PAPER

A novel technique for the formation of embryoid bodies inside liquid marbles†

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The significant and inherent limitations associated with commonly used methods of *in vitro* embryoid body (EB) formation motivate the development of novel, facile, efficient and reproducible techniques. In this study we report the possibility of using ''liquid marbles'' as facile and efficient micro bioreactors for *in vitro* EB formation. To exploit liquid marbles as micro biological reactors, embryonic stem cells (ES cells) were inoculated into liquid marbles containing embryonic cell growth medium. Herein we show how the confined internal space of a liquid marble, along with the porous and non-adhesive properties of the highly hydrophobic liquid marble shell, can provide the necessary conditions for the formation of uniform EBs inside liquid marbles. Factors such as the powder particle size, the liquid marble volume and the cell seeding density inside each liquid marble were also investigated to evaluate the effects of varying experimental conditions on the efficiency of EB formation within a liquid marble.

Introduction

The term ''liquid marble'' was first introduced to describe an interfacial phenomenon whereby upon the contact of a small drop of liquid with hydrophobic powder particles, the drop becomes covered by a multilayer of powder particles of micro- to nano-sizes.[1,2] The powder particles encase the liquid drop, forming a powder shell which prevents the liquid drop from coming into contact with the supporting substrate, while allowing gases to freely transport across the shell. Liquid marbles also have the flexibility of a liquid drop; when small enough, they stably retain a near-spherical shape on the supporting liquid or solid surface. Whilst many studies have investigated the fundamental interfacial properties of liquid marbles,[3–6] several studies have also focused on the exploitation of liquid marbles as miniature reactors.[7–10] Tian *et al.* investigated the possibility of using liquid marbles for gas sensing applications.[7,8] Lin's group also reported some novel powder materials for making liquid marbles into controllable miniature reactors.[9,10] Recently, Arbatan *et al.* explored the use of liquid marbles to perform immunohaemotological assays[11] and to culture cancer spheroids.[12] These works clearly demonstrate the promising potential of liquid marbles for

biological and biomedical applications. The current report aims to demonstrate yet another particularly important biological application of liquid marbles – their use as platforms for the culturing of embryoid bodies (EBs) from embryonic stem cells (ES cells).

Embryonic stem cells are pluripotent cells that are directly derived from the inner cell mass of preimplantation embryos, and have the unique potential to continuously proliferate *in vitro*.[13] Thanks to their unique capability of long-term self-renewal and their ability to differentiate into a variety of specific cell lineages, stem cells are of paramount importance in regenerative tissue studies and cell-based therapies.[14] ES cells can form EBs with all three somatic germ layers (mesoderm, ectoderm and endoderm) under well defined *in vitro* conditions, and have the potential to differentiate into different types of tissues including hematopoietic,[15,16] endothelial,[17] cardiac,[18,19] and neuronal[20] tissues. An existing biological challenge is to find efficient methods to yield EBs from ES cells. In this endeavour, liquid marbles are introduced as micro bioreactors to explore the efficient production of EBs from ES cells.

ES cells can differentiate into a variety of cell lineages, but only after they aggregate and form 3D cell structures known as EBs.[21] EB formation facilitates subsequent multicellular interactions and the formed EBs have the potential to differentiate into derivatives of all three germ layers. Structurally, EBs consist of ectodermal, mesodermal and endodermal tissues, mimicking the structure of a developing embryo. Hence, EB formation is of paramount importance for the *in vitro* investigation of embryonic development, and differentiation between mouse and human ES cells.[22,23]

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Several methods are currently being used for *in vitro* EB formation.[24–26] These methods are mostly based on preventing the adhesion of ES cells to the surface of the culturing dish, hence maintaining the cells in suspension so that they can adhere to one another and form EBs. In these methods, EB formation is generally initiated either by removing the Leukemia Inhibitory Factor (LIF) from the medium, or by culturing ES cells in the absence of MEF (Mouse Embryonic Feeder) layer. The most common methods of inducing EB formation are the suspension culture method (*e.g.* culturing cells in anti-adhesive bacterial-grade dishes) and the hanging drop method.[27,28] Furthermore, forced aggregation methods have been reported; these methods include the use of a round-bottomed 96-well plate, the use of methylcellulose semisolid media and the use of bioreactors.[28–30]

The EB formation methods currently in use have some disadvantages, preventing them from producing EBs in high yield and also inhibiting quality. For instance, although the suspension technique has been successfully used to generate EBs that can differentiate into various cell types, it only provides limited control over EB size, shape and uniformity, as it relies on the accidental aggregation of ES cells. Consequently, considerable heterogeneity in size and shape will be observed. Moreover, the possibility of EB attachment to the dish, mainly arising due to defects in the surface chemistry of the culture dish, causes greater heterogeneity and may result in the loss of EBs from the suspension culture.[28–31] In contrast, culturing EBs in methylcellulose semisolid media, in which single cells tend to remain separated and isolated by the matrix of methylcellulose, enables the reproducible formation of EBs from single ES cells. The main drawbacks of this method are that the methylcellulose matrix may slow down the mass transfer of factors added during the EB formation experiment, and the handling of a semi-solid solution by pipette is also challenging. The obtained yield is often low because of the inherent instability of the prolonged single cell culture of the pluripotent stem cells. Further complication may also be encountered when isolating EBs from the hydrogel for further use.[28–32]

The hanging drop technique is the most commonly used small scale technique for EB formation. The method is based on dispensing equal numbers of ES cells into physically separated droplets of media suspended from the lid of a Petri dish, which leads to gravity induced EB formation.[23] Under normal conditions, the hanging drop method only yields a single EB from each hanging drop. Furthermore, medium exchange of the hanging drops is laborious and may disturb the conditions necessary for EB formation. The reproducibility of the size and quality of EBs formed by the hanging drop method is heavily dependent on the operator's level of skill and experience.[33]

The development of bioreactors has made EB formation more facile and controllable compared with the methods discussed above. The use of bioreactors such as the spinner flask offers the possibility for large scale production of EBs, thanks to their simple design, scalable configuration and ability to produce homogeneous culture conditions. Bioreactors also facilitate the measurement of different environmental factors such as oxygen tension, pH and medium exchange.[25] However, a significant drawback of this method is the shear stress induced by the rotation of the impellers which may damage the cells, disrupt cell–cell signalling, damage the fragile cellular components and affect the subsequent cell differentiation.[34]

The same drawbacks apply to the rotating suspension technique, in which cell suspensions are rotated on a horizontal rotating device equipped with a membrane for gas exchange, and ports for media exchange and sampling at different speeds.[35] Although the rotation culture system improves oxygen supply and enables the production of high density cultures, the method may not be suitable for assessing multiple experimental samples in parallel. Also this system requires specialized culture equipment and larger volume bioreactors, which makes this system very costly.

In this study, we report the use of liquid marbles as low-cost, high-yield and easy-to-control micro bioreactors for the formation of EBs. Factors including liquid marble shell properties, liquid marble size and the cell seeding density are investigated in order to understand their effects on the size, uniformity and quality of the embryoid bodies formed.

Experimental

Murine-derived embryonic stem cell lines (Oct4b2) which possess an Oct4-green fluorescent protein (Oct4-GFP) reporter were used, the expression of Oct4-GFP being correlated to the stem cell pluripotency. Oct4b2 cells were maintained in knockout medium (Gibco, Australia) supplemented with 20% knockout serum replacement (Gibco), 1% nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 1% Glutamax (Gibco), 1% penicillin–streptomycin (Gibco) and 1000 U ml^{-1} murine leukemia inhibitory factor (mLIF, Chemicon, Australia). Cells were seeded on a 0.1% gelatin-coated dish at 37 °C in humidified air with 5% CO_2. Cells were passaged every 2–3 days after 70–80% of confluency. In order to induce EB formation, ES cells were dissociated with Tryple^{TM} express and suspended in a medium that did not contain LIF (EB medium).

Polytetrafluoroethylene (PTFE) powder with particle sizes of 35 and 100 μm were acquired from Sigma-Aldrich. To create the liquid marbles, a powder bed was prepared inside a Petri dish and a spatula was used to gently make a curved gully at the centre of the powder bed (Fig. 5). A micropipette was then used to dispense the required volume of EB medium containing a predetermined number of cells on the powder bed. The Petri dish was then gently shaken in a circular motion so the powder particles covered the surface of the liquid drop. The Petri dish was then placed in a larger Petri dish containing sterile water and both Petri dishes were capped. The use of the second Petri dish was necessary to prevent liquid marble evaporation. The liquid marble content was then monitored

Table 1 PCR primer sequences

Primer	Primer sequences (forward-reverse) 5′→3′	NCBI accession no.	PCR product size bp
Nestin	TCTGGAAGTCAACAGAGGTGG/ACGGAGTCTTGTTCACCTGC	NM_016701.3	350
Brachyury	CATGTACTCTTTCTTGCTGG/GGTCTCGGGAAAGCAGTGGC	NM_009309.2	313
Foxa2	TGGTCACTGGGGACAAGGGAA/GCAACAACAGCAATAGAGAAC	NM_010446.2	289
β-Actin	CACCACACCTTCTACAATGAGC/TCGTAGATGGGCACAGTGTGGG	NM_007393.3	242

on days 3 and 7 of incubation, using an optical–fluorescent microscope (Nikon, Eclipse Ti), to examine the cell growth and EB formation. To prepare the samples, a micropipette was adjusted to the required volume, equal to that of the liquid marble. Once the liquid core was drawn from the liquid marble, it was gently placed inside a small Petri dish and the cells were examined using the microscope.

The EB formation efficiency inside a liquid marble (LM technique) was further evaluated and compared with the suspension culture method (LS technique) as a common traditional technique used for EB formation. In order to do this, the total number of viable cells was counted and the variation in EB size and morphology alterations were estimated during cultivation. Throughout this set of experiments, the initial cell seeding density was kept constant at 20 000 cells ml^{-1}. One set of cells were seeded onto a low attachment well-plate (cat# 351178, BD, suspension method), while another set of cells were seeded inside liquid marbles and were allowed to form EBs for 3, 7 and 10 days. In order to count single cells, EBs were dissociated by the addition of Tryple (Life Technologies Pty Ltd, Australia) for 10–15 min. Cell counting was then performed for each of the above mentioned culture techniques at different time intervals using trypan blue dye and a haemocytometer.

RNA isolation and cDNA synthesis were also carried out in another set of experiments. As for the quantitative reverse-transcription (RT) polymerase chain reaction (PCR), EBs were allowed to form inside the liquid marbles and were then harvested for RNA isolation using a RNeasy kit (QIAGEN Inc.) at days 3 and 7. They were subsequently treated with Turbo DNase (Ambion), according to the manufacturer's instructions, in order to remove any remaining genomic DNA contamination. The PCR amplification consisted of a total of 35 cycles of denaturation at 95 °C for 2 min, followed by annealing at an appropriate temperature for 30 s and extension at 72 °C for 30 s, with a first denaturation step at 95 °C for 4 min and a final extension step at 74 °C for 10 min. The names of the primers of the three germ layers, annealing temperatures and product sizes are presented in Table 1; undifferentiated ES cells were employed as negative control. The house keeping β-actin was used as an internal control.

Results and discussion

Multiple liquid marbles containing mouse embryonic cells were formed and incubated for ten days, using the liquid marble method reported previously.[12] The effect of powder particle size, liquid marble size and the cell seeding density inside the liquid marbles was investigated. The liquid cores of the liquid marbles were drawn on day 3 and day 7 in order to monitor the cell growth, cell aggregation and EB formation, using optical and fluorescent microscopy. In all cases, embryoid body formation was clearly observable on day 3. However, the shape, uniformity and number of the acquired EBs were significantly affected by altering the experimental conditions. To optimize the experimental conditions, the following experiments were carried out:

1. Monitoring the effect of powder particle size: two different particle sizes of the polytetrafluoroethylene (PTFE) powders (35 μm and 100 μm) were used to investigate the effect of powder particle size on EB formation inside liquid marbles, while the cell seeding density and the liquid marble size were kept constant. EB formation was observed on day 3 in liquid marbles made from both 35 and 100 μm PTFE powders. However, it was observed, through repeated experiments, that liquid marbles formed from 35 μm PTFE powder promoted EB formation more efficiently compared with liquid marbles made from 100 μm particles, in terms of shape and uniformity of the EBs, number of EBs formed and their surface compactness (Fig. 1). The corresponding microscopic images show a layer of aggregated but less compact cells on the surfaces of the EBs formed within liquid marbles made from 100 μm PTFE particles. The aggregated cell layer was uneven and as thick as few individual cells combined. The reason for the significant influence of the liquid marble shell particle size on EB quality is unclear and requires further study. A possible explanation, however, could be the different mechanical properties of the shell of liquid marble formed from powder particles of different particle size. Arbatan and Shen previously reported that the force required to pierce a glass slide through a layer of PTFE powder of different particle sizes over water is different.[36] This force is smaller when the particle size of the powder is smaller. It may be possible that a mechanically weaker PTFE powder layer on water could further reduce the cell adhesion, despite the fact that the powder particles all have the same chemical structure regardless of the powder particle size. On the other hand, it is well known that the focal adhesion of cells on soft substrates is irregular and unstable compared to those attached on a hard substrate.[37] From our observations, the marble shells consisting of 35 μm powder particles provided a liquid marble shell that was less adhesive to cells than the marble shells consisting of 100 μm powder
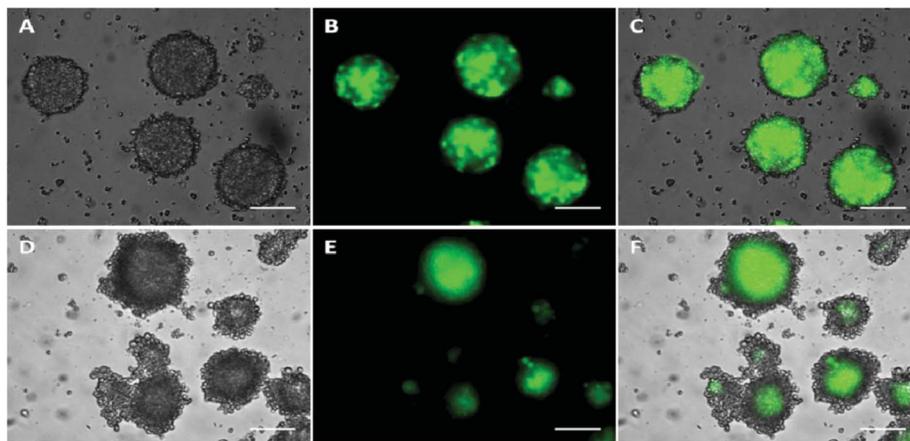
Fig. 1 The difference in morphology and shape in EBs formed in liquid marbles made using (1A–C) 35 μm PTFE powder and (1D–F) 100 μm PTFE powder, scale bar = 100 μm. 1A and 1D are bright field images, while 1B and 1E are fluorescent microscopy images. 1C and 1F are obtained by overlaying the bright field and fluorescent images.

particles. This lower cell adhesion encourages stronger cell aggregation and the formation of EBs.

2. The effect of liquid marble size (volume): two different liquid marble sizes were investigated to monitor the effect of liquid marble size on EB formation and properties: either 50 μL or 300 μL of cell culture medium were used to form the liquid marbles. The liquid marble size seems to have a clear influence on the shape and number of EBs formed inside liquid marble micro bioreactors. When keeping the number of cells and the powder particle size constant, liquid marbles of 300 μL yielded a higher number of EBs that were more uniform than those harvested from the 50 μL liquid marbles. These results are in complete accordance with expectations, since a larger liquid marble contains a greater quantity of

growth medium (6 times greater in this set of experiments), so it can provide a more nutrient-rich cell growth medium than a smaller liquid marble (Fig. 2). It should be noted that, unlike conventional methods such as the hanging drop method which requires daily exchange of growth medium, in our EB formation approach we did not perform growth medium exchange. Therefore it is important in our approach to provide the cells and EBs with enough necessary nutrients for the duration of the experiment (generally from 3 to 7 days). Based on our observations, the quantity of nutrients of a 300 μL liquid marble is sufficient to keep the ES cells alive and active inside the liquid marble, throughout the experiment's time frame.
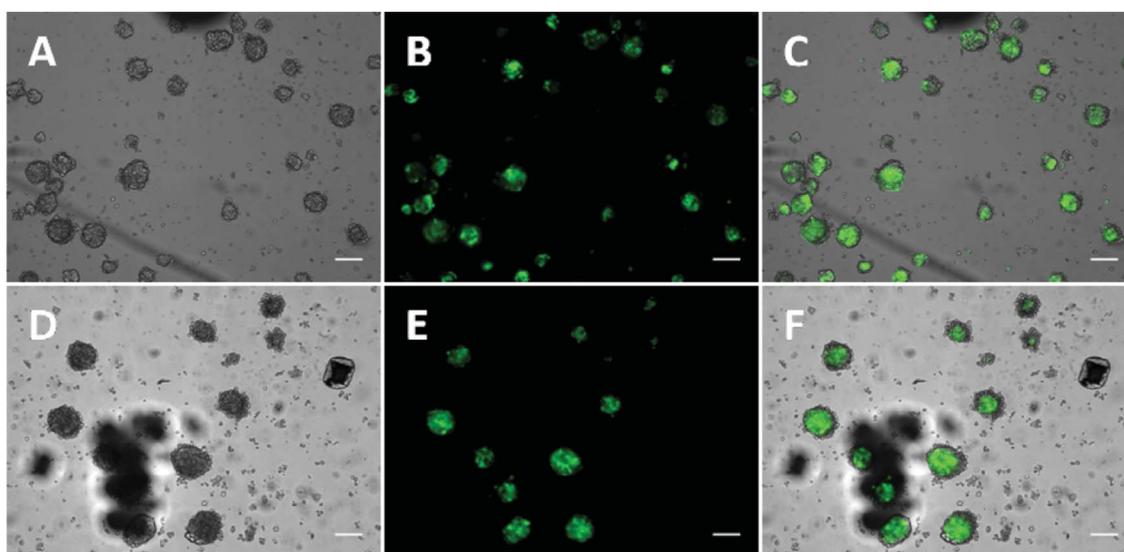
Fig. 2 Higher number of EBs formed in a 300 μl liquid marble (2A–C) compared with a 50 μl liquid marble (2D–F). Both liquid marbles were made from 100 μm PTFE powder and initially inoculated with 10 000 cells. 2A and 2D are optical microscopy images, while 2B and 2E are fluorescent microscopy images. 2C and 2F are obtained by overlaying the corresponding optical and fluorescent images.
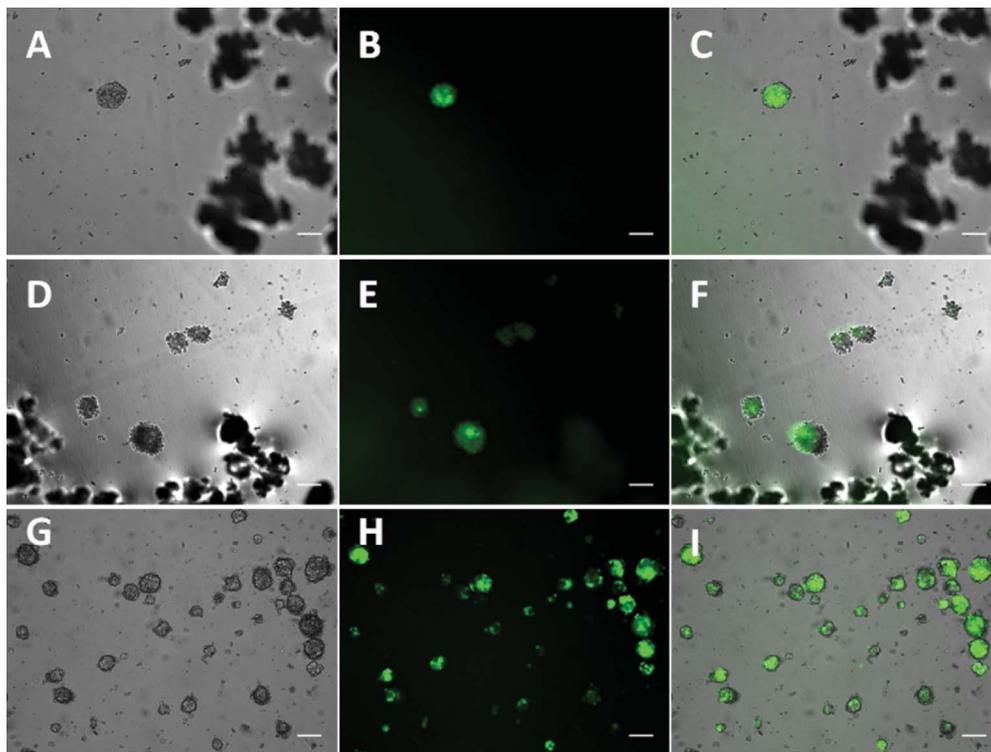
Fig. 3 Comparison of the number of the EBs formed in liquid marbles inoculated with ES cells, with various cell seeding densities: 1000 (3A–C), 5000 (3D–F) and 10 000 (3G–I) cells, (scale bar = 100 μm). 3A, 3D, and 3G are bright field images, while 3B, 3E, 3H are fluorescent microscopy images. 3C, 3F, and 3I are obtained by overlaying the bright field and fluorescent images.

3. The effect of cell seeding density: the cell seeding density inside liquid marbles also plays an important role in the yield and uniformity of the formed EBs. Liquid marbles of the same size, containing four different cell seeding densities (1000, 5000, 10 000 and 20 000 cells per liquid marble), were made and investigated. EB formation in marbles containing 1000, 5000, and 10 000 ES cells are shown in Fig. 3, while the best result which was obtained when using 20 000 cells can be seen in Fig. 4. As can be seen in these figures, although EB formation was observed in all cases, liquid marbles having a higher cell seeding density led to the formation of a greater number of EBs compared with liquid marbles containing lower cell seeding densities. A liquid marble provides a

confined space in which cells are free to interact effectively with one another, while having minimal contact with the powder particles thanks to the anti-adhesive property of the hydrophobic powder shell. On the other hand, EB formation partly depends on the accidental impact of ES cells with one another. It is therefore expected that a higher density of cells will provide a higher chance of impact, hence a higher number of EBs.

4. EB formation efficiency inside liquid marbles was compared with the liquid suspension technique as the chosen control method for (a) examining the morphology of the EBs, (b) counting the number of viable cells in the EBs and (c) measuring the size variation in the EBs formed by these two
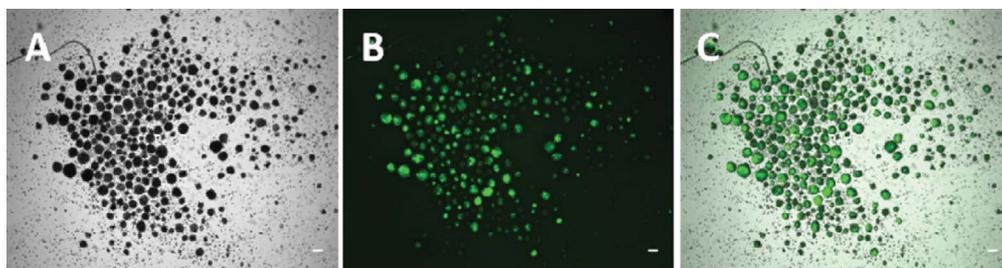
Fig. 4 The high number of EBs formed inside a liquid marble micro bioreactor under optimized conditions on day 3. The liquid marble was 300 μL in volume and was formed using 35 μm PTFE powder, with a cell density of 20 000 ES cells. A is a bright field image, B is a fluorescent microscopy image and C is composed of overlaid corresponding bright field and fluorescent images. Note that the EBs are located in intimate proximity to one another. Bar = 100 μm.
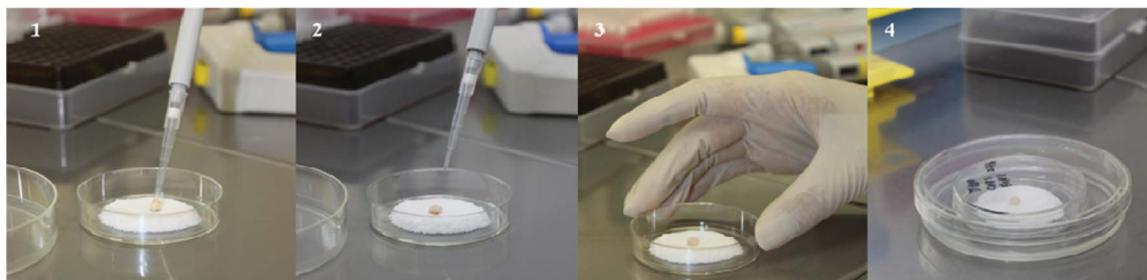
Fig. 5 Steps towards the formation of liquid marbles containing stem cells. The liquid containing the suspended stem cells in the growth medium are placed on the hydrophobic powder bed where the liquid beads up, due to the high hydrophobicity of the powder. The drop is then rolled on the powder bed to cover it with the hydrophobic powder. The Petri dish containing the resulting liquid marble is then placed inside a second Petri dish that is half filled with sterile water, and it is placed in the incubator.

methods. The morphology of EBs formed *via* both methods (LM and LS) after 3, 7 and 10 days of being in culture can be seen in Fig. 6. Our observations suggest that the efficiency of EB formation is significantly affected by the culture method. For instance, the formation of a large number of EBs in both LM and LS methods on day 3 is observed as shown in Fig. 6 and 7. Yet it is clear that the EBs obtained by the LM method are morphologically more uniform and of a narrower size distribution compared to those formed in LS. As for the LS technique, EBs with a wider size distribution and non-uniform morphologies were observed over time (Fig. 6B and D). With continuous growth of EBs in both LM and LS methods, EBs with necrotic cores are observed (darker EBs) as the diameter of EBs increases (Fig. 7). Moreover, as can be seen in Fig. 8, when the average diameter of EBs formed by LM and LS methods are compared at different times, the obtained standard deviation confirms that there is broad size distribution among EBs formed by the LS method. Furthermore, the size variation was found to be much smaller for EBs formed by

the LM method. To conclude, although EBs can be generated using the low adhesion suspension culture method, the LM technique in comparison produces more homogeneous EBs, while the former technique suffers from a large variation in size and morphology of the obtained EBs, limiting its yield.

5. The viability of cells inside EBs was also quantified by counting the number of viable cells from cells dissociated from EBs. It was found that there is a significant difference in the viability of cells obtained from the LM and LS methods. In addition, the number of cells increased by factors of 29 and 24 in LM compared to 13 and 11 in LS after 5 and 10 days of being in culture respectively, as shown in (Fig. 9) (initial number of cells was 20 000). The number of viable cells decreased over time for both the LM and LS methods, most probably because nutrient diffusion becomes the limiting factor as the size of EBs increases, as nutrients cannot reach the cores of the EBs.

6. Isolation of RNA was also carried out in another set of experiments using a RNeasy kit (QIAGEN Inc.) on days 3 and 7 of EB culturing. The brachyury expression is known as a mesoderm marker associated with gastrulation, the FOXA2 expression is also known as an endoderm marker, whereas the
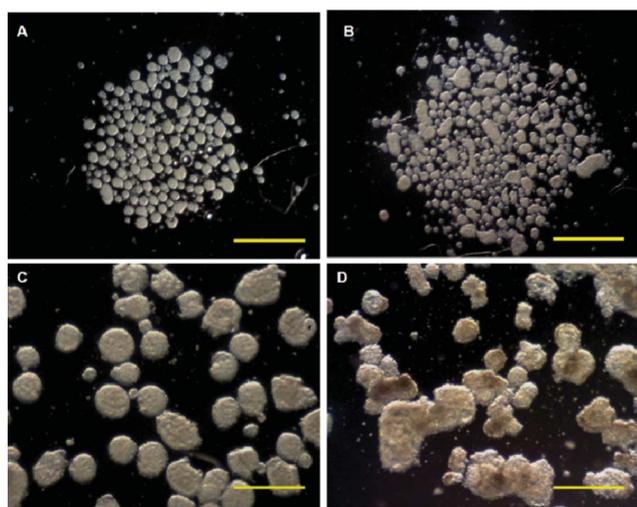
Fig. 6 Bright field images of EBs formed using liquid marble (A, C) and a suspension culture well plate (B, D) for 3 and 7 days in culture, scale bar: 500 μm.
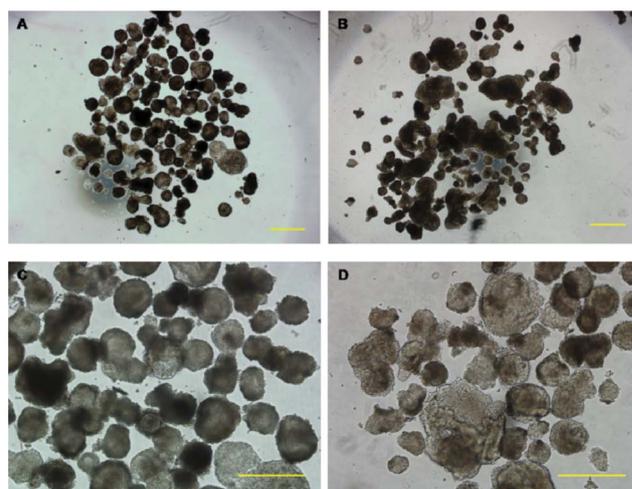
Fig. 7 Bright field images of EBs formed using liquid marble (A, C) and a low adhesion culture plate (B, D) after 10 days in culture, scale bar: 500 μm.
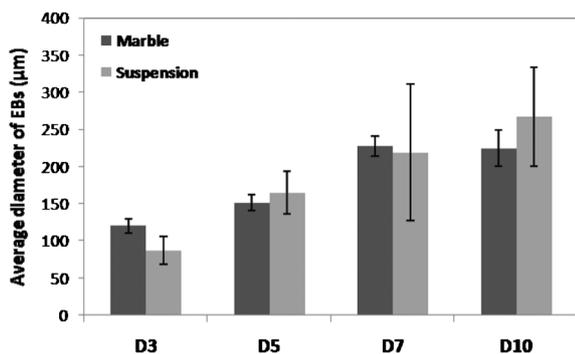

Fig. 8 Average diameter of EBs formed by LM and LS methods. Error bars represent the standard deviation.

nestin expression is an ectoderm marker.[38,39] Fig. 10 shows the RT-PCR of EBs collected at days 3 and 7. Markers of all three germ layers were observed in the EBs which confirms the *in vitro* differentiation potential of EBs that are formed using the liquid marble technique. The presence of these three germ layers demonstrates the potential of these EBs to differentiate into multipotent stem cells (progenitors), which will eventually progress into terminally differentiated cells. Further research into the differentiation of EBs inside liquid marbles is beyond the scope of this paper, but is currently underway in our group and will be reported in another paper.

In summary, when seeded with 2×10^4 ES cells, 300 µL liquid marbles made from PTFE powder with a 35 µm particle size resulted in the formation of hundreds of uniform EBs (Fig. 4). Large liquid marbles made from smaller PTFE powder particles were also found to be easier to handle and manipulate; it was easier to pierce the liquid marble shell of such marbles, to draw the liquid core for microscopy studies. After inoculation of ES cells into a liquid marble, green fluorescence protein expression from the EBs was observed for the first 3 days, indicating that these cells were able to maintain their pluripotency in liquid marbles prior to their differentiation. Some preliminary study on the feasibility of EB differentiation inside liquid marbles was also carried out. Once left in the incubator for 7 days, some EBs were naturally
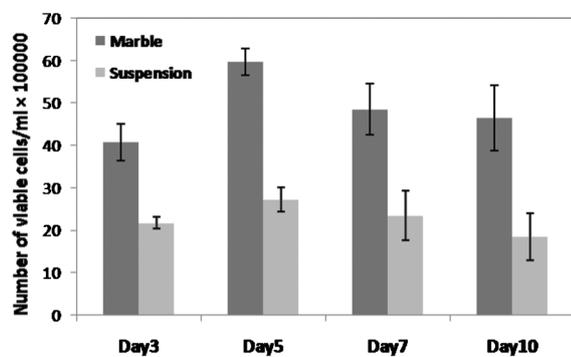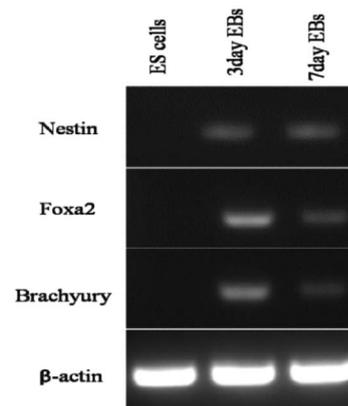

Fig. 10 RT-PCR for the expression of 3 germ layers for 3-day and 7-day old EBs formed inside the liquid marble.

differentiated to beating cardiomyocyte-like cells inside liquid marbles (see ESI†). This is a promising observation, which encourages future studies on the one-pot formation and differentiation of EBs inside liquid marbles as bioreactors. Therefore, we speculate that the addition of appropriate differentiation factors to the liquid marbles upon formation of EBs on day 3 could result in the formation of differentiated cells of desired cell types. The influence of the physical properties of liquid marble on EB differentiation and beating EB characteristics is currently under investigation.

Conclusion

The formation of EBs inside liquid marbles made from hydrophobic PTFE particles was investigated in this study. Liquid marbles are introduced as a novel and efficient means for *in vitro* preparation of EBs that could subsequently be differentiated into different cell lineages, such as cardiovascular cells. Although further studies are required to fully comprehend the biochemical aspects of the interactions between ES cells as well as EBs, and the hydrophobic powder particles, smaller powder particles generally resulted in more efficient liquid marble micro bioreactors for the formation of EBs. The cell seeding density (CSD) and liquid marble size (LMS) can also affect the efficiency of the method; the greater the CSD and LMS, the higher the efficiency of EB formation inside the liquid marbles. Liquid marble micro bioreactors provide a facile new method for the highly efficient production of EBs. Optimization of this method shows that it is capable of producing EBs of more homogeneous size and shape compared to EBs produced by the liquid suspension method. Miniaturization of bioreactors using the liquid marble concept also has a clear economical advantage over the existing methods.

Fig. 9 Number of viable cells in EBs after 3, 5, 7 and 10 days in liquid marble and in suspension culture.

Acknowledgements

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