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Agarose multi-wells for tumour spheroid formation and anti-cancer drug test

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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.

1. 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 against the tumour progression. However, the most previously used cell based assays for cancer drug studies are based on 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.

2. Experimental

2.1. Fabrication of multi-well array

Fig. 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 a ratio of

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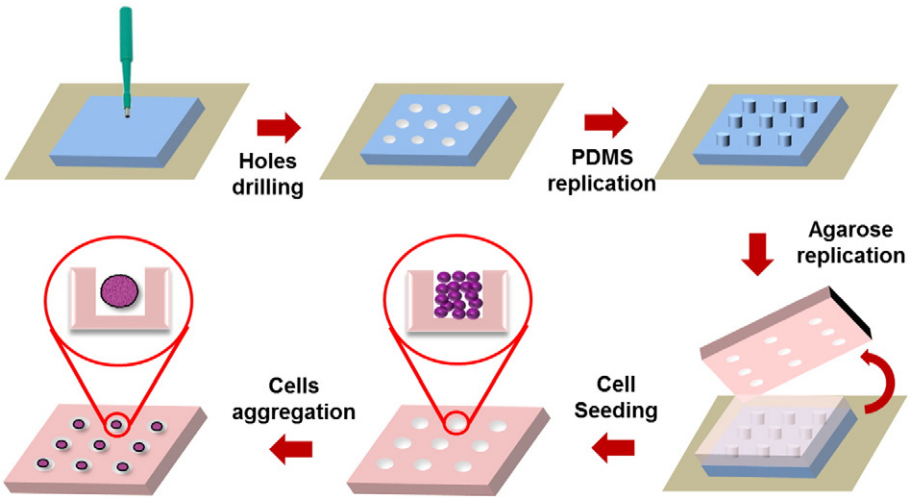

Fig. 1. Schematic fabrication process flow of agarose multi-well arrays and tumour spheroid formation.

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 an 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 mould. 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 mould. 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 mould, resulting in a multi-well device for spheroid culture.

For comparison, PDMS multi-well arrays were prepared similarly, while PEGDA multi-well arrays were produced by UV assisted casting with a PEGDA solution (Mw = 250, Sigma) mixed with 1 v/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 a 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 a 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 an 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, a new culture medium containing 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 2 μM 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 cell number divided by total cell number.

3. Results and discussion

3.1. Material 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. Fig. 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 of 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 a microscope, as shown as Fig. 2(b). Agarose is a typical hydrogel, low-cost, transparent and not toxic to cells [15] 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

Fig. 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