Microfluidic technologies to engineer mesenchymal stem cell aggregates—applications and benefits

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Abstract

Three-dimensional cell culture and the forming multicellular aggregates are superior over traditional monolayer approaches due to better mimicking of *in vivo* conditions and hence functions of a tissue. A considerable amount of attention has been devoted to devising efficient methods for the rapid formation of uniform-sized multicellular aggregates. Microfluidic technology describes a platform of techniques comprising microchannels to manipulate the small number of reagents with unique properties and capabilities suitable for biological studies. The focus of this review is to highlight recent studies of using microfluidics, especially droplet-based types for the formation, culture, and harvesting of mesenchymal stem cell aggregates and their subsequent application in stem cell biology, tissue engineering, and drug screening. Droplet-based microfluidics can be used to form microgels as carriers for delivering cells and to provide biological cues to the target tissue so as to be minimally invasive. Stem cell-laden microgels with a shape-forming property can be used as smart building blocks by injecting them into the injured tissue thereby constituting the cornerstone of tissue regeneration.

Keywords Mesenchymal stem cells · Microfluidics · Bioink · Multicellular aggregates · Droplet

Introduction

There are significant differences between two (2D) and three-dimensional (3D) cell cultures for mimicking the physiological microenvironment cell experience *in vivo* (Kim, 2005; Lin and Chang, 2008). These differences are important for *in vitro* studies in order to correctly recreate *in vivo* conditions due to the influence that the cell culture environment plays on biological phenomenon such as stem cell fate. For an instance, the monolayer environment in 2D culture tends to alter gene expression and prevents cell differentiation (Kim, 2005; Chen et al., 2017). Culturing cells on rigid surfaces may enhance proliferation but inhibit cell differentiation since the interactions are limited (Knight and Przyborski, 2015; Okuyama et al., 2010). Furthermore the proliferation rate of cells in terminal differentiation state is higher in three-dimensional cultures than monolayer cultures (Langenbach et al., 2013).

Gene expression in 3D cultures is much closer to clinical expression profiles than in 2D monolayers (Jin et al., 2010). The cell culture substrate also influences cell morphology, phenotype, and focal adhesions in monolayer culture (Higuchi et al., 2013; Samal et al., 2019). One of the parameters determining stem cell fate is mechanical stiffness and elastic modulus of the extracellular matrix surrounding cells which is quite different in 2D and 3D cell cultures and may increase cell proliferation rate (Higuchi et al., 2013; Ito et al., 2016; Cesarz and Tamama, 2015; Lee and Cha, 2018; Naruse, 2018). The modulus of elasticity for cells in 2D monolayers is in gigapascal (GPa) range while cell spheroids along with surrounding ECM have a combined modulus of elasticity less than 0.1 KPa (Cesarz and Tamama, 2015).

The organ-specific functions of many cell types are dependent on three-dimensional cell culture, and cells cannot maintain their functions within a monolayer culture. This phenomenon can be interpreted by complex interactions facilitated in 3D microenvironments, while 2D cultures have a limited number of cell-cell or cell-matrix interactions (Knowlton et al., 2016; Charwat and Egger, 2018). The mutual influence of cell-cell contacts and cell adhesion junctions in coordinating cellular cytoskeleton and collective cell migration has been previously investigated, and the mathematical and

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experimental results showed that cell cytoskeleton could be organized by the interaction of cells which in turn causes directional response to biochemical factors (Shamloo, 2014).

Three-dimensional cell culture approaches can be classified as scaffold-based and scaffold-free strategies. In general, scaffold-free cell delivery can be divided into three basic methods which are single-cell delivery (Mao et al., 2017; Kamperman et al., 2017a; Lienemann et al., 2017; Qiu et al., 2018; Carvalho et al., 2015; Mei et al., 2019), cell sheet engineering, and microtissue technology (Kelm and Fussenegger, 2010). Microtissues are cell aggregates with a spheroidal shape and diameters between 100 and 500 μm . Microtissues might be transferred into damaged tissue to enable pre-vascularization or the induction of angiogenesis after implantation (Torres et al., 2018). Spheroid cell and microtissue delivery have many advantages compared to conventional cell suspensions since cell survival and function are increased (Yap et al., 2013). As an example, the strong interaction between integrin, extracellular matrix, and bone morphogenetic protein 2 (BMP2) signaling is enhanced in osteogenic differentiation within microtissues (Langenbach et al., 2013).

There are several traditional methodologies for three-dimensional cell culture and these include the hanging drop method, growth in spinner flasks, three-dimensional scaffolds, and non-adherent surfaces (Landry et al., 1985; Lazar et al., 1995; Hamilton et al., 2001; Chua et al., 2005; Nyberg et al., 2005; Kelm et al., 2006; Lin et al., 2006; Elkayam et al., 2006; Bartosh et al., 2010; Laschke et al., 2013; Yamaguchi et al., 2014; Albritton et al., 2016; Amaral and Pasparakis, 2016; Shao et al., 2019). Each of these approaches have advantages and disadvantages for instance, the hanging drop approach is simple and easy, but the droplets formed in this way are not uniform and changing culture medium for cell culture would be a challenge (Egger et al., 2018).

Microfluidic devices and in particular droplet-based platforms have been widely used for the formation of multicellular aggregates during the last few decades due to the many advantages they hold with respect to traditional methods. Some of the unique attributes of microfluidics allow the fabrication of 3D architectures with controlled spatial relations between the cells, the presence of flow-induced signaling and transduction as well as the capacity to introduce the chemical gradients necessary to reproduce the *in vivo* microenvironment (Carvalho et al., 2015).

Herein, a brief description of the mechanism of the formation of multicellular aggregates is presented. Microfluidic devices are introduced, and their wide range of applications in the creation and growth of stem cell aggregates, and in particular mesenchymal stem cells is presented. Moreover, droplet-based microfluidic devices and their advantages are highlighted to produce stem cell aggregates as smart building blocks in tissue engineering.

Formation of multicellular aggregates

In general, three necessary factors for the formation of an artificial tissue are cells that the cells should be localized, an extracellular matrix (ECM) should be both present and possessing sufficient mechanical stability, and the availability of interstitial fluid for the delivery of nutrients and biological effectors to the cells (Kim et al., 2004). Cell spheroids are the aggregation of one or more different cell types along with extracellular matrix; in other words, they are scaffold-free tissues. The formation of multicellular spheroids occurs as follows. At first, integrin and ECM bind together through the enhanced cell to cell connections via hemophilic cadherin binding (Cesarz and Tamama, 2015; Silva et al., 2019). E-cadherin is a transmembrane protein for regulating cell adhesion (Chan et al., 2013). A delay phase follows cell aggregation and then cadherin proteins bind together, and multicellular aggregates form. It should be noted that the formation of multicellular spheroids via ECM fiber and cadherin depends on cell type and may vary from cell to cell (Lin and Chang, 2008).

Three-dimensional spheroids for mesenchymal stem cell culture and differentiation

Mesenchymal stem cells are pluripotent stem cells with differentiation capacity into various lineages involving chondrocytes, osteocytes, adipocytes, myocytes, hepatocytes, astrocytes, and neurons (Wang et al., 2009). In addition to bone marrow, periosteum muscle and adipose tissues are two sources of mesenchymal stem cells (Winter et al., 2003). Culturing pluripotent stem cells in spheroids under appropriate conditions can lead to the generation of different germ layers (Langenbach et al., 2013; Heinemann and Wiesmann, 2013). Cesarz and Tamama have shown that mesenchymal stem cells cultured in spheroids have enhanced anti-inflammatory, angiogenic, and regenerative tissue effects and improved cell survival after transplantation (Cesarz and Tamama, 2015).

Cell spheroids can proliferate or differentiate and are representative of *in vivo* physiological situations (Patra et al., 2013; Kojima et al., 2009). Physical properties of cell spheroids such as size, shape, and the distance between individual cells may influence cell fate (Wang et al., 2009). For instance, the condensation of cells by reduction of intercellular spaces would be promising for chondrogenesis since the aggregation of mesenchymal stem cells leads to higher differentiation while inhibition of cell aggregation delays chondrogenic differentiation (Winter et al., 2003). It has been found that small and round spheroids are prone to differentiate to chondrocytes while large and extended spheroids can differentiate into osteogenic tissue (Cesarz and Tamama, 2015; Chan et al., 2013; Wilson et al., 2014; Fu et al., 2014). The number of cells

within a cell aggregate may activate chondrogenic differentiation of mesenchymal stem cells, which is also indicative of aggregate size (Sarem et al., 2019).

Benefits and applications of microfluidic platforms for the formation of cell aggregates

Several microfluidic platforms with different geometries have been developed to encapsulate a defined number of cells. Some of them produce cell aggregates continuously while other designs work statically (Altmann et al., 2014). Each configuration may use various stimulations, including gravity, rotation, acoustic, or magnetic force. These platforms can also be classified based on cell types, including stem cells, cancer cells, or co-culture of different cell types for recapitulating functions of a tissue (Visone et al., 2016). A category on governing forces acting on microfluidics and materials developed to prepare cell aggregates is presented in the “Multicellular aggregate formation within microfluidics” section.

Recently, digital microfluidic devices have been developed for automated cell spheroid culture with the aid of the hanging drop technique (Aijian and Garrell, 2015). These microfluidic devices can be utilized for the long-term culture of spheroids by in situ medium exchange. The flexibility of liquid handling protocols, addition or extraction of any solution, and the ability to extract solution from a well for further analysis are considered as benefits when using these types of microfluidic platforms. The hanging drop method has also been optimized to improve the therapeutic potential of mesenchymal stem cells in three dimensions (Tung et al., 2011).

Another advantage of microfluidics for the production of cell spheroids is drug testing on cancer cells (Ananthanarayanan et al., 2014; Rimann et al., 2014; Kwak et al., 2018). Multicellular spheroids have many advantages compared to animal models for related cancer research which have many non-positive ethical issues (Wu et al., 2008; Shi et al., 2016; Fabisiewicz and Grzybowska, 2017; Assal et al., 2016). Also, cancer cells within a spheroid are more resistant to anticancer drugs and can recapitulate in vivo conditions better than two-dimensional monolayer cultures (Chen et al., 2010; Ching, Bansal and Bhandari, 2017). Using microfluidics for the formation of cell aggregates would result in the control of cell numbers and thus the rate of oxygen needed for the cells (Wu et al., 2008; Kashaninejad et al., 2016). Organoid-on-a-chip and body-on-a-chip are new technologies to monitor the kinetics of tumor progression and metastasis in vitro (Skardal et al., 2016; Porto et al., 2016; Jackson and Lu, 2016; Marsano et al., 2016; Ashammakhi et al., 2018). Examples of microfluidic devices are shown in Fig. 1, (a) for producing mesenchymal stem cell spheroids (Wang et al., 2009) and (b) a heart on a chip (Marsano et al., 2016).

Multicellular aggregate formation within microfluidics

I. Geometrical control of cell aggregate formation within microfluidics

One of the main goals of producing stem cell aggregates is promoting their differentiation by controlling the shape, size, and the number of cells within each spheroid (Kim et al., 2011; Khoury et al., 2010; Torisawa et al., 2007; Hsiao et al., 2009; Liu et al., 2019). In this manner, stem cell aggregates may be differentiated without the imposition of mechanical stress (Kim et al., 2011). The establishment of a concentration gradient with the aid of unique geometry is another approach adopted to simulate the differentiation of stem cells (Khoury et al., 2010). In one study, a microdomain on a glass substrate (Fig. 1) was fabricated based on photolithography and micropatterning techniques (Wang et al., 2009). The authors checked gene expression patterns corresponding to the maintenance of the stemness of mesenchymal stem cells, mesoderm-specific transcript, and mesenchymal stem cell membrane marker. After the formation of a 3D spheroid and during the induction of osteogenesis or adipogenesis, genes showed remarkable downregulation compared to the monolayer culture.

II. Force-combined aggregate formation within microfluidics

The creation of cell spheroids usually needs an external force such as gravity, centrifugal force, acoustic, electrical or magnetic force, and other stimulations (Cottet et al., 2019; Alhasan et al., 2016; Lee et al., 2012; Yoon et al., 2013; Ota et al., 2011a; Sudeepthi et al., 2019). Some researchers combined these forces with the fluid flow in a microfluidic device to form multicellular aggregates. Ota et al. implemented the rotational flow for developing a lab-on-a-chip platform to create three-dimensional spheroids (Ota et al., 2010; Ota and Miki, 2011; Ota et al., 2011a; Ota et al., 2011b). Based on their results, the time needed for the formation of cell spheroids was about 2 min, and their device had the capability of producing only a relatively small number of cell spheroids and involved generation of significant shear stress-induced rotational flow which is not suitable for stem cells. Another simple way for forming cell aggregates in culture involves using gravity in combination with gentle reverse flow thereby producing highly elastic spheroids (Lee et al., 2012; Kim et al., 2012; Kim et al., 2012). In this approach after the formation of multicellular aggregates, the chip should be placed upright, and the surplus medium should be removed.

Harvesting is one of the significant challenges after forming cell aggregates. This procedure should not induce stress on the cells in order to maintain their viability within

Fig. 1 **a** Left: Schematic diagram of microdomain patterned template on glass substrates and right: General method for the formation of spheroids and induction into adipocytes and osteoblasts, reproduced after (Wang et al., 2009). **b** Schematic view of a 3D heart on a chip microdevice, reproduced after (Marsano et al., 2016)

the spheroid. While a significant amount of research effort has dealt with culturing spheroids in a microfluidic device, the ease of removal of spheroids from the device is still a challenge. Relatively successful methods for removing spheroids from the microchannels have been developed based on the utilization of membranes resistant to cell adhesion, concave microwells, and fluid flow (Torisawa et al., 2007; Kang et al., 2010; Anada et al., 2010; Patra et al., 2016).

III. Hydrogel-assisted cell aggregate formation within microfluidics

Hydrogels hold great promise for the establishment of mesenchymal stem cell aggregates and drug testing (Carvalho et al., 2016; Kim et al., 2016; Chen et al., 2010; Lee et al., 2011; Yu et al., 2010). There are several crosslinking approaches for the formation of hydrogels. It has been shown that enzymatic crosslinking is superior over ionic or UV crosslinking for on-chip preparation of hydrogels of polymer conjugates due to significantly higher cell survival (Henke et al., 2016). Injectability is another aspect of hydrogels to deliver biological factors to the target tissue. Droplet-based microfluidic devices have been widely used to form injectable microgels. Several natural and synthetic hydrogels such as alginate, matrigel, dextran, hyaluronic acid, PEGDA, and their mixtures are being used within microfluidics for the formation

of cell-laden microgels (Yu et al., 2010; Liu et al., 2012; Wang et al., 2015a; Sabhachandani et al., 2016; Rossow et al., 2017). Some of these works are described in the next section.

IV. Co-culture of different cell types using microfluidics

Multicellular aggregates may be homogenous consisting of one cell type or heterogeneous having diverse cell populations. It is recommended to culture cell spheroids comprising various cell sources to recapitulate in vivo conditions better. Many types of research are focused on co-cultures of cells within a particular tissue (Winter et al., 2003; Okuyama et al., 2010; Yang et al., 2012; Kojima et al., 2009). Co-cultures of different cell types can also be used for targeted differentiation. As vascularization is a challenge in tissue engineering, one promising approach for improving vascularization after transplantation involves co-culture of specific tissue cells. Different combinations of human primary osteoblasts, human dermal microvascular endothelial cells, and normal human dermal fibroblasts were cultured in a study by Walser et al. (2013). The spheroids were then transplanted into dorsal skinfold chambers of mice. The results showed that the co-culture of human dermal microvascular endothelial cells with human osteoblasts could improve the formation of vessel-like structures and the viability of cells were increased to monolayer-derived cultures.

Drug screening for tumor spheroids is another benefit of co-culture of different cell types. The behavior of spheroids composed of more than one cell type is closer to the native tissue than that demonstrated by spheroids composed of one cell type, and therefore their reaction to drug stimulation would be closer to reality (Ota et al., 2011b; Labusca, 2015; Jeong et al., 2016; Sart et al., 2016; Du et al., 2018). Resistant three-dimensional cancer structures can be modeled by introducing appropriate tumor microenvironment which is suitable for anticancer drug screening (Ziółkowska et al., 2013; Sun et al., 2014; Kashaninejad et al., 2016; Wu et al., 2016; Hsiao et al., 2009).

Droplet-based microfluidics

Droplets are generated in a two-phase flow consisting of a continuous and a dispersed phase. Two governing forces for the formation of a droplet are surface tension and viscous stress. The continuous phase induces viscous stress on the immiscible phase, which is balanced by the surface tension, and a droplet is formed at the interface of the two streams (Yu et al., 2010). In fact, if the dispersed phase is injected into the stream of a continuous phase and a droplet is formed in a T junction, a flow-focusing channel or co-flow channel, then a set of diverse regimes can be observed which includes plug flow, dripping, jetting, and deformed interface flow (Salim et al., 2008; Basova and Foret, 2015; Mazzitelli et al., 2013; Verboet et al., 2014). Figure 2 shows the formation of a droplet within a microchannel at the interface of two immiscible phases.

Droplet-based microfluidic platforms are considered as microreactors with a wide volume range from femtoliter to milliliter and thus can synthesize particles for biological applications (Basova and Foret, 2015; Chen et al., 2019a, b). In order to obtain a stable emulsion with a spherical shape and minimized interfacial area in a droplet-based microfluidic device, parameters such as size, shape, and monodispersity of the droplets and their resistance to coalescence should be considered (Basova and Foret, 2015; Kim et al., 2013; Schultz and Furst, 2011). Controlling the interaction of particles and cells with reagent was precisely and spatiotemporally performed by developing a continuous flow microfluidic platform that enabled transient treatment of chemical reagents of the cells and particles. In this device, the meeting three solutions resulted in a laminar co-flow and therefore, transfer of cells and particles across the laminar streams was achieved by wall effect inertial lift forces (Dudani et al., 2014).

The ability of droplet-based microfluidics to function as bioreactors has drawn attention for using them to form three-dimensional matrices. They have the unique ability to compartmentalize cells into monodisperse and defined structures by controlling the number of cells in each droplet (Chan et al., 2013; Allazetta and Lutolf, 2015; Huang et al., 2016; Choi et al., 2017). One of the main applications of these bioreactors is the encapsulation of cells and the formation of multicellular aggregates (Sart et al., 2016). Rapid and high throughput formation of droplets and microbeads in droplet-based microfluidics along with cell culture within microbeads allow for the transport of oxygen and nutrients specifically in double emulsion droplets to ensure cell viability (Wang and Wang, 2014; Hendriks et al., 2015; Wang et al., 2017; Zhu et al., 2019). Another benefit of using droplet-based microfluidics that should be taken into account is the formation of stem cell niches surrounding multicellular aggregates which can be formed with high precision and might recreate in vivo-like conditions (Clausell-Tormos et al., 2008; Guiró and Arinze, 2015). As a proof of concept, mesenchymal stem cells within droplets are shown to secrete more extracellular matrix proteins compared to cells grown within static culture or transverse flow (Sart et al., 2016; Sart et al., 2019).

Droplet-based microfluidics for the formation of multicellular aggregates

Regarding the advantages of droplet-based microfluidics, in this section, we present the various applications of these types of microfluidic techniques for the establishment of multicellular aggregates. A schematic view representing some of the droplet-based microfluidic devices is depicted in Fig. 3. A droplet is generated at the interface of two immiscible phases—the aqueous phase containing cells along with the culture medium (mostly a hydrogel) and an oil phase which is biocompatible. Droplets should have desired properties such as appropriate size and uniformity in shape (Basova and Foret, 2015; Jang et al., 2016) since by increasing the diameter of a spheroid, the diffusion of oxygen would be limited to the center of the spheroid, and the rate of death would be increased (Yoon et al., 2013).

The focus of many studies is on incorporating various hydrogels for the generation of microgel structures that could be solid spheres, hard shell and soft core, and hollow type microgels (Ashida et al., 2013; Sakai et al., 2014; Khanmohammadi et al., 2016). Multicellular aggregates encapsulated within microgels serve as bioinks inducing

Fig. 2 Droplet formation sequence in a flow-focusing microchannel by demonstrating two-phase contours

Fig. 3 Examples of droplet-based microfluidics for the formation of cell aggregates. **a** Droplet generating in flow-focusing design for the encapsulation of cells, reproduced after (Clausell-Tormos et al., 2008). **b** (A) A schematic design of the microfluidic device for the encapsulation of cells and magnetic nanoparticles, (B) droplet formation part, and (C) bead separation channel, reproduced after (Yoon et al., 2013). **c** Top: An overview of the droplet-based microfluidic device for the formation of different shapes of cell aggregates, bottom: Emulsification and crosslinking reaction coupled in this microfluidic device, reproduced after (Wang et al., 2015)

organoid formation (Pajoumshariati et al., 2018; Chen et al., 2019b; Abdulghani and Morouço, 2019). They can be engineered as three-dimensional building blocks for the production of macromaterials with the capability of recreating the shape of a damaged tissue. If the degradation rate of a hydrogel is equal to the regeneration of the damaged tissue, multicellular aggregates can pop out of the hydrogel and help the damaged tissue to be recovered. Combining micro and macromaterials stimulate microniche generation, enabling stem cell differentiation into a specific lineage (Kamperman et al., 2017a; Imaninezhad et al., 2018). A summary about the use of droplet-based microfluidic devices for the formation of different types of microgels is presented as Table 1.

A microfluidic device has been developed by Alessandri et al., for the encapsulation of cells within alginate based on droplet formation in a calcium bath (Alessandri et al., 2013). The advantage of their work involved the use hollow spheres instead of the bulk hydrogel. The authors found that the survival rate following encapsulation is identical to trypsinized cells cultured in a petri dish. Thus neither shear nor exposure to the calcium bath was harmful to the cells. It was found that the size and porosity of microcarriers and shear force are

essential factors in designing a microfluidic device for the generation of the cell containing microspheres. Shear force may damage the cells on the surface of a microcarrier and insufficient exchange of nutrients to the encapsulated cells can be a risk for internal cells. Wang et al. developed a microfluidic device for generating porous microcarriers and a three-dimensional cell culture consisting of an external-internal connected scaffold and biopolymer matrix fillers for the cell culture (Wang et al., 2015a). In this work, cells acquired spherical shape after 3 days of culture, they were mature after 7 days, and cell spheroids were formed in most of the microcarriers.

One of the challenges in working with droplet-based microfluidics is recovering cell spheroids or multicellular aggregates from the oil phase. On the other hand, the oil phase should be biocompatible and have regular mechanical properties (Wagner et al., 2016). Extracting droplets in an oil phase with a dominant viscous force is difficult since the oil phase drives droplets to move together with the surrounding oil. Clausell-Tormos et al. used perfluorocarbon as the carrier oil because of its compatibility with PDMS devices (Clausell-Tormos et al., 2008). This oil can also dissolve greater

Table 1 A summary on different types of microgels, crosslinking method, cell type, and type of microfluidic device for the formation

Microgel type	Microgel material	Crosslinking method	Cell type	Type of microfluidic device	Reference
Spheroid	Alginate/gelatin	Ionic	Co-culture of crypt cells and Peyer's patch immune cells	Droplet based	Pajoumshariati et al. (2018)
Spheroid	4-arm polyethylene glycol acrylate (4-arm PEGAc)	Michael-type addition, 2-amino butane dithiol (DTBA)	U251 glioblastoma cells	T junction, droplet based	Imanizhad et al. (2018)
Microcapsule	Polyethylene glycol as continuous phase, sodium alginate, and dextran as the shell phase and cell suspended sodium carboxymethylcellulose as the core phase	Ionic	Porcine adipose-derived stem cell	Double emulsion	Zhu et al. (2019)
Spheroid	Polyethylene glycol diacrylate (PEGDA)/NIPAM	PEGDA with alcohol and HMPP as photo initiation and UV crosslinking, NIPAM with alcohol, NIPAM Bis as crosslinking agent and HMPP as photo-initiator	HepG2	Hanging drop	Shao et al. (2019)
Spheroid	Alginate as the inner phase, cell culture medium as the outer phase	Ionic	Human mesenchymal stem cells	Double emulsion	Chan et al. (2013)
Hard shell and soft core	Dex-HA-TA	Enzymatic, HRP and H ₂ O ₂	Human mesenchymal stem cells	Droplet-based microfluidics	Kamperman et al. (2017B)
Hollow microcapsules	Dex-TA	Enzymatic, HRP and H ₂ O ₂	Pancreatic beta cell line MIN6	Droplet-based microfluidics	Kamperman et al. (2017c)
Hard shell and soft core	Gel-MA	Photocrosslinking	NIH3T3 fibroblasts (ATCC)	Flow-focusing microfluidic device	Kim et al. (2016)
Hard shell and soft core, single cell	Alginate	Ionic	Murine pre-adipocyte cells (OP9s)	Droplet-based microfluidic device	Mao et al. (2017)

amounts of O₂ and CO₂ than water and can facilitate respiratory gas delivery. Collecting hydrogels conventionally without on-chip extraction leads to the aggregation of microparticles due to the residual oil drop. For solving this problem, a passive method in microfluidic Stokes flow is presented by utilizing the interfacial tension between the oil and the microparticles (Huang and He, 2014). This microfluidic device consists of a non-planar flow-focusing design for generating aqueous droplets of sodium alginate and a planar extraction channel with an additional inlet for an aqueous extracting solution and two outlets. Combining hydrogel precursor and magnetic nanoparticles is another novel idea eliminating the separation of cell spheroids from oil phase which is the focus of some studies thus far (Yoon et al., 2013; Al-Hetlani and Amin, 2019).

Conclusions

In recent years, the application of microfluidic technology for the formation of three-dimensional cell aggregates has drawn a considerable amount of attention. In this paper, a review of applications of microfluidic devices for the creation of multicellular aggregates and in particular mesenchymal stem cells is presented. Microfluidic devices are high throughput platforms with unique properties which are appropriate for the precise formation of cell spheroids along with their culture and harvesting. Several microfluidic designs with different advantages and shortcomings have been developed. Recent studies implementing such microfluidic technology in the formation of multicellular aggregates and in tissue engineering and stem cell biology are presented. Droplet-based microfluidics are the most efficient of the microfluidic methods for producing uniform cell spheroids. Rapid formation of uniform-sized multicellular spheroids can pave the way for studying the effect of multiple drugs in tumor and spheroid cell masses. It can solve many of the questions about different diseases in diverse organs. This review was aimed to provide some thoughts amongst the readers by presenting some of the most recent developments in 3D and spheroid cell culture technology based on microfluidic design.

References

- Abdulghani S, Morouço PG (2019) Biofabrication for osteochondral tissue regeneration: bioink printability requirements. *J Mater Sci Mater Med* 30(2):20
- Aijian AP, Garrell RL (2015) Digital microfluidics for automated hanging drop cell spheroid culture. *J Lab Autom* 20:283–295
- Albritton JL, Roybal JD, Paulsen SJ, Calafat NJ, Flores-Zaher JA, Farach-Carson MC et al (2016) Ultrahigh-throughput generation and characterization of cellular aggregates in laser-ablated microwells of poly (dimethylsiloxane). *RSC Adv* 6:8980–8991
- Alessandri K, Sarangi BR, Gurchenkov VV, Sinha B, Kießling TR, Fetter L et al (2013) Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proc Natl Acad Sci* 110:14843–14848
- Alhasan L, Qi A, Al-Abboodi A, Rezk AR, Chan PPY, Iliescu C, et al. 2016. Rapid enhancement of cellular spheroid assembly by acoustically-driven microcentrifugation. *ACS Biomater Sci Eng*
- Al-Hetlani E, Amin MO (2019) Continuous magnetic droplets and microfluidics: generation, manipulation, synthesis, and detection. *Microchim Acta* 186(2):55
- Allazetta S, Lutolf MP (2015) Stem cell niche engineering through droplet microfluidics. *Curr Opin Biotechnol* 35:86–93
- Altmann B, Löchner A, Swain M, Kohal RJ, Giselbrecht S, Gottwald E et al (2014) Differences in morphogenesis of 3D cultured primary human osteoblasts under static and microfluidic growth conditions. *Biomaterials* 35:3208–3219
- Amaral AJ, Pasparakis G (2016) Rapid formation of cell aggregates and spheroids induced by a “smart” boronic acid copolymer. *ACS Appl Mater Interfaces* 8(35):22930–22941
- Anada T, Masuda T, Honda Y, Fukuda J, Arai F, Fukuda T et al (2010) Three-dimensional cell culture device utilizing thin membrane deformation by decompression. *Sensors Actuators B Chem* 147:376–379
- Ananthanarayanan A, Nugraha B, Triyatni M, Hart S, Sankurati S, Yu H (2014) Scalable spheroid model of human hepatocytes for hepatitis C infection and replication. *Mol Pharm* 11:2106–2114
- Ashammakhi N, Elkhannas E, Hasan A (2018) Translating advances in organ-on-a-chip technology for supporting organs. *J Biomed Mater Res B Appl Biomater* 107(6):2006–2018
- Ashida T, Sakai S, Taya M (2013) Competing two enzymatic reactions realizing one-step preparation of cell-enclosing duplex microcapsules. *Biotechnol Prog* 29(6):1528–1534
- El Assal R, Gurkan UA, Chen P, Juillard F, Tocchio A, Chinnasamy T et al (2016) 3-D microwell array system for culturing virus infected tumor cells. *Scie Rep* 6:39144
- Bartosh TJ, Ylöstalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K et al (2010) Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their anti-inflammatory properties. *Proc Natl Acad Sci* 107:13724–13729
- Basova EY, Foret F (2015) Droplet microfluidics in (bio) chemical analysis. *Analyst* 140:22–38
- Carvalho MP, Costa EC, Miguel SP, Correia IJ (2016) Tumor spheroid assembly on hyaluronic acid-based structures: a review. *Carbohydr Polym* 150:139–148
- Carvalho MR, Lima D, Reis RL, Correlo VM, Oliveira JM (2015) Evaluating biomaterial-and microfluidic-based 3D tumor models. *Trends Biotechnol* 33:667–678
- Cesarz Z, Tamama K (2015) Spheroid culture of mesenchymal stem cells. *Stem Cells Int* 2016:9176357
- Chan HF, Zhang Y, Ho YP, Chiu YL, Jung Y, Leong KW (2013) Rapid formation of multicellular spheroids in double-emulsion droplets with controllable microenvironment. *Sci Rep* 3
- Charwat V, Egger D (2018) The third dimension in cell culture: from 2D to 3D culture formats, in cell culture technology. Springer, pp 75–90
- Chen J, Elsayed MY, Wei Y, Mousa N (2017) Advances in micro-and nanotechnologies for stem cell-based translational applications. In: *Advances in Stem Cell Therapy*. Springer, pp 277–302
- Chen M et al (2019a) Naked liquid marbles: a robust three-dimensional low-volume cell culturing system. *ACS Appl Mater Interfaces*
- Chen MC, Gupta M, Cheung KC (2010) Alginate-based microfluidic system for tumor spheroid formation and anticancer agent screening. *Biomed Microdevices* 12:647–654

- Chen R, Sun Z, Chen D (2019b) Droplet-based microfluidics for cell encapsulation and delivery, in microfluidics for pharmaceutical applications. *Elsevier*, pp 307–335
- Ching SH, Bansal N, Bhandari B (2017) Alginate gel particles—a review of production techniques and physical properties. *Crit Rev Food Sci Nutr* 57(6):1133–1152
- Choi A, Seo K, Kim DW, Kim BC, Kim DS (2017) Recent advances in engineering microparticles and their nascent utilization in biomedical delivery and diagnostic applications. *Lab Chip* 17(4):591–613
- Chua KN, Lim WS, Zhang P, Lu H, Wen J, Ramakrishna S et al (2005) Stable immobilization of rat hepatocyte spheroids on galactosylated nanofiber scaffold. *Biomaterials* 26:2537–2547
- Clausell-Tormos J, Lieber D, Baret JC, El-Harrak A, Miller OJ, Frenz L et al (2008) Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms. *Chem Biol* 15:427–437
- Cottet J et al (2019) Dielectrophoresis-assisted creation of cell aggregates under flow conditions using planar electrodes. *Electrophoresis*
- Du X et al (2018) Droplet array-based 3D coculture system for high-throughput tumor angiogenesis assay. *Anal Chem* 90(5):3253–3261
- Dudani JS, Go DE, Gossett DR, Tan AP, Di Carlo D (2014) Mediating millisecond reaction time around particles and cells. *Anal Chem* 86:1502–1510
- Egger D et al (2018) Dynamic cultivation of mesenchymal stem cell aggregates. *Bioengineering* 5(2):48
- Elkayam T, Amitay-Shaprut S, Dvir-Ginzberg M, Harel T, Cohen S (2006) Enhancing the drug metabolism activities of C3A—a human hepatocyte cell line-by tissue engineering within alginate scaffolds. *Tissue Eng* 12:1357–1368
- Fabisiewicz A, Grzybowska E (2017) CTC clusters in cancer progression and metastasis. *Med Oncol* 34:12
- Fu CY, Tseng SY, Yang SM, Hsu L, Liu CH, Chang HY (2014) A microfluidic chip with a U-shaped microstructure array for multicellular spheroid formation, culturing and analysis. *Biofabrication* 6:015009
- Guirao K, Arinze TL (2015) Bioengineering models for breast cancer research. *Breast Cancer: Basic Clin Res* 9:57
- Hamilton GA, Westmoreland C, George E (2001) Effects of medium composition on the morphology and function of rat hepatocytes cultured as spheroids and monolayers. *In Vitro Cell Dev Biol Anim* 37:656–667
- Heinemann S, Wiesmann HP (2013) 3-D osteoblast culture for biomaterials testing. *J Dev Biol Tissue Eng* 5:7–12
- Hendriks J, Visser CW, Henke S, Leijten J, Saris DB, Sun C et al (2015) Optimizing cell viability in droplet-based cell deposition. *Sci Rep* 5:11304
- Henke S, Leijten J, Kemna E, Neubauer M, Fery A et al (2016) Enzymatic crosslinking of polymer conjugates is superior over ionic or UV crosslinking for the on-chip production of cell-laden microgels. *Macromol Biosci* 16(10):1524–1532
- Higuchi A, Ling QD, Chang Y, Hsu ST, Umezawa A (2013) Physical cues of biomaterials guide stem cell differentiation fate. *Chem Rev* 113:3297–3328
- Huang G, Li M, Yang Q, Li Y, Liu H, Yang H et al (2016) Magnetically actuated droplet manipulation and its potential biomedical applications. *ACS Appl Mater Interfaces*
- Hsiao AY, Ys T, Tung YC, Sud S, Taichman RS, Pienta KJ et al (2009) Microfluidic system for formation of PC-3 prostate cancer co-culture spheroids. *Biomaterials* 30:3020–3027
- Huang H, He X (2014) Interfacial tension based on-chip extraction of microparticles confined in microfluidic stokes flows. *Appl Phys Lett* 105:143704
- Imaninezhad M et al (2018) Templated macroporous polyethylene glycol hydrogels for spheroid and aggregate cell culture. *Bioconjug Chem* 30(1):34–46
- Ito K, Sakuma S, Kimura M, Takebe T, Kaneko M, Arai F (2016) Temporal transition of mechanical characteristics of HUVEC/MSC spheroids using a microfluidic chip with force sensor probes. *Micromachines* 7:221
- Jackson E, Lu H (2016) Three-dimensional models for studying development and disease: moving on from organisms to organs-on-a-chip and organoids. *Integr Biol* 8(6):672–683
- Jang M, Yang S, Kim S (2016) Microdroplet-based cell culture models and their application. *BioChip J* 10:310–317
- Jeong SY, Lee JH, Shin Y, Chung S, Kuh HJ (2016) Co-culture of tumor spheroids and fibroblasts in a collagen matrix-incorporated microfluidic chip mimics reciprocal activation in solid tumor micro-environment. *PLoS One* 11:e0159013
- Jin HJ, Cho YH, Gu JM, Kim J, Oh YS (2010) A multicellular spheroid formation and extraction chip using removable cell trapping barriers. *Lab Chip* 11:115–119
- Kamperman T, Henke S, Van den Berg A, Shin S, Tamayol A et al (2017a) Single cell microgel based modular bioinks for uncoupled cellular micro-and macroenvironments. *Adv Healthc Mater* 6(3)
- Kamperman T, Henke S, Visser C, Karperien M, Leijten J (2017b) Centering single cells in microgels via delayed crosslinking supports long-term 3D culture by preventing cell escape. *Small* 13(22)
- Kamperman T, Henke S, Zoetebier B, Ruiterkamo N, Wang R et al (2017c) Nanoemulsion-induced enzymatic crosslinking of tyramine-functionalized polymer droplets. *J Mater Chem B*
- Kang E, Choi YY, Jun Y, Chung BG, Lee SH (2010) Development of a multi-layer microfluidic array chip to culture and replate uniform-sized embryoid bodies without manual cell retrieval. *Lab Chip* 10:2651–2654
- Kashaninejad N, Nikmaneshi MR, Moghadas H, Kiyomarsi Oskouei A, Rismanian M, Barisam M et al (2016) Organ-tumor-on-a-chip for chemosensitivity assay: a critical review. *Micromachines* 7:130
- Kelm JM, Djonov V, Ittner LM, Fluri D, Born W, Hoerstrup SP et al (2006) Design of custom-shaped vascularized tissues using microtissue spheroids as minimal building units. *Tissue Eng* 12:2151–2160
- Kelm JM, Fussenegger M (2010) Scaffold-free cell delivery for use in regenerative medicine. *Adv Drug Deliv Rev* 62:753–764
- Khanmohammadi M, Sakai S, Ashida T, Taya M (2016) Production of hyaluronic-acid-based cell-enclosing microparticles and microcapsules via enzymatic reaction using a microfluidic system. *J Appl Polym Sci* 133(16)
- Khoury M, Bransky A, Korin N, Konak LC, Enikolopov G, Tzchori I et al (2010) A microfluidic traps system supporting prolonged culture of human embryonic stem cells aggregates. *Biomed Microdevices* 12:1001–1008
- Kim C, Lee KS, Bang JH, Kim YE, Kim MC, Oh KW et al (2011) 3-dimensional cell culture for on-chip differentiation of stem cells in embryoid body. *Lab Chip* 11:874–882
- Kim JB, Stein R, O'Hare MJ (2004) Three-dimensional in vitro tissue culture models of breast cancer—a review. *Breast Cancer Res Treat* 85:281–291
- Kim JB (2005) Three-dimensional tissue culture models in cancer biology. *Semin Cancer Biol* 15(5):365–377
- Kim JH, Jeon TY, Choi TM, Shim TS, Kim SH, Yang SM (2013) Droplet microfluidics for producing functional microparticles. *Langmuir* 30:1473–1488
- Kim S, Oh J, Cha C (2016) Enhancing the biocompatibility of microfluidics-assisted fabrication of cell-laden microgels with channel geometry. *Colloids Surf B: Biointerfaces* 147:1–8
- Kim T, Doh I, Cho YH (2012) On-chip three-dimensional tumor spheroid formation and pump-less perfusion culture using gravity-driven cell aggregation and balanced droplet dispensing. *Biomicrofluidics* 6:034107

- Knight E, Przyborski S (2015) Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. *J Anat* 227:746–756
- Knowlton S, Cho Y, Li XJ, Khademhosseini A, Tasoglu S (2016) Utilizing stem cells for three-dimensional neural tissue engineering. *Biomater Sci* 4:768–784
- Kojima R, Yoshimoto K, Takahashi E, Ichino M, Miyoshi H, Nagasaki Y (2009) Spheroid array of fetal mouse liver cells constructed on a PEG-gel micropatterned surface: upregulation of hepatic functions by co-culture with nonparenchymal liver cells. *Lab Chip* 9:1991–1993
- Kwak B et al (2018) Mass fabrication of uniform sized 3D tumor spheroid using high-throughput microfluidic system. *J Control Release* 275: 201–207
- Labusca L (2015) Scaffold free 3D culture of mesenchymal stem cells; implications for regenerative medicine. *J. Transpl. Stem Cell Biol.* 2(1):1–5
- Landry J, Bernier D, Ouellet C, Goyette RA, Marceau N (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* 101:914–923
- Langenbach F, Naujoks C, Smeets R, Berr K, Depprich R, Kübler N et al (2013) Scaffold-free microtissues: differences from monolayer cultures and their potential in bone tissue engineering. *Clin Oral Investig* 17:9–17
- Laschke M, Schank T, Scheuer C, Kleer S, Schuler S, Metzger W et al (2013) Three-dimensional spheroids of adipose-derived mesenchymal stem cells are potent initiators of blood vessel formation in porous polyurethane scaffolds. *Acta Biomater* 9:6876–6884
- Lazar A, Mann HJ, Rimmel RP, Shatford RA, Cerra FB, Hu WS (1995) Extended liver-specific functions of porcine hepatocyte spheroids entrapped in collagen gel. *In Vitro Cell Dev Biol Anim* 31:340–346
- Lee D, Cha C (2018) The combined effects of co-culture and substrate mechanics on 3D tumor spheroid formation within microgels prepared via flow-focusing microfluidic fabrication. *Pharmaceutics* 10(4):229
- Lee K, Kim C, Yang JY, Lee H, Ahn B, Xu L et al (2012) Gravity-oriented microfluidic device for uniform and massive cell spheroid formation. *Biomicrofluidics* 6:014114
- Lee KH, Kim S-H, Ryoo JH, Wong SF, Lee S-H (2011) Diffusion-mediated in situ alginate encapsulation of cell spheroids using microscale concave well and nanoporous membrane. *Lab Chip* 11: 1168–1173
- Lienemann PS, Rossow T, Mao AS, Vallmajo-Martin Q, Ehrbar M, Mooney D (2017) Single cell-laden protease-sensitive microniche for long-term culture in 3D. *Lab Chip* 17(4):727–737
- Lin RZ, Chou LF, Chien CCM, Chang HY (2006) Dynamic analysis of hepatoma spheroid formation: roles of E-cadherin and β 1-integrin. *Cell Tissue Res* 324:411–422
- Lin RZ, Chang HY (2008) Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 3: 1172–1184
- Liu K, Deng Y, Zhang N, Li S, Ding H, Guo F et al (2012) Generation of disk-like hydrogel beads for cell encapsulation and manipulation using a droplet-based microfluidic device. *Microfluid Nanofluid* 13:761–767
- Liu W et al (2019) A microfluidic platform for multi-size 3D tumor culture, monitoring and drug resistance testing. *Sens Actuators B Chem*
- Mao AS, Shin JW, Utech S, Wang H, Uzun O, Li W et al (2017) Deterministic encapsulation of single cells in thin tunable microgels for niche modelling and therapeutic delivery. *Nat Mater* 16(2):236
- Marsano A, Conficconi C, Lemme M, Occhetta P, Gaudiello E, Votta E et al (2016) Beating heart on a chip: a novel microfluidic platform to generate functional 3D cardiac microtissues. *Lab Chip* 16:599–610
- Mazzitelli S, Capretto L, Quinci F, Piva R, Nastruzzi C (2013) Preparation of cell-encapsulation devices in confined microenvironment. *Adv Drug Deliv Rev* 65:1533–1555
- Mei C et al (2019) Three-dimensional spherical gelatin bubble-based scaffold improves the myotube formation of H9c2 myoblasts. *Biotechnol Bioeng*
- Naruse K (2018) Mechanomedicine. *Biophys Rev* 10(5):1257–1262
- Nyberg SL, Hardin J, Amiot B, Argikar UA, Rimmel RP, Rinaldo P (2005) Rapid, large-scale formation of porcine hepatocyte spheroids in a novel spheroid reservoir bioartificial liver. *Liver Transpl* 11: 901–910
- Okuyama T, Yamazoe H, Mochizuki N, Khademhosseini A, Suzuki H, Fukuda J (2010) Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device. *J Biosci Bioeng* 110:572–576
- Ota H, Yamamoto R, Deguchi K, Tanaka Y, Kazoe Y, Sato Y et al (2010) Three-dimensional spheroid-forming lab-on-a-chip using micro-rotational flow. *Sens Actuators B Chem* 147:359–365
- Ota H, Kodama T, Miki N (2011a) Rapid formation of size-controlled three-dimensional hetero-cell aggregates using micro-rotation flow for spheroid study. *Biomicrofluidics* 5:034105
- Ota H, Kodama T, Yamato M, Okano T, and Miki N. 2011b. Micro-rotation flow chamber rapidly forming collagen gel-mediated hetero-spheroids, in *Micro-NanoMechatronics and Human Science (MHS), 2011 International Symposium on*, pp. 94–98
- Ota H, Miki N (2011) Microfluidic experimental platform for producing size-controlled three-dimensional spheroids. *Sensors Actuators A Phys* 169:266–273
- Pajoumshariati SR et al (2018) Microfluidic-based cell-embedded microgels using nonfluorinated oil as a model for the gastrointestinal niche. *ACS Appl Mater Interfaces* 10(11):9235–9246
- Patra B, Chen YH, Peng CC, Lin SC, Lee CH, Tung YC (2013) A microfluidic device for uniform-sized cell spheroids formation, culture, harvesting and flow cytometry analysis. *Biomicrofluidics* 7: 054114
- Patra B, Peng CC, Liao WH, Lee CH, Tung YC (2016) Drug testing and flow cytometry analysis on a large number of uniform sized tumor spheroids using a microfluidic device. *Sci Rep* 6
- Porto DA, Rouse TM, San-Miguel A, Lu H (2016) Microfluidic platforms for quantitative biology studies in model organisms. In: *Microfluidic Methods for Molecular Biology*. Springer, pp 1–18
- Qiu X et al (2018) Microfluidic channel optimization to improve hydrodynamic dissociation of cell aggregates and tissue. *Sci Rep* 8(1): 2774
- Rimann M, Latenser S, Gvozdenovic A, Muff R, Fuchs B, Kelm JM et al (2014) An in vitro osteosarcoma 3D microtissue model for drug development. *J Biotechnol* 189:129–135
- Rossow T, Lienemann PS, Mooney D (2017) Cell microencapsulation by droplet microfluidic templating. *Macromol Chem Phys* 218(2)
- Sabhachandani P, Motwani V, Cohen N, Sarkar S, Torchilin V, Konry T (2016) Generation and functional assessment of 3D multicellular spheroids in droplet-based microfluidics platform. *Lab Chip* 16: 497–505
- Sakai S, Ashida T, Ogino S, Taya M (2014) Horseradish peroxidase-mediated encapsulation of mammalian cells in hydrogel particles by dropping. *J Microencapsul* 31(1):100–104
- Salim A, Fourar M, Pironon J, Sausse J (2008) Oil–water two-phase flow in microchannels: flow patterns and pressure drop measurements. *Can J Chem Eng* 86:978–988
- Samal P et al (2019) Grow with the flow: when morphogenesis meets microfluidics. *Adv Mater*:1805764
- Sarem M et al (2019) Cell number in mesenchymal stem cell aggregates dictates cell stiffness and chondrogenesis. *Stem Cell Res Ther* 10(1): 10

- Sart S, Agathos SN, Li Y, Ma T (2016) Regulation of mesenchymal stem cell 3D microenvironment: from macro to microfluidic bioreactors. *Biotechnol J* 11:43–57
- Sart S, et al. 2019. Mapping structure and biological functions within mesenchymal bodies using microfluidics bioRxiv p 514158
- Schultz KM, Furst EM (2011) High-throughput rheology in a microfluidic device. *Lab Chip* 11:3802–3809
- Shamloo A (2014) Cell-cell interactions mediate cytoskeleton organization and collective endothelial cell chemotaxis. *Cytoskeleton* 71: 501–512
- Shao C et al (2019) Droplet microarray on patterned butterfly wing surfaces for cell spheroid culture. *Langmuir*
- Shi W, Weng D, Niu W (2016) Nanoparticle drug delivery systems and three-dimensional cell cultures in cancer treatments and research. *Cancer Transl Med* 2:154
- Silva TP et al (2019) Design principles for pluripotent stem cell-derived organoid engineering. *Stem Cells Int* 2019:4508470
- Skardal A, Shupe T, Atala A (2016) Organoid-on-a-chip and body-on-a-chip systems for drug screening and disease modeling. *Drug Discov Today* 21(9):1399–1411
- Sudeepthi A, Sen AK, Yeo L (2019) Aggregation of a dense suspension of particles in a microwell using surface acoustic wave microcentrifugation. *Microfluid Nanofluid* 23(5):76
- Sun D, Lu J, Chen Z, Yu Y, Li Y (2014) A novel three-dimensional microfluidic platform for on-chip multicellular tumor spheroid formation and culture. *Microfluid Nanofluid* 17:831–842
- Torisawa Y, Chueh B, Huh D, Ramamurthy P, Roth TM, Barald KF et al (2007) Efficient formation of uniform-sized embryoid bodies using a compartmentalized microchannel device. *Lab Chip* 7:770–776
- Torres A et al (2018) Guiding morphogenesis in cell-instructive microgels for therapeutic angiogenesis. *Biomaterials* 154:34–47
- Tung Y-C et al (2011) High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *Analyst* 136(3):473–478
- Verboet PE, Borovinskaya O, Meyer N, Günther D, Dittrich PS (2014) A new microfluidics-based droplet dispenser for ICPMS. *Anal Chem* 86:6012–6018
- Visone R, Gilardi M, Marsano A, Rasponi M, Bersini S, Moretti M (2016) Cardiac meets skeletal: what's new in microfluidic models for muscle tissue engineering. *Molecules* 21:1128
- Wagner O, Thiele J, Weinhart M, Mazutis L, Weitz DA, Huck WT et al (2016) Biocompatible fluorinated polyglycerols for droplet microfluidics as an alternative to PEG-based copolymer surfactants. *Lab Chip* 16:65–69
- Walser R, Metzger W, Görg A, Pohlemann T, Menger M, Laschke M (2013) Generation of co-culture spheroids as vascularisation units for bone tissue engineering. *Eur Cell Mater* 26:222–233
- Wang J, Cheng Y, Yu Y, Fu F, Chen Z, Zhao Y et al (2015a) Microfluidic generation of porous microcarriers for three-dimensional cell culture. *ACS Appl Mater Interfaces* 7:27035–27039
- Wang J, Li Y, Wang X, Wang J, Tian H, Zhao P et al (2017) Droplet microfluidics for the production of microparticles and nanoparticles. *Micromachines* 8(1):22
- Wang W, Itaka K, Ohba S, Nishiyama N, Chung UI, Yamasaki Y et al (2009) 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials* 30:2705–2715
- Wang Y, Wang J (2014) Mixed hydrogel bead-based tumor spheroid formation and anticancer drug testing. *Analyst* 139:2449–2458
- Wang Y, Zhao L, Tian C, Ma C, Wang J (2015b) Geometrically controlled preparation of various cell aggregates by droplet-based microfluidics. *Anal Methods* 7:10040–10051
- Wilson JL, Najia MA, Saeed R, McDevitt TC (2014) Alginate encapsulation parameters influence the differentiation of microencapsulated embryonic stem cell aggregates. *Biotechnol Bioeng* 111:618–631
- Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H et al (2003) Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. *Arthritis Rheum* 48:418–429
- Wu J, Chen Q, Liu W, He Z, Lin JM (2016) Recent advances in microfluidic 3D cellular scaffolds for drug assays. *TrAC Trends Anal Chem*
- Wu LY, Di Carlo D, Lee LP (2008) Microfluidic self-assembly of tumor spheroids for anticancer drug discovery. *Biomed Microdevices* 10: 197–202
- Yamaguchi Y, Ohno J, Sato A, Kido H, Fukushima T (2014) Mesenchymal stem cell spheroids exhibit enhanced in-vitro and in-vivo osteoregenerative potential. *BMC Biotechnol* 14:1
- Yang S, Fu C, Tseng S, Srinivasu V, Shilpa S, Chang H, et al. 2012. NUSAS: negative pressure driving HEPG2/3T3 cells mixing/gradient co-culture inside U trapper array on rapid multicellular spheroid assembling system, in *Micro Electro Mechanical Systems (MEMS), 2012 IEEE 25th International Conference on*, pp. 1077–1080
- Yap KK, Dingle AM, Palmer JA, Dhillon RS, Lokmic Z, Penington AJ et al (2013) Enhanced liver progenitor cell survival and differentiation in vivo by spheroid implantation in a vascularized tissue engineering chamber. *Biomaterials* 34:3992–4001
- Yoon S, Kim JA, Lee SH, Kim M, Park TH (2013) Droplet-based microfluidic system to form and separate multicellular spheroids using magnetic nanoparticles. *Lab Chip* 13:1522–1528
- Yu L, Chen MC, Cheung KC (2010) Droplet-based microfluidic system for multicellular tumor spheroid formation and anticancer drug testing. *Lab Chip* 10:2424–2432
- Zhu K et al (2019) All-aqueous phase microfluidics for cell encapsulation. *ACS Appl Mater Interfaces* 11(5):4826–4832
- Ziółkowska K, Stelmachowska A, Kwapiszewski R, Chudy M, Dybko A, Brzózka Z (2013) Long-term three-dimensional cell culture and anticancer drug activity evaluation in a microfluidic chip. *Biosens Bioelectron* 40:68–74

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