, the spheroid grew until day 10 due to cell proliferation. After 10 days, the size of the spheroid remained almost the same (about 1.4 mm). Interestingly, the size of the spheroid decreases during the first 4 days. This decrease can probably be attributed to the sharp change from single cells to spheroid aggregation and the compact of cells due to strong cell-cell interaction. It is also interesting to note that increasing the cell seeding density has no effect on the final size of the spheroid which was always about 1.4 mm, due probably to the limitation of diffusion length. In fact, when the size of the spheroid becomes too large, there will be no sufficient nutrient diffused to the center area. The spheroid can be considered as a self-organized entity in which the proliferation of cells should be strongly affected or regulated

by cell-cell interaction in the whole entity. More details analyses have to be developed for further studies.

3.3 Combretastatin A-4 effects on tumour spheroids growth

To study the anticancer drug effect, CA4 was added in the medium after culture for 3 days, which is a stilbene inhibits tubulin polymerization at the colchicine-binding site of tubulin and high cytotoxic against a variety of human cancer cells including some multidrug resistant cancer cell lines [17, 18]. As expected, the spheroid stopped to grow and some of the cells were detached from the spheroid (Fig.4). The live/dead cell staining images showed that in the culture medium with the drug, many cells were dead after three days drug treatment, whereas no matter on day 5 or day 7, almost all cells were alive in the medium without CA4. But for the different time of addition drug, there was difference on drug effect. From the live/dead staining results, for drug adding on day 2, there were dead cells on both the area next to the edge and the central area of the spheroid after three days, but for drug adding on day 4, there were fewer dead cells compared to earlier drug adding, which were mainly distributed only on the edge area of the spheroid on day 7. In the process of the tumour spheroid growth, beginning from single separated cells, cells keep aggregation until day 4, shown as Figure 3. It means that when the drug was added on day 2, the tumour spheroid was not organization enough, which will let drug penetrate into the center of spheroid to effect on the cells, whereas for drug added on day 4, the tumour sphere was compact and became a self-organized entity, it will be more difficult for drug to effect in the center of spheroid. It has also been reported that these cell–cell contacts and ensuing communication in closely packed 3D cell structures can influence the response of cells to drugs [19, 20].

4. Conclusion

We have demonstrated a simple but reliable technique for the fabrication of agarose multi-wells and their usefulness in anti-cancer drug tests. In 2 mm diameter agarose wells, 1.4 mm diameter U87-MG tumour spheroids can be formed and cultured for many days. Interestingly, the size of the tumour spheroids does not change with the further increase of seeding cell density. Anti-cancer drug CA4 added at different stages of tumour spheroid formation have different drug effect. More systematic studies are expected for more clear and mechanistic understanding of spheroid formation and anti-cancer drug effect on tumours.

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Figure caption

Figure 1. Schematic fabrication process flow of agarose multi-well arrays and tumour spheroid formation.

Figure 2. Microphotographs of tumour spheroids in multi-wells made of PDMS, PEGDA and agarose, respectively. Scale bar: 0.5 mm.

Figure 3. (a) Tumour spheroid formation in an agarose well at different time (D1, D2 to D13 represent the first, the second and the thirteenth day during culture); scale bar: 0.5 mm; (b) Size variation of the spheroid as a function of time.

Figure 4. Effect of anticancer drug CA4 on the morphology of tumour spheroids and cell viability. (a) Morphology change of the tumour spheroid with or without CA4 adding for drug added at day 2 and day 4 respectively. Scale bar: 0.5 mm. (b) Bright field and live/dead cell staining microphotographs of the spheroid after three days (at day 5 and day 7) with or without CA4 treatment added at day 2 and day 4 respectively. Scale bar: 0.5 mm.

Figure 1

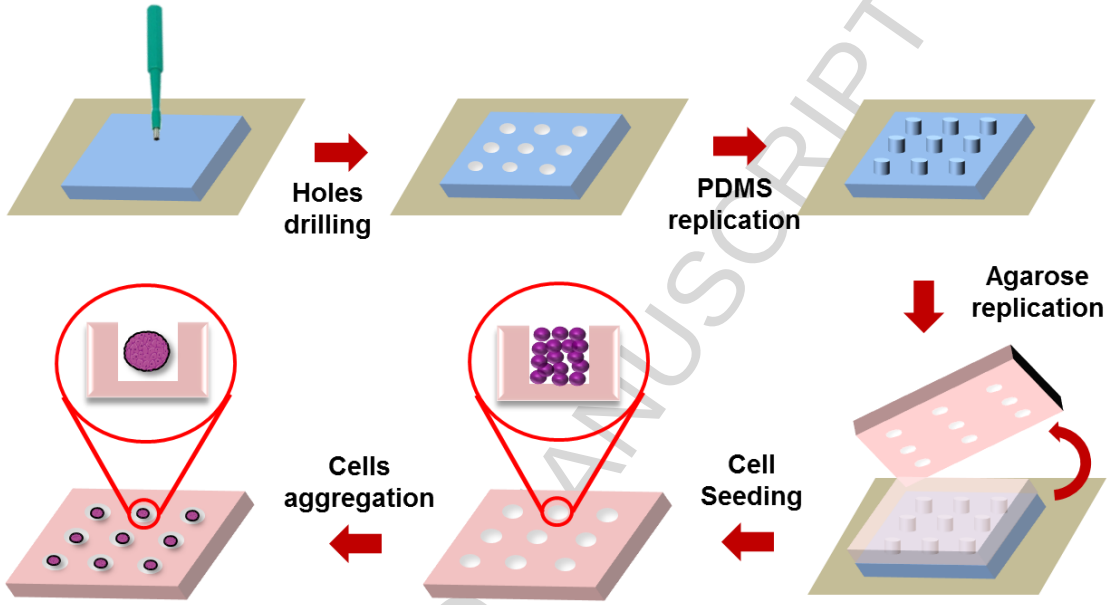


Figure 2

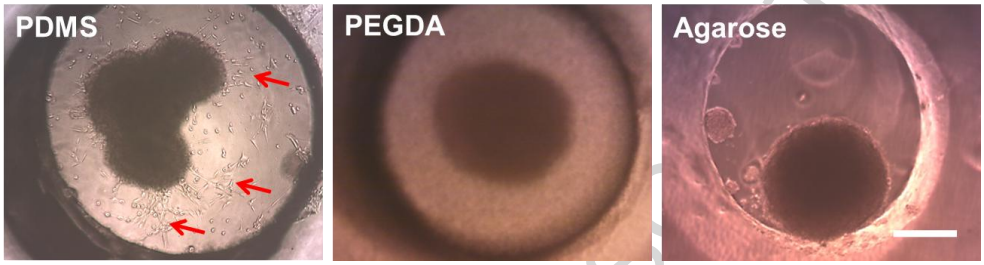


Figure 3

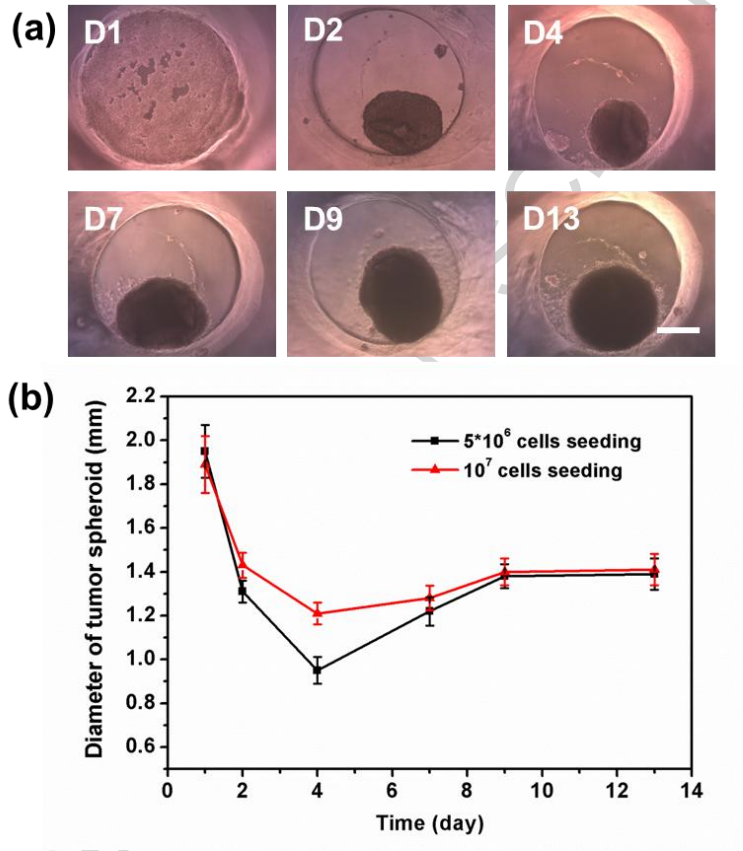
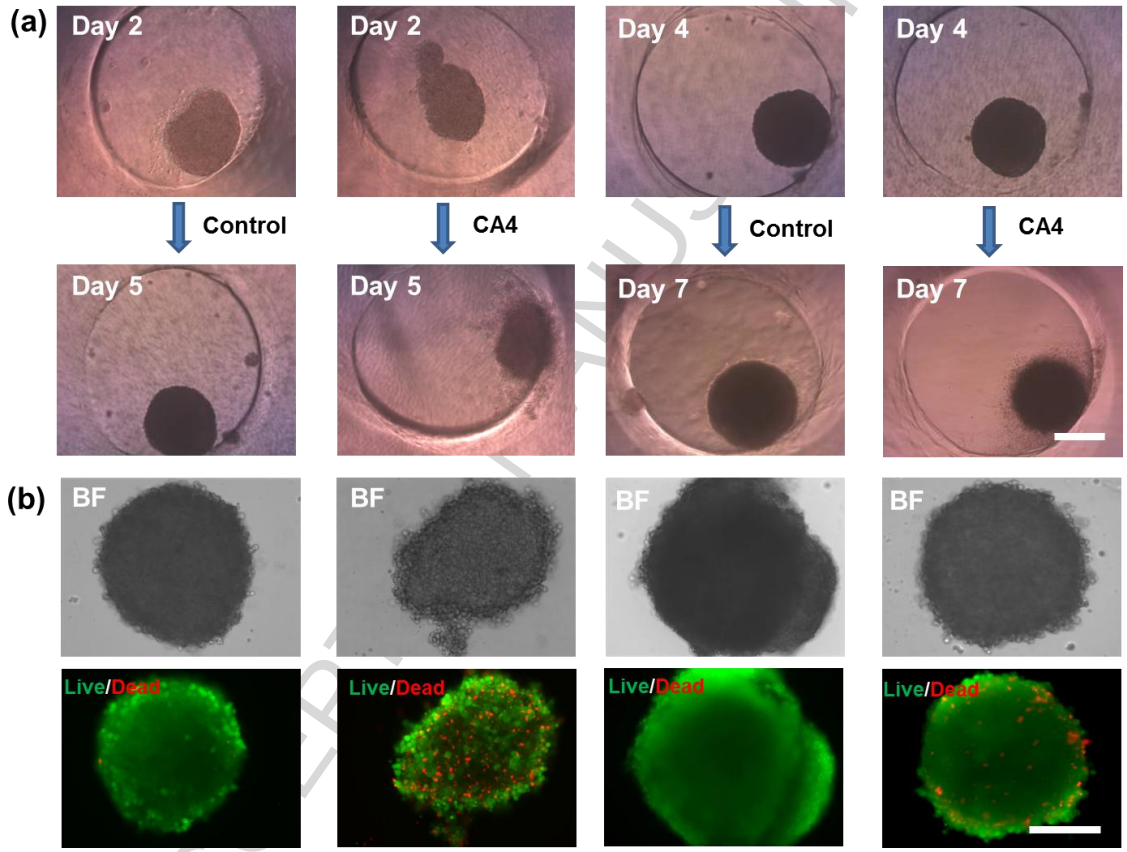
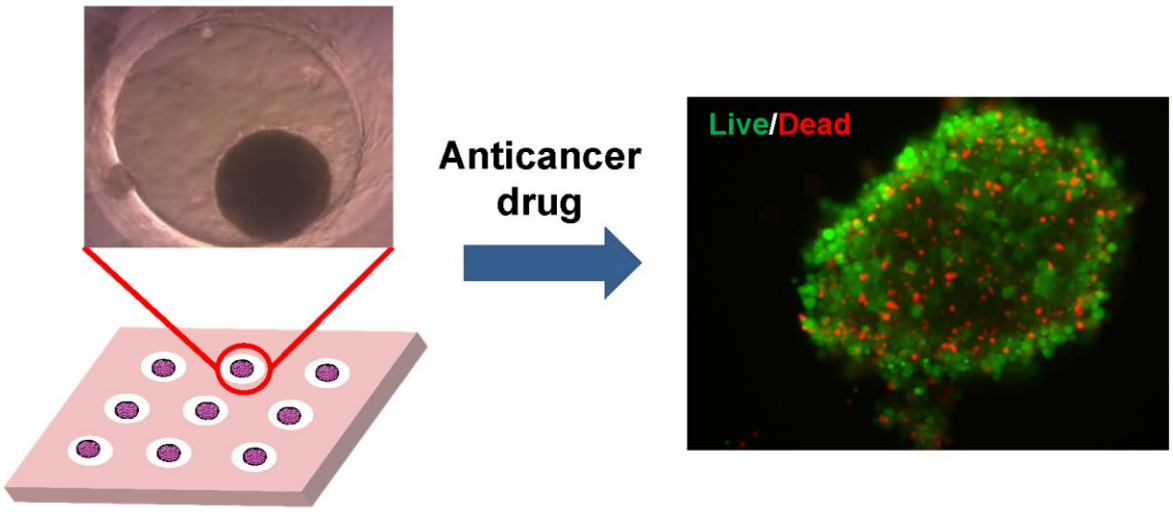


Figure 4





Graphical abstract

Highlights

- A facile technique to fabricate agarose multi-wells is developed.
- Millimeter size tumor spheroids could be generated in 2 mm diameter agarose wells.
- The size of tumour spheroids did not increase after a certainly limit.
- The effect of anticancer drug is more efficient at early days