Tumor Inside a Pearl Drop

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Liquid marbles are exploited in this study as efficient miniature bioreactors inoculated with Hep G2 (hepatocellular carcinoma) cells to culture cell spheroids in vitro. This method successfully yields numerous cell spheroids, via a facile and straight-forward route, thanks to the constrained internal structure of liquid marbles, along with their non-adhesive shell. Since in vitro cultured cancer cell spheroids are of paramount importance in biomedical science and applications, this novel approach may open new avenues in the field of cancer research.

Cancer cell spheroids (CCSs) cultured in vitro are believed to be capable of reflecting the in vivo physiology of tumors more realistically than two dimensional cell cultures.[1] Therefore, in vitro cultured CCSs are frequently exploited as models[2] to study the physiology of tumors, and several methods have been hitherto developed to culture them.[3] Among all the available methods in the literature,[3] the hanging drop method[4] used by Kelm et al.[5] is the most common method over others, yielding a single homogeneous multicellular CCS in each drop, with similar architecture alike in vivo tumors. In a typical hanging-drop spheroid-culture experiment,[5] 20–50 μL cell suspensions is dispensed into each of the 60 wells of a MicroWell MiniTray, followed by inversion of the tray which leads to 60 hanging drops. The first inherent experimental challenge of the method, however, appears right here; since in order to form a sufficiently stable hanging drop for easy experimental handling, the volume of the drop must be carefully controlled, because the stability of a hanging drop is governed by the balance of the liquid surface tension force, that holds the drop around the opening of each well, and the gravitational force of the drop, that pulls it off the well. Although efficient in yielding spheroids, the hanging drop technique is well known to be laborious, and requires a great deal of manipulation.

Herein a novel approach is reported for in vitro CCSs formation; to culture cancer cell spheroid inside liquid marbles, also known as pearl drops.[6] Liquid marbles[7] were first introduced in 2001 by Aussillous and Quéré,[7a] as liquid drops covered with hydrophobic powder particles. A liquid marble can be simply made by gently rolling a drop of liquid, placed on a hydrophobic powder bed, so the hydrophobic powder particles cover the liquid surface. The as prepared liquid marble can then be regarded as a non-wetting drop of liquid that can be transferred, manipulated, or even collapsed when needed.

One of the main applications suggested for liquid marbles is to use them as miniature reactors, capable of containing chemical and biological reactions.[8–11] In this study, we show for the first time that liquid marbles can be used to contain living cells, and present how liquid marbles can be effectively exploited in in vitro CCS formation. Liquid marbles of varied size can be easily formed using a variety of readily available hydrophobic powders. In this study,chemically inert polytetrafluoroethylene (PTFE) powder was chosen as the non-adhesive material utilized in the liquid marble shell. Our recent study shows that the inert PTFE particles do not significantly change important interfacial properties of liquid marbles, such as their surface tension.

Hypothetically, there are three major factors which make liquid marbles convenient for cells to live in: 1) A porous shell allow O_2 and CO_2 exchange between cell culture medium and surrounding environment; 2) hydrophobic powder particles that forms the shell and provides a non-adhesive surface, which encourages the cells to suspend in the medium and, 3) a confined liquid core volume that promotes effective contact between cells, hence leads to better aggregation. To support the above-mentioned hypothesis, liquid marbles of varied sizes were formed.

A schematic comparison of liquid marble bioreactor versus the hanging drop method can be seen in (**Figure 1**, note that the powder particles on the liquid marble shell are not drawn). The dimensions of PTFE powder particles are shown in the micrograph (**Figure 2**); the observed PTFE powder particles sizes are in reasonable agreement with the material specification from the supplier (100 μm). The selection of this particle size is based on our previous study,[10] which showed that liquid marbles made of 100 μm PTFE particles were more stable than those made of smaller particles. In case of Hep G2 cells, cell aggregation could be clearly observed after 24 hours, while formation of numerous three dimensional cell aggregates were observed using confocal microscopy by the end of day 10, as shown in **Figure 3**.

The successful cell aggregation observed after one day and 10 days inside liquid marbles proves that our hypothesis was right - liquid marbles can indeed conveniently accommodate cells to survive and form aggregates. As was hypothesized, the confined volume of a liquid marble promotes the intimate intercellular interaction, resulting in aggregation. On the other hand, the cell-PTFE particle interactions at the liquid marble shell is biochemically discouraged by the low surface tension and anti-adhesion nature of PTFE particles.

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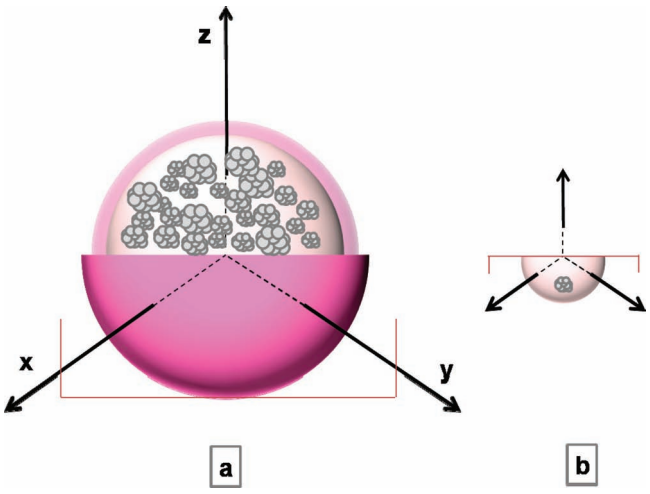

Figure 1. Schematic comparison of cancer cell spheroid (CCS) formation in; a) a liquid marble (the powder particles are not drawn), b) a hanging drop. Unlike hanging method which has a stringent droplet size limitation, liquid marbles can accommodate a larger quantity of liquid, therefore allowing formation of a large number of cell aggregates. Besides, liquid marble size can be varied by aspirating or dispensing fluid held within the marble using a micropipette, where nutrient and waste exchange is permissible. Liquid marble also allows cell aggregates to be incubated inside the marble for a long period time until spheroids are formed.

To use liquid marble for CCSs formation has several advantages: 1) Quick cell aggregates formation, as CCSs are formed in time similar to the hanging drop method. 2) The yield of CCSs formation is high, since a single liquid marble micro bio-reactor produces numerous spheroids. 3) the liquid marble method is simple and easy to operate; it does not require instrumentation other than customary cell culture equipment. 4) Human intervention is kept to a minimum; once the liquid marbles are prepared, they can be placed in an incubator until the spheroids are formed. 5) The flexible structure of liquid marbles enables the extraction, replenishment, or change of the liquid core of the liquid marble to be carried out easily, should they be needed. This significantly increases the flexibility for the manipulation of the medium in cell culture experiments. 6) The liquid marble bioreactors are cost-effective; they are much cheaper than some of the conventional bioreactors.

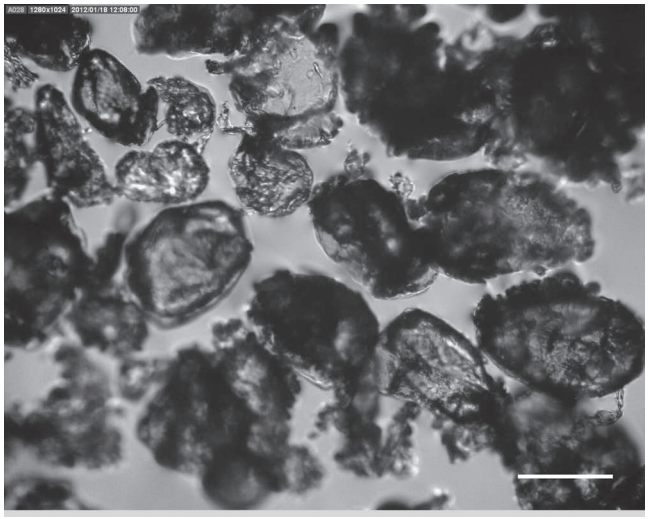

Figure 2. Optical microscopy image of PTFE powder particles on the surface of a liquid marble. (Scale bar = 100 µm).

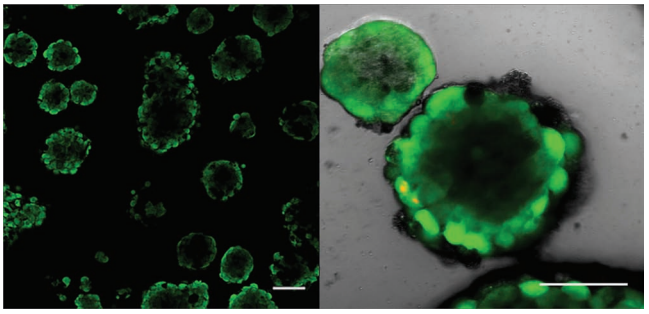

Figure 3. Hep G2 cells inside a liquid marble on day 10; viable cell aggregates stained by Calcein AM (green) can be observed. (Scale bar = 50 um). Note the formation of dark lining around the cell aggregates indicated the transition of cell aggregates into tumor spheroid. (Scale bar = 50 µm).

In this application, liquid marble micro bio-reactors also present two difficulties: 1) It is more difficult to perform an entire medium exchange and, 2) the liquid core of the marble must be evacuated to enable the microscopic monitoring of the cell growth. To overcome the first difficulty, a different medium control method was used. We added fresh medium into the liquid marbles during the 10-day experiment, instead of performing an entire medium exchange of the liquid marbles. This method worked very well; the CCSs formation did not seem to have been suppressed. To overcome the second difficulty, we think it possible to design magnetic hydrophobic powder to form the marble shell,[8d] which enables opening and closing of the north pole of the liquid marble by means of magnetic force. In situ microscopic observation would then be enabled using an upright microscopy; the liquid marble could then be closed after the observation.

The method outlined in this paper can certainly be tailored to also culture aggregates of other cell types. For instance, as embryonic cells in suspension tend to form cell aggregates similar to cancer cells. Hence, this method can be potentially used in embryonic body formations with obvious implications in tissue engineering. Overall, this study shows that liquid marbles made of inert PTFE particles can serve not only as novel platforms for formation of CCSs, but also as potential means to understand the mechanisms through which cells interact with one another, leading to the formation of cell aggregation. Encapsulation of living cells in a liquid marble can open new avenues in biomedical science and tissue engineering.

Experimental Section

In a typical CCSs formation experiment, 100–400 µL drops of medium containing Hep G2 cells (~10^4 per 100 µL) were placed in a Petri dish containing a PTFE powder bed (particle size = 100 µm, see Figure 2). The liquid drops were then gently rolled on the powder bed until they were covered completely with powder particles to form liquid marbles. This Petri dish containing the

liquid marble on powder bed was then placed in a larger Petri dish, half filled with water, to provide an atmosphere saturated with water vapour to suppress the evaporation of the medium from the liquid marble. This experimental setup eliminates the problem of water core evaporation from water marbles reported in the literature.[11] The Petri dish was then capped and placed inside an incubator, to provide the cells with suitable growth conditions. In order to monitor cell aggregation, the liquid core of a marble was extracted out of the marble using a micropipette for confocal imaging.

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