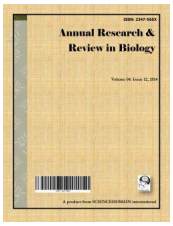

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3D Oral Squamous Cell Carcinoma Microtissues Grown in Calcium Alginate Microbeads

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Authors' contributions

This work was carried out in collaboration between all authors. Author SCW wrote the protocol and the first draft of the manuscript. Author CFS designed the study and performed the statistical analysis. Authors MKA and SCC managed the analyses of the study. Authors WYL and KST managed the literature review. All authors read and approved the final manuscript.

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ABSTRACT

3D microtissue models, especially cancer microtissue model, are potentially applicable for new drug testing because 3D microtissue reveals more realistic drug response, characterizes the disease and mimics the tumor in human body. In this paper, we report the integration of oral squamous cell carcinoma (OSCC) cell line (ORL-48) into 3D microtissue after two weeks of microencapsulation in calcium alginate microbeads that were produced based on the flicking technique. The microtissues contained highly proliferative cells as indicated by Alamar blue assay. The viable microtissues formed were extracted from the calcium alginate shell by means of alginate lyase. As revealed by

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field emission scanning electron microscopy (FE-SEM), the extracted 3D microtissues were characterized by inhomogeneous microtissue surface but good cell integrity via self-secreted extracellular matrix proteins. DAPI staining showed the proliferative behavior of cells in multilayer structure. These microtissues were able to spread into 2D monolayer after being transferred to grow on petri dishes. The 3D cultured microtissues of ORL-48 could be potentially useful for cancer therapeutic drug assessment *in-vitro*.

Keywords: 3D cell culture; calcium alginate; microbeads; microtissue; ORL-48; oral squamous cell carcinoma.

1. INTRODUCTION

In routine two-dimensional (2D) animal cell culture, cells are tightly spread and coupled to the culture flask in monolayer which is contrary to cells *in vivo* [1]. In 2D culture, cell to cell interaction is mainly concentrated at the boundary of the flattened cells [2] and the spatial organization of the cells in 2D is distorted when compared with the three-dimensional (3D) tissue *in vivo* [3]. Cells grown in 2D spread involuntarily by producing limited amount of extracellular matrix proteins due to the contactless spreading of cells [4]. Thus, 3D cell culture promotes cells growth in all directions with neighboring and surrounding cells which is believed to provide a better cell model for cancer research because 3D culture restores specific morphological, multi-stratified structure and biochemical features similar to the corresponding tissue *in vivo* [5]. Hence, modeling the intricacy of cancer using 2D culture on plastic ware is far from representing the tumor within the patient [6]. 3D microtissues reveal a more realistic drug response, predictively characterizes the disease and mimics the tumor in human body [7]. Previous report demonstrated that, culturing cancer cells in 2D monolayer conditions do not respond well to the cancer therapeutics due to dissimilar manner of the cancer cells in 3D models [6]. Thus, 3D cell culture systems are useful in drug discovery because their advantages in revealing more physiologically information and more predictive data for *in vivo* tests [8]. It is now widely accepted that the tumor spheroids, a three-dimensional model more closely resembles the initial avascular stages of small solid tumors *in vivo* [9,10].

Head and neck cancers are the sixth most common malignant tumors worldwide, with more than 650,000 new cases and 350,000 deaths each year [11]. Oral squamous cell carcinoma (OSCC) is common among the most devastating head and neck cancer subtypes especially in South-East and South-Central Asia. In countries such as Bangladesh, Pakistan, Sri Lanka and India, OSCC is the most common cancer among men with a five year survival rate of 50–60% [12]. Consequently, the establishment of 3D microtissue *in vitro* models to study tumors will help in the understanding of molecular events related with the development of these cancers [13].

Microbeads or microcapsules have wide applications in biomedical engineering field that include drug delivery, encapsulation of biomolecules, tissue padding and tissue regeneration [14-18]. Different strategies have been applied to produce 3D microtissues [19]. The cells are usually encapsulated in calcium alginate microbeads that can be easily removed once the microtissues are formed. Calcium alginate is a white milky colored, gelatinous and water insoluble substance that can be created through the addition of aqueous sodium alginate to aqueous calcium chloride [18]. The porosity of calcium alginate provides exchange of gases, nutrients and catabolites for the encapsulated cells which support the growth of microtissues in encapsulation [20]. Alginate is currently recognized as a ready for clinical application material by the US Food and Drug Administration (FDA) [20,21].

Microencapsulation based on flicking technique is a promising technique developed by our group in producing high volume of microtissues of keratinocytes within a narrow distribution of size (230–280 µm) [22]. This method for generation of microbeads of cells is highly reproducible. In this work, flicking technique will be applied to encapsulate oral squamous cell carcinoma (ORL-48) cells [13,23] in calcium alginate microbeads leading to the microtissue formation. The biochemical, proliferation and biophysical structures of the microtissues will be investigated using phase contrast microscopy, DAPI staining, live and dead cells staining, Alamar blue assay, Field Emission Scanning Electron Microscopy (FE-SEM) and 3D microtissue replating. The

contribution of this research that has not been reported elsewhere is expected to be useful for generating 3D cancer microtissues for therapeutic drug assessment.

2. MATERIALS AND METHODS

2.1 ORL-48 Cell Culture

Oral squamous cell carcinoma (ORL-48) cell lines were provided by Cancer Research Malaysia (CRM). The cells were cultured in a 75 cm^2 tissue-treated culture flask containing DMEM (Dulbecco's Modified Eagle's Medium, Gibco®) supplemented with penicillin (100 units/ml, Sigma Aldrich, UK), fungizone (2.5 mg/l, Invitrogen, USA), streptomycin (100 mg/ml, Invitrogen, USA) and 10% fetal bovine serum (Invitrogen, USA). Upon reaching confluency, the media was removed from the cell culture flask and the flask was washed three times with Hank's Balanced Salt Solution (HBSS, Life Technologies, USA). After removing the HBSS solution, 3 ml of trypsin (0.5 mg/ml, Invitrogen, USA) was deposited into the flask and the flask was incubated in a humidified 5% CO_2 for about 5 mins. Subsequently, the flask was examined under a phase contrast microscope (Eclipse TS100, Nikon, Tokyo, Japan) to ensure that all the cells were detached from the surface of the culture flask. 6 ml of DMEM (containing 10% fetal bovine serum) was then deposited to halt the trypsinization process. Subsequently, the cell suspensions were transferred to a 15 ml polypropylene tube and centrifuged for 5 min at 269 ×g. The supernatant were discarded and the cells were re-suspended in 100 µl of 2 w/v% concentration sodium alginate solution. Then, the cell-sodium alginate solution with an approximate cell density of 9.42×10^7 cells/ml was ready for the microencapsulation experiments.

2.2 Preparation of Solutions and Microencapsulation Setup in Sterile Conditions

Sodium alginate powder (W201502-1KG, Sigma Aldrich, St. Louis, MO, USA) and calcium chloride anhydrous, granular (C1016-500G, Sigma Aldrich, St. Louis, MO, USA) are the materials used to prepare the biopolymer. 2 w/v% concentration of sodium alginate solution and 1 w/v% concentration of calcium chloride solution were used for gelation of calcium alginate (Ca-Alg) microbeads. The calcium chloride solution was filtered using a 0.2 µm PTFE membrane Acrodisc® syringe filter (Pall® Life Sciences, Port Washington, NY) before used. A flicking device was coupled to a syringe pump (NE-4002X, New Era, Farmingdale, NY, USA) to microencapsulate cells in Ca-Alg microbeads based on the flicking technique as described previously [22]. An ethylene tetrafluoroethylene (ETFE) cable tie with a length of 2.5 cm was tied to the shaft of a direct current geared motor to be constructed into a flicking device or flicker. The flicker produces rebounding force on the syringe needle to force the ejected droplets of cell suspension sodium alginate from the syringe needle to disperse and drop into a petri dish (60 × 15 mm) containing 4 ml of 1 w/v% calcium chloride solution. The distance between the needle tip and surface of calcium chloride solution was fixed at 5 cm. Tiny droplets of cell-suspension sodium alginate solution were submerged to calcium chloride solution to form cell encapsulated calcium alginate microbeads in the petri dish.

2.3 Microencapsulation of ORL-48 Cells

In the microencapsulation experiment, the cell-alginate solution was filled in a 0.5 ml BD (Becton, Dickinson and Company, United States) insulin syringe (29 Gauge) and the syringe was inserted to the syringe pump holder. The orifice of the tapped needle tip was faced downward to the calcium chloride solution containing in the petri dish. The flicking speed of the flicker and flow rate of the syringe pump were set at 80 rpm and 4 µl/min, respectively to produce microbeads with a size ranging from 230-280 µm that are suitable to encapsulate cells [22]. After 5 mins of flicking, the cell-alginate microbeads were left to be further polymerized in the calcium chloride bath for another 5 mins. When the microbeads of calcium alginates were formed, the calcium chloride solution was removed and then the microbeads were submerged into 1.5 ml of DMEM. The cell encapsulated microbeads were incubated at 37°C in a 5% CO_2 humidified incubator. The culture media (DMEM) was refreshed every two days. The growth of cells in the microbeads was monitored every 24 hours in an inverted phase contrast microscope (TS100, Nikon, Tokyo, Japan) linked to a digital Go-5 CCD camera (QImaging, Surrey, Canada).

2.4 DAPI Staining

DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to the deoxyribonucleic acid (DNA), and was used to determine the distribution of cells in the

calcium alginate microbeads. It is able to stain both live and fixed cells [24]. 0.5 µg/ml of DAPI (Sigma Aldrich, St. Louis, MO, USA) stain was prepared in HBSS solution. The cells encapsulated in the calcium alginate microbeads (3, 9 and 15 days of culture) were washed with HBSS before they were stained with DAPI solution for 20 mins. After 20 mins of staining, the DAPI solution was removed and the cells encapsulated microbeads were washed with HBSS solution. The stained cells in calcium alginate microbeads were observed in a BX53 Olympus fluorescence microscope mounted with a DP72 CCD camera (Olympus, Tokyo, Japan).

2.5 Live and Dead Cells Staining

LIVE/DEAD® Kits for mammalian cells (Molecular Probes® Product Brands) purchased from Life Technologies were used to stain the viable and dead cells of microtissue (after 15 days of culture) encapsulated in calcium alginate microbeads. 5 µl of calcein-AM and 20 µl of ethidium homodimer-1 (EthD-1) were mixed with 10 ml of HBSS solution to form 2 µM of calcein-AM and 4 µM of EthD-1 mixed solution. The container of the mixed solution was wrapped with aluminum foil and sonicated for 10 mins in an ultrasonic bath sonicator. The mixed solution was used to stain the cells of microtissue encapsulated in calcium alginate microbeads. The microtissue containing in the microbeads were washed with HBSS solution before staining. The calcium alginate microbeads were stained with the diluted calcein-AM and EthD-1 solution for 20 mins in dark. After 20 mins of staining, the staining solution was removed and the cells encapsulated microbeads were washed with HBSS solution again. Then, the stained microtissue in alginate microbeads were observed in a BX53 fluorescence microscope (Olympus, Tokyo, Japan) immediately. The purpose of carrying out live and dead cells stainings were to determine whether the aggregated cells (microtissue) in the calcium alginate microbeads were viable.

2.6 Alamar® Blue Assay

Negative control, control and samples of microencapsulated cells were treated with Alamar blue assay to investigate the proliferation of ORL-48 cells in microencapsulation after 15 days of culture. Before the experiment, the microencapsulated cells were washed three times in HBSS. After washing, three pieces of microcapsules containing cells were added to each of the eight wells in a single column of a 96 well plate. For the control, three pieces of alginate microcapsules without cells were also added to each of the eight wells in another column. The third column of the 96 well plate was set as the negative control. Then, the negative control, control and samples were added with 90 µl of DMEM media and 10 µl of Alamar blue (Invitrogen, Carlsbad, California, USA). The 96 well plate was incubated in a CO_2 perfused incubator at 37°C for 24 hours. After incubation, the absorbance of the Alamar blue assay in the 96 well plate at 570 nm was scanned using a Multiskan™ GO microplate spectrophotometer (Thermo Fisher scientific, Waltham, Massachusetts, USA). The absorbance data was acquired using SkanIt™ Software and exported in Microsoft Excel format. The absorbance was computed and expressed in mean ± standard deviation (SD). Similar experiments were repeated three times. Statistical significant differences between control, negative control and sample were determined using student's t-test in Microsoft Excel for $p<0.05$.

2.7 Extraction of 3D Microtissues with Alginate Lyase

Calcium alginate microbeads containing 3D microtissues of ORL-48 after 15 days of culture were soaked in a DMEM medium containing 0.2 mg/ml of alginate lyase (A1603, Sigma Aldrich, St. Louis, MO, USA) for degradation of alginate capsules [25]. Within 2 mins of soaking, the degradation of the calcium alginate was observed and recorded in a phase contrast microscope.

2.8 FE-SEM Scanning of the Extracted 3D Microtissues

Field emission scanning electron microscopy (FE-SEM, JSM-7600F, JOEL, Tokyo, Japan) was used to observe the morphology of the 3D microtissues. The extracted 3D microtissues by alginate lyase were washed three times with HBSS followed by 4% of formaldehyde (F8775, Sigma Aldrich, St. Louis, MO, USA) fixation for 24 hours at 4°C. After fixation, the 3D microtissues were air dried and sputter coated with gold nanoparticles before FE-SEM scanning. An auto fine coater (JFC–1600, JOEL, Tokyo, Japan) was used to coat the specimens with a gold layer at a thickness of 8 nm for 30 seconds in automatic mode. The parameter settings of the FE-SEM during imaging were 2 kV

of acceleration voltage with a secondary electron image (SEI) detector that provides 150×, 300× and 1500 × of magnifications.

2.9 3D Microtissues Replating

3D microtissues replating is a simple and easy method to investigate viability of the extracted 3D microtissues after alginate lyase. The extracted 3D microtissues were washed three times with HBSS and transferred to a tissue-treated polystyrene petri dish (35 × 10 mm) for replating. 2 ml of fresh DMEM was added into the culture petri dish and the behavior of the 3D microtissues in the culture dish was monitored.

3. RESULTS AND DISCUSSION

The size of microbeads produced by the flicking technique was small enough to serve as a condition required for microencapsulation of cells with a flicking speed of 80 rpm and flow rate of 4 µl/min to produce microbeads size in the range of 230-280 µm (Fig. 1). The microencapsulated cells were able to grow due to the suitable size of small-diameter (less than 300 µm) microbeads. This size range of microbeads could decrease mass transfer resistance caused by the encapsulation material in which allowing greater oxygen and nutrient availability for cells dwelling inside the microbeads [26,27]. Nonetheless, higher cell growth rate is an advantage of small microbeads over the larger microbeads for cells encapsulation because larger microbeads have more empty space at the same cell concentration [27]. In addition, smaller microbeads size might minimize or overcome the formation of a necrotic region at the center of cells resulting from insufficient nutrition and oxygenation [25].

Fig. 2 shows the results of ORL-48 cells grown in the calcium alginate microbeads with a cell density of 9.42×10^7 cells/ml. On the first day of microencapsulation, individual ORL-48 cells were closely packed at high density in the calcium alginate microbeads (Fig. 2(a)). Every cell is adhered to the adjacent or neighboring cells. Fig. 2(b) shows the ORL-48 cells grown in the calcium alginate bead and turned into aggregation after 3 days of culture. As time progressed from 5 to 11 days of culture, more aggregation of cells were observed as shown in Fig. 2(c-f). The progressive formation of microtissues was obvious when individual cells can hardly be distinguished on the day 13 and 15 of culture (Fig. 2(g-h)). On day 15 of culture, thick and dark cloudy microtissues could be seen in the phase contrast microscopy indicating the maturation of the 3D microtissue formed. The use of alginate for cell culture is encouraging and plausible because it is a biodegradable polymer that mimics the extracellular matrix and supports both cell functions and metabolism [18,28]. However, cells produced catabolites and waste into culture media over prolong culture period. This caused the media to turn acidic and thus, increased the biodegradability for some of the alginate microbeads over time. As a result, this led to the rupture of alginate membrane and leaking of cells from the microcapsule. This problem can be greatly reduced by replenishing the media every two days to reduce the acidity of the media caused by the metabolism of the cells. A few hundreds of microtissues with good cell integrity were able to be harvested after the issue was addressed.

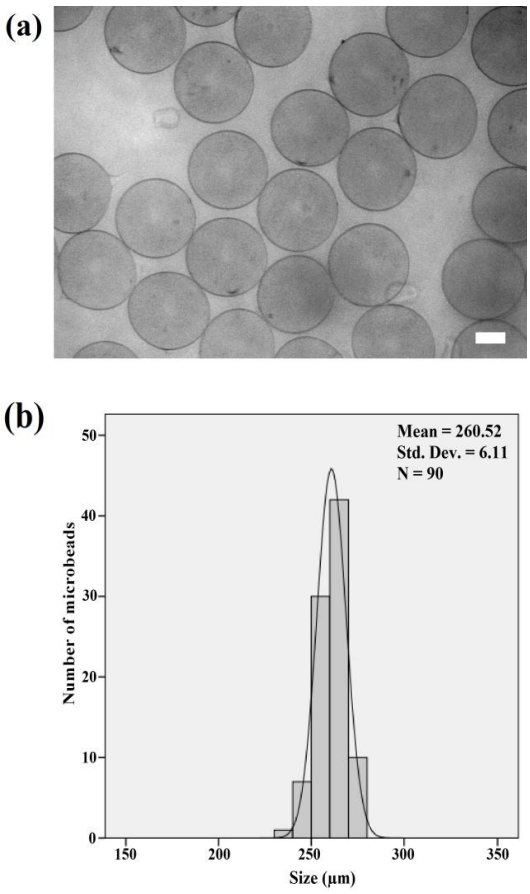

Fig. 1. The calcium alginate microbeads size used for cells encapsulation, (a) optical photomicrograph of microbeads and (b) size distribution graph of microbeads. (Scale bar: 100 µm)

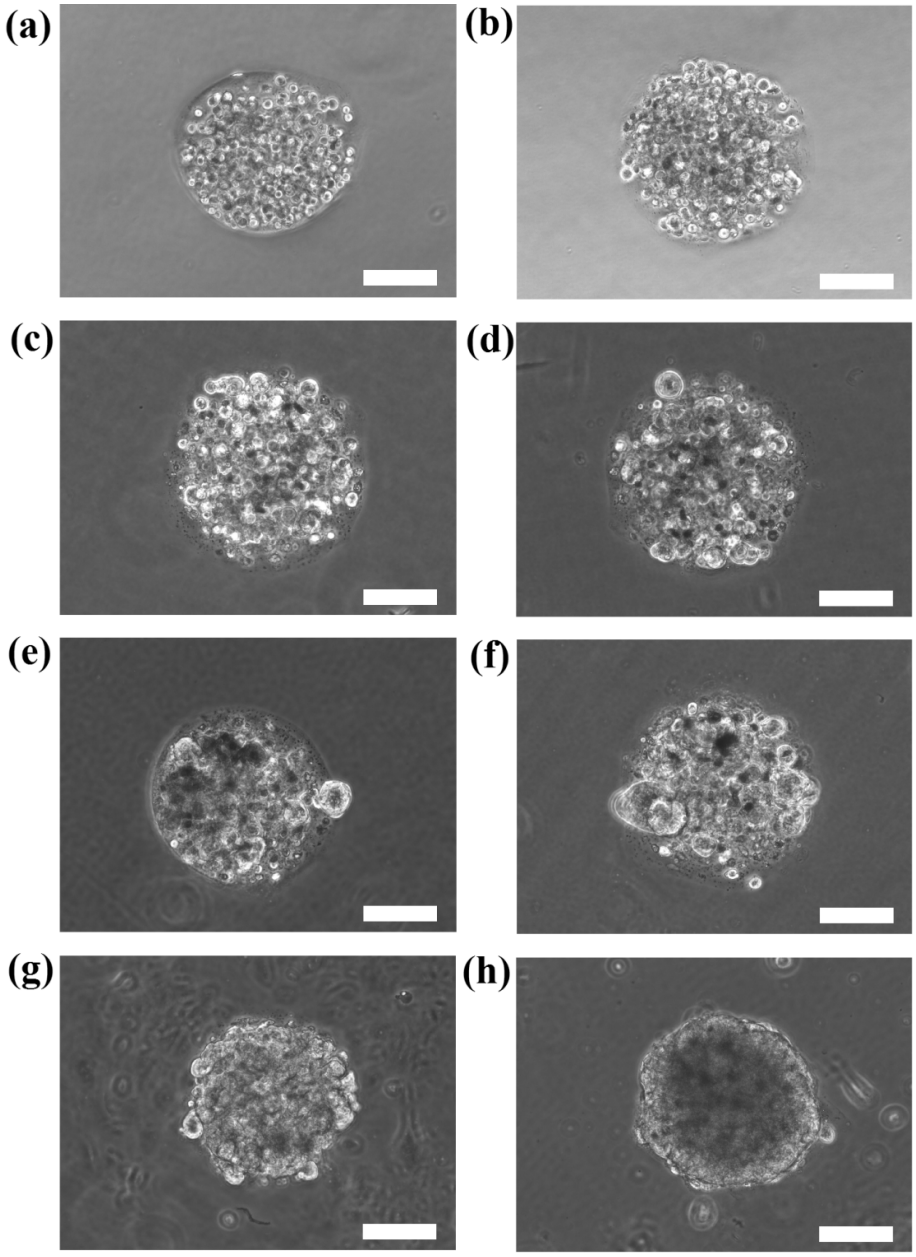

Fig. 2. The encapsulated ORL-48 cells in microbeads of calcium alginate at Day (a) 1, (b) 3, (c) 5, (d) 7, (e) 9, (f) 11, (g) 13 and (h) 15 of culture. (Scale bar: 100 µm)

Fig. 3 (a-c) shows the results of DAPI staining on ORL-48 cells in a calcium alginate microbead grown after 3, 9 and 15 days, respectively. The blue dots in the DAPI staining represent the nucleus of the ORL-48 cells. On day 3 of culture (Fig. 3(a)), individual punctuated dots of nucleus in loose spaces can be clearly identified. On day 9 of culture, the number of the blue dots or nuclei seemed to increase and filled up the empty spaces of microbeads that were observed on day 3 of culture (Fig. 3(b)). On day 15 of culture (Fig. 3(c)), these dots were indistinguishable due to the formation of highly and closely packed cell aggregations with very limited spaces in the microtissue. The DAPI staining results were useful to indicate the proliferative behavior of cells in the microencapsulation that were in good agreement with the densely packed microtissues observed in the phase contrast microscopy.

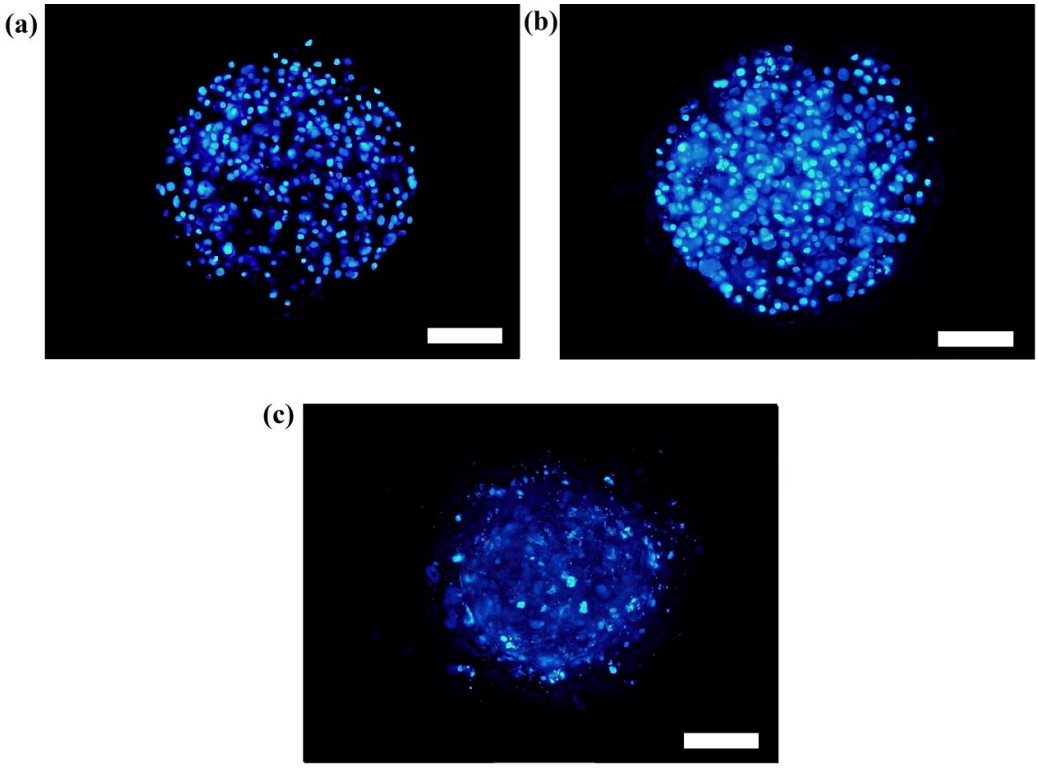

Fig. 3. DAPI stainings of ORL-48 cells encapsulated in the microbeads of calcium alginate at Day (a) 3, (b) 9 and (c) 15 of culture. (Scale bar: 100 µm)

Fig. 4 shows the result of live and dead cell stainings of the 3D microtissue of ORL-48 encapsulated in a calcium alginate microbead after 15 days of culture. Green staining indicates live cells and red staining indicates death cells. As shown in Fig. 4, majority of the cells in the microtissues cultured in the calcium alginate microbeads were stained in green which indicated the cells were alive. The viability result of microtissue suggested that the cells received enough nutrients and gases allowing them to be alive in the encapsulation after prolong period of culture and a constant change of media. Nonetheless, the high viability of cells determined could be attributed to the gentle flicking mechanism and polymerization process. These processes had presented safe encapsulation of the cells. In our experiment, the flicking technique has successfully produced high throughput and viable 3D microtissues of oral squamous cell carcinoma cells (N = 80 per min of flicking). This is a positive achievement in comparison to the micro-nozzle array method which yielded a lower viability (~70 %) of cells [17] that could be due to the post cleaning treatment with lipase and high productivity of microbeads generated that was inversely proportional to the number of cells encapsulated.

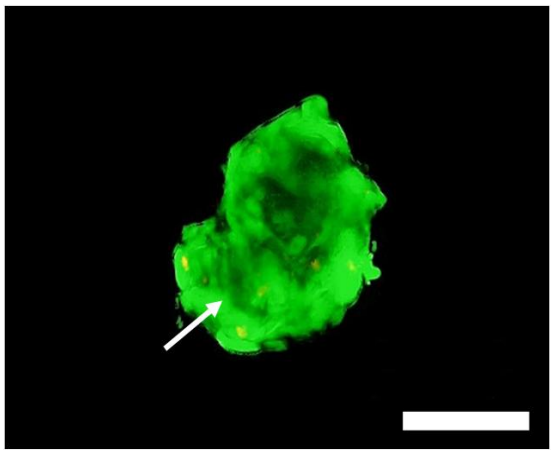

Fig. 4. Live and dead cell stainings of ORL-48 cells encapsulated in calcium alginate microbeads after 15 days of culture. Green stain of calcein-Am characterizes the live cells as indicated by an arrow (Scale bar: 100 µm)

The absorbance of the Alamar blue for the negative control (media) and control (alginate microcapsules) at 0.5367 ± 0.0765 and 0.5059 ± 0.0668 show no statistically differences (p= 0.4056), respectively. In comparison with the negative controls and controls, the results of Alamar blue assay in Fig. 5(a) indicated that the ORL-48 cells in the microtissue sample were highly proliferative at an absorbance of 0.7539 ± 0.0853. The absorbance of the sample is statistical significant different from the control (p = 0, p <0.05) and negative control (p = 0, p < 0.05). This result is supported by a previous work [12] which revealed that ORL-48 derived from stage IV tumors exhibited with high growth rate and has a short doubling time of 15 hours. The absorbance can be visually observed in the 96 well plate as shown in Fig. 5(b). The highly metabolic cells converted non-toxic purple resazurin in media into pink resorufin via the reduction reactions. The amount of colorimetric indicator produced is proportional to the quantity of live cells.

The calcium alginate microbead membrane was completely dissolved within 1 min of immersion in alginate lyase. Before alginate lyase, the 3D microtissue was encapsulated spherically in the calcium alginate microbead as shown in Fig. 6(a). However, the released 3D microtissue from the alginate capsule was slightly expanded into irregular shape without confinement of the spherical alginate capsule as shown in Fig. 6(b). Clearly, the microtissues encapsulated in the microbeads may not be spherical in shape but their appearances seen were defined by the shape of the alginate microbeads. The extracted microtissues were characterized by good integrity of cells with no fragment of loose tissue [25]. The microtissues remained intact even after gentle mechanical disturbance or handling which indicated strong cell-cell adhesion in the microtissues. The extracellular matrix proteins were strongly developed and established amongst the cells during formation of the microtissues. Enzymatic dissociation of cells using EDTA-trypsin could be a means to prove that the cells were bound by the ECM proteins [29].

In order to further analyze the surface morphology of 3D ORL-48 microtissues and to understand the cell adhesion to the matrix, FE-SEM was used to investigate the shape and surface morphology of the microtissues released from the alginate capsules. Fig. 7(a) and Fig. 7(b) show the FE-SEM image of 3D ORL-48 microtissue at ×150 and ×300 magnifications,

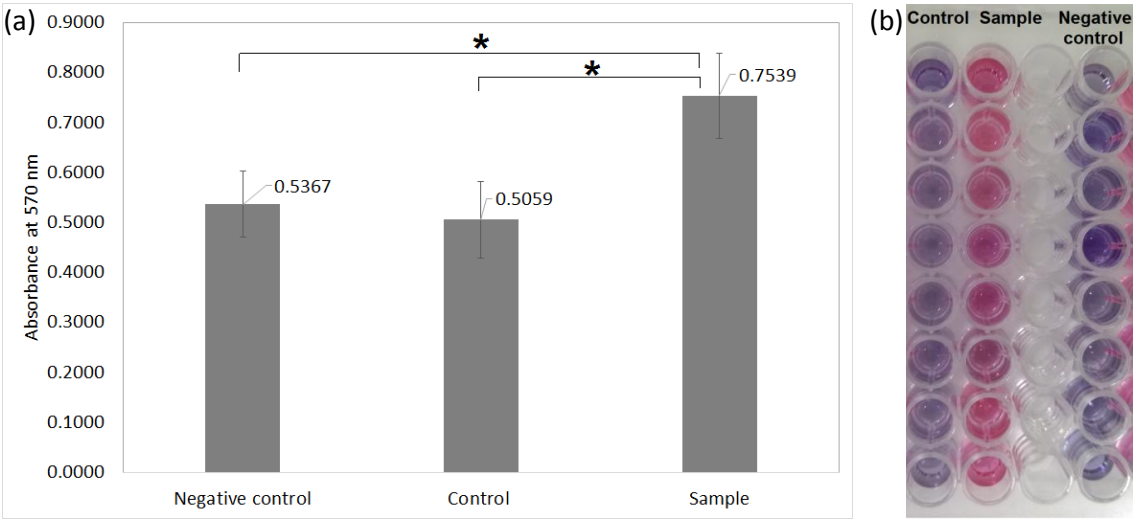

Fig. 5. (a) Absorbance graph for negative control, control and sample of microencapsulated cells after 24 hours culture in Alamar blue. The absorbance is expressed in mean ± SD. (b) The Alamar blue assay for the control, negative control and sample in a 96 well plate. Pink is the colorimetric indicator after conversion of Alamar blue by cells. Purple characterizes the original absorbance of Alamar blue mixed with DMEM media

**indicates significance of p < 0.05 (p= 0) as assessed by Student's t-test*

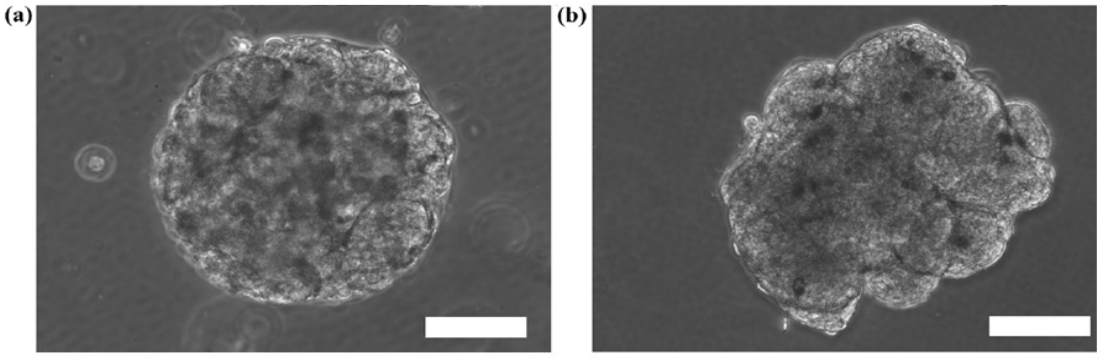

Fig. 6. The phase contrast images of microtissues of ORL-48: (a) before and (b) after application of alginate lyase. (Scale bar: 100 µm)

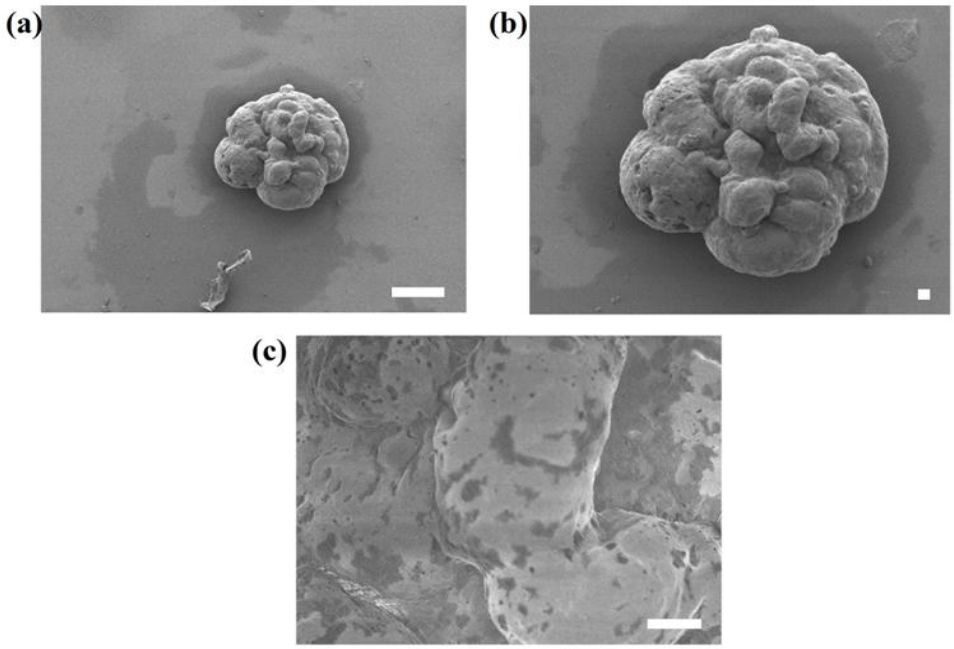

Fig. 7. FE-SEM images of 3D ORL-48 microtissue with different magnifications: (a) ×150 (Scale bar: 100 µm), (b) ×300 (Scale bar: 10 µm) and (c) ×1500 (Scale bar: 10 µm)

respectively. Similar to previous research [30,31], the FE-SEM results showed that the microtissues harvested from the calcium alginate microbeads were in the form of spheroids. In the FE-SEM image, the surface of the 3D spheroids of ORL-48 was not characterized by individualized cells protruding. The cells were well fused in the extracellular matrix that was indicated by the heterogeneous surface of microtissue (Fig. 7(c)). As reported in [32], solid tumors (*in vivo*) are organ-like structures that are heterogeneous and structurally complex. Solid tumors are characterized by malignant cells, ECM and several types of normal cells [33]. The multi structural tissues organization and denser cell density in tumors was suggested to be a cause of drug resistance [32,33]. Thus, a 3D *in vitro* microtissue model might reveal a more realistic drug response and mimics the tumor *in vivo*.

3D microtissue of ORL-48 cells replating was performed to rapidly investigate the viability and migration functionality of the cells of microtissue after alginate lyase. On the first day of replating, the microtissue (Fig. 8(a)) was suspended in the culture medium. After 24 hours of replating, the

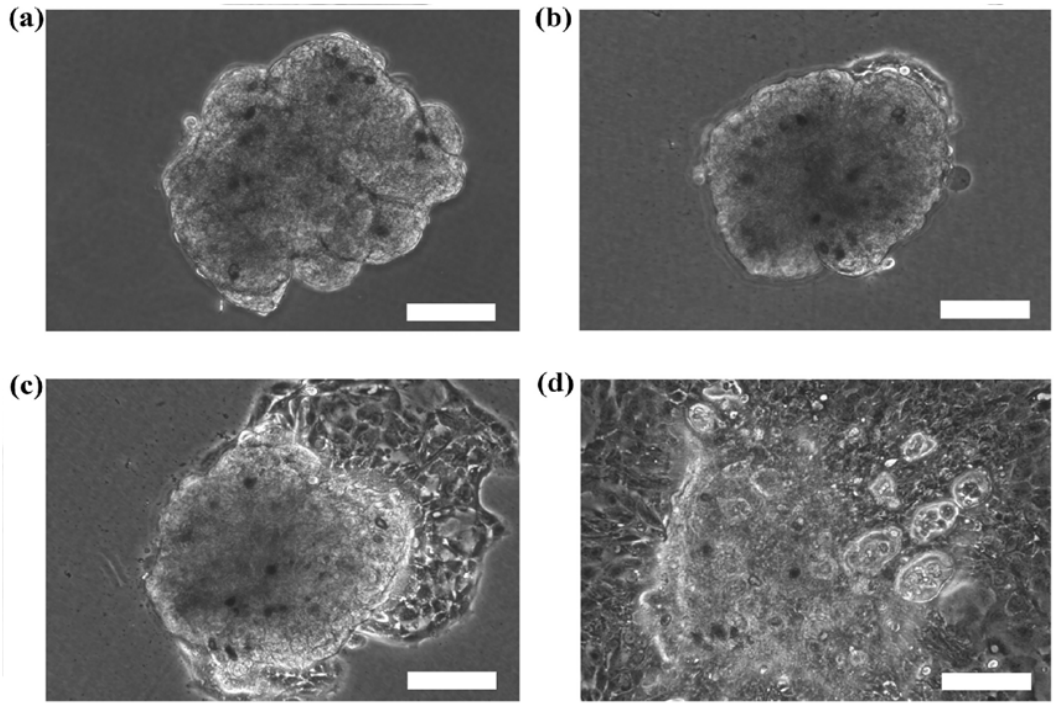

Fig. 8. The phase contrast photograph of replating extracted 3D ORL-48 microtissue at: (a) Day 1, (b) Day 2, (c) Day 3 and (d) Day 4. (Scale bar: 100 µm)

microtissue attached to the surface of the culture dish and cells started to migrate out of the microtissue as shown in Fig. 8(b). The attachment of microtissue on culture dish was confirmed by gently shaking the culture dish and the microtissue did not detach or float in the culture medium. Fig. 8(c) shows that more 2D monolayer of cells were formed and spread out from the 3D microtissue on Day 3. The 3D microtissue was completely disintegrated and grew into 2D monolayer of cells on Day 4 as shown in Fig. 8(d). This experiment showed that the cells after experiencing the alginate lyase process were viable and retained their basic ability to migrate and proliferate. The cells of 3D microtissue preferably attached to a tissue-culture treated dish. This could be due to the stiffness and the strong negatively charged surface of the tissue-culture treated dish that was able to attract the deposition of positively charged amino acids to support cells adhesion [34]. If the OSSC spheroid model is to be applied and treated with an inhibitor to the actin filament polymerization such as cytochalasin-B, this replating method can be applied in future work to provide information on the effects of the inhibitor to the migratory ability of the cancer cells out of the microtissues. Hence, the overall cancer microtissues model will be useful for screening cancer therapeutic agent.

4. CONCLUSION

A 3D cell model of oral squamous cell carcinoma cell line was successfully produced based on the microencapsulation technique of flicking. This model is potentially useful for regenerative medicine and cancer therapeutic drug research. With the microencapsulation of flicking technique, ORL-48 cells encapsulated in calcium alginate microbeads were able to grow into microtissues after being cultured for approximately two weeks. The live and dead cell stainings indicated that the cells in the microtissues were highly viable after the alginate lyase process and the cells were reported to be functional via the replating experiment. The ORL-48 cells grown in the microtissues were highly proliferative as indicated by the Alamar blue assay. The DAPI staining indicated the proliferative behavior of the cells in multi-layer structure during the microtissues formation. The DAPI result also showed that the cells were densely packed and highly integrated in the microtissues. The microtissues were not ideally spherical as what was observed in the

entrapment of microbeads. FE-SEM microscopy confirmed the organization of cells into 3D spheroids of cells surrounded by extracellular matrix. We believe that the viable cancer microtissues are suitable to be applied for studying novel cancer therapeutics and investigating oral squamous cell carcinoma pathogenesis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Souza GR, Molina JR, Raphael RM, Ozawa MG, Stark DJ, Levin CS, Bronk LF, Ananta JS, Mandelin J, Georgescu MM, et al. Three-dimensional tissue culture based on magnetic cell levitation. Nat Nanotechnol. 2010;5(4):291-296.
2. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3d cell culture. Biotechnology and Bioengineering. 2009;103(4):655-663.
3. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science. 2001; 294(5547):1708-1712.
4. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. Cell. 2007;130:601-610.
5. Kunz-Schughart L, Freyer JP, Hofstaedter F, Ebner R. The use of 3-D cultures for high throughput screening: The multicellular spheroid model. J Biomol Screen. 2004;9(4):273-285.
6. Lovitt CJ, Shelper TB, Avery VM. Advanced cell culture techniques for cancer drug discovery. Biology (Basel). 2014;3(2):345-367.
7. Asghar W, Assal RE, Shafiee H, Pitteri S, Paulmurugan R, Demirci U. Engineering cancer microenvironments for *in vitro* 3-D tumor models. Materials Today. 2015; 18(10):539-553.
8. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol. 2014;12(4):207-218.
9. Jiang Y, Pjesivac-Grbovic J, Cantrell C, Freyer JP. A multiscale model for avascular tumor growth. Biophysical Journal. 2005;89(6):3884-3894.
10. Carver K, Ming X, Juliano RL. Multicellular tumor spheroids as a model for assessing delivery of oligonucleotides in three dimensions. Molecular Therapy Nucleic Acids. 2014;3:e153.
11. Polz-Gruszka D, Macieląg P, Fołtyn S, Polz-Dacewicz M. Oral squamous cell carcinoma (OSCC) - molecular, viral and bacterial concepts. Journal of Pre-Clinical and Clinical Research. 2014;8(2):61-66.
12. Fadlullah MZ, Chiang IK, Dionne KR, Yee PS, Gan CP, Sam KK, Tiong KH, Wen Ng AK, Martin D, Lim KP, et al. Genetically-defined novel oral squamous cell carcinoma cell lines for the development of molecular therapies. Oncotarget. 2016; 7(19):27802-27818.
13. Hamid S, Lim KP, Zain RB, Ismail SM, Lau SH, Mustafa WM, Abraham MT, Nam NA, Teo SH, Cheong SC. Establishment and characterization of Asian oral cancer cell lines as *in vitro* models to study a disease prevalent in Asia. Int J Mol Med. 2007; 19(3):453-460.
14. umer H, Nigam H, Tamboli AM, Nainar MSM. Microencapsulation: Process, techniques and applications. International Journal of Research in Pharmaceutical and Biomedical Sciences. 2011;2(2):474-481.
15. Sakai S, Ito S, Inagaki H, Hirose K, Matsuyama T, Taya M, Kawakami K. Cell-enclosing gelatin-based microcapsule production for tissue engineering using a microfluidic flow-focusing system. Biomicrofluidics. 2011;5(1):13402.
16. Bhujbal SV, de Haan B, Niclou SP, de Vos P. A novel multilayer immunoisolating encapsulation system overcoming protrusion of cells. Sci Rep. 2014;4:6856.
17. Sugiura S, Oda T, Izumida Y, Aoyagi Y, Satake M, Ochiai A, Ohkohchi N, Nakajima M. Size control of calcium alginate beads containing living cells using micro-nozzle array. Biomaterials. 2005;26(16):3327-3331.

18. Andersen T, Auk-Emblem P, Dornish M. 3D Cell Culture in alginate hydrogels. Microarrays. 2015;4:133-161.
19. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: Advantages and applications. J Cell Physiol. 2015;230(1):16-26.
20. Paredes Juarez GA, Spasojevic M, Faas MM, de Vos P. Immunological and technical considerations in application of alginate-based microencapsulation systems. Front Bioeng Biotechnol. 2014; 2:26.
21. de Vos P, Lazarjani HA, Poncelet D, Faas MM. Polymers in cell encapsulation from an enveloped cell perspective. Adv Drug Deliv Rev. 2014;67-68:15-34.
22. Wong SC, Soon CF, Leong WY, Tee KS. Flicking technique for microencapsulation of cells in calcium alginate leading to the microtissue formation. J Microencapsul. 2016;33(2):162-171.
23. Hidayatullah Fadlullah MZ, Chiang IK, Dionne KR, Yee PS, Gan CP, Sam KK, Tiong KH, Wen Ng AK, Martin D, Lim KP, et al. Genetically-defined novel oral squamous cell carcinoma cell lines for the development of molecular therapies. Oncotarget; 2016.
24. Crissman HA, Hirons GT. Staining of DNA in live and fixed cells. Methods Cell Biol. 1994;41:195-209.
25. Sakai S, Ito S, Ogushi Y, Hashimoto I, Hosoda N, Sawae Y, Kawakami K. Enzymatically fabricated and degradable microcapsules for production of multicellular spheroids with well-defined diameters of less than 150 microm. Biomaterials. 2009;30(30):5937-5942.
26. Bressel TAB, Paz AH, Baldo G, Lima EOC, Matte U, Saraiva-Pereira ML. An effective device for generating alginate microcapsules. Genetics and Molecular Biology. 2008;31(1):136-140.
27. Sugiura S, Oda T, Aoyagi Y, Matsuo R, Enomoto T, Matsumoto K, Nakamura T, Satake M, Ochiai A, Ohkohchi N, et al. Microfabricated airflow nozzle for microencapsulation of living cells into 150 micrometer microcapsules. Biomed Microdevices. 2007;9(1):91-99.
28. Ghidoni I, Chlapanidas T, Bucco M, Crovato F, Marazzi M, Vigo D, Torre ML, Faustini M. Alginate cell encapsulation: new advances in reproduction and cartilage regenerative medicine. Cytotechnology. 2008;58(1):49-56.
29. Thong KT, Soon CF, Tee KS. The Effects of enzyme to the dissociation of cells in monolayer and 3D microtissue on the liquid crystal substrate. International Federation for Medical and Biological Engineering (IFMBE) Proceedings. 2015; 46:231-234.
30. Schmidt M, Scholz CJ, Polednik C, Roller J. Spheroid-based 3-dimensional culture models: Gene expression and functionality in head and neck cancer. Oncology Reports. 2016;35(4):2431-2440.
31. Lou YR, Kanninen L, Kaehr B, Townson JL, Niklander J, Harjumaki R, Jeffrey Brinker C, Yliperttula M. Silica bioreplication preserves three-dimensional spheroid structures of human pluripotent stem cells and HepG2 cells. Sci Rep. 2015;5:13635.
32. Tredan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute. 2007;99(19):1441-1454.
33. Grantab RH, Tannock IF. Penetration of anticancer drugs through tumour tissue as a function of cellular packing density and interstitial fluid pressure and its modification by bortezomib. BMC Cancer. 2012;12:214.
34. Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton. 2005;60(1):24-34.

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