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Microtechnology-based three-dimensional spheroid formation

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1. ABSTRACT

This article highlights the current state of three-dimensional spheroid/tissue formation technologies offering a new experimental platform that is both as reproducible as conventional *in vitro* experiments and highly correlated to *in vivo* conditions. Three-dimensional tissue exhibit higher biological functions and reflect the *in vivo* context more precisely than classical two-dimensional cultures or monolayers. The applications cover highly efficient drug screening, regenerative medicine and fundamental biological research. Numerous three-dimensional spheroid and tissue formation devices have been developed over the past six decades. In early studies, mass production of spheroids using shaking, and non-adhesive surfaces and scaffolds in flasks was conducted. With the emergence of micro/nano fabrication technology and fundamental understanding of micro/nano fluidics, micro/nano devices capable of forming three-dimensional spheroids in a well-controlled manner have been extensively studied. The cell species composing spheroids include hepatic cells, cancer cells, primary cells and, more recently, stem cells. Formation of hetero-spheroids composed of different cells has also been attempted to further resemble the *in vivo* conditions. These new trends in spheroid research are particularly highlighted in this review.

2. INTRODUCTION

Cell species in the human body, including liver cells and stem cells, form and maintain three-dimensional structures by communicating with neighboring cells and/or extracellular matrix (ECM) (1). These interactions caused by connections between cells and/or ECM are associated with the formation of complex signaling networks and increased synthesis of physiologically active substances (2) (3). This leads to discrepancies between *in vivo* and *in vitro* conditions. As *in vivo* experiments are difficult to perform, due to large individual variations and the necessity of approval for animal experiments, it is imperative to optimize the *in vitro* culture environment to more closely resemble the *in vivo* environment, i.e., maintaining the biological functions of human body cells (4) (5), thereby allowing more precise predictions in clinical trials.

Monolayer culture on a petri dish or flask coated with biomicromolecules, such as collagen and fibronectin is commonly used in bio-research (6) (7), industry (8) and medical fields (9) because of its ease of handling and observing cells reactions, such as growth, morphological changes, fluorescence reactions, etc., under a microscope. However, *in vitro* primary cultured cells derived from animals lose their specific functions in short-term culture

Table 1. Mass production devices for spheroid/tissue formation

Category		Method	Advantages	Disadvantages	References
Mass production	Large systems	Oscillatory agitation	Mass production	Requires special equipment	(12)
			Easy to scale up	Cannot control spheroid size or number	
			Formation of mixed hetero-spheroids		
		Rocker	Mass production	Requires special equipment	(39) (40)
			Easy to scale up	Cannot control spheroid size or number	
			Formation of mixed hetero-spheroids		
		Spinner flask	Mass production	Requires special equipment	(23)
			Easy to scale up	Cannot control spheroid size or number	
			Formation of mixed hetero-spheroids	High shear force	
		NASA rotary system	Mass production	Require special equipment	(43)
			Easy to scale up		
			Control of size (limited)		
			Easy to control culture condition		
			Formation of mixed hetero-sphroids		
	Dish-based cultures	Hanging drop	Inexpensive	Scale up difficult	(44) (45)
			Control of size (limited)	Cannot control spheroid size or number	
			Formation of mixed hetero-sphroids	Dependent on skill	
			Rapid formation of spheroids		
		Primaria dish	Inexpensive	Cannot control spheroid size or number	(4)
			Simple to perform	Only allows formation of specific cell types	
			Good spheroid viabilty		
	Scaffolds (PGS, PUF, Matrigel, Agarose, Collagen)	3-D scaffold	Mass production	Requires special equipment	(1) (32) (33) (7) (48)
			Easy to scale up	Only allows formation of specific cell types	
			Three dimensional support of spheroid	Spheroids can flow out	
			Constant supply of nutrition		

when they are cultured as monolayers, probably as a result of differences in *in vitro* and *in vivo* communication networks (4).

In order to realize *in vitro* culture conditions that maintain *in vivo* cell functions, numerous attempts have been made to form cell aggregates, known as spheroids. A representative cell species that maintains and develops function in a spheroid form is the hepatocyte (10). Hepatocytes account for 80% of the liver and are responsible for more than 500 specific functions related to metabolic and detoxification processes in the liver. During tissue formation from isolated cells, they increase in cell density and strengthen their metabolic capacity, exhibiting urea synthesis, bile acid secretion and albumin secretion (2) (3). Hepatic spheroids therefore possess much of the potential activities of the liver. Thus, *in vitro* spheroid- and tissue-forming devices would be useful for assisting in the development of artificial livers (8) (11) (12), achieving reliable and predictable *in vitro* data on drug trials in humans and other animals (13) (14) (15) (16) and investigating differences between cells and tissues (17) (18).

The first report on three-dimensional tissue formation was by Moscona in 1952, and it revealed that cells dissociated from embryonic tissue aggregated during culture (19). In the 1950s, tissue cultures were formed on dishes using specimens from adults and pigs (20) (21). During the 1960s, when useful cell media, enzymes, disposable plastic devices and culture conditions were developed, more complex tissue culture systems than two-dimensional culture, including mass culture (22), co-culture and three-dimensional culture, were gradually developed (22). In the 1980s, spinner flasks, which can mix and aggregate cells on non-adhesive surfaces, were exploited (23-27). From the 1980s to the early 1990s, various scaffold culture devices to form three-dimensional spheroids were developed based on biomacromolecules and polymers such as agarose (28) (29), polyHEMA (30) (31), proteoglycans (32), polyurethane foam (PUF) (33) and positively charged polystyrene (34). Using the above-mentioned three-dimensional spheroid formation methods, translational research into tumor tissues composed of cancer cells from a cell line was performed in the 1980s (35) (36). In the 1990s, primary cultured cells, such as hepatocytes, were used in scaffold-based spheroid formation devices for fundamental study of tissue engineering (37) and clinical applications as spare organs. Scaffold-based spheroid-forming devices were useful for clinical applications because of their potential for mass production.

A lot of mass production models have been developed and used before spheroid formation devices based on micro-technology. Mass production models can produce thousands of spheroids in a single test (Table 1). Representative devices are based on oscillatory and agitation culture (12) (49), shaking flasks (38), rockers (39) (40) and the NASA rotary system (41) (42). These devices, which are large and require numerous components, form spheroids as a result of random contact in cell suspensions. Although spheroid size cannot be controlled due to the random nature of the cell contact, Thielecke *et al.* reported that the NASA rotary system was able to create numerous spheroids and that the diameter of 90% of the formed spheroids was less than 150 micrometers (43). When hepatic spheroids are more than 180 micrometers in diameter, the center of the spheroids undergoes necrosis due to a lack of oxygen and nutrition. The lack of an ability to control size may lead to decreased reproducibility of results and difficulty in investigating morphological and functional changes of individual spheroids.

The hanging drop method (44) (45) and Primaria dishes (4) do not require large systems or components.

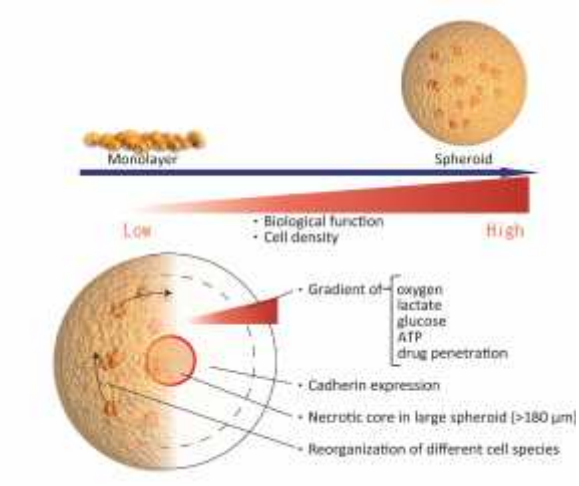

Figure 1. Spheroid characteristics. Spheroids show increased biological function and increased cell density. Hepatic spheroids form from cell aggregates, and accumulate and express E-cadherin. Pathophysiological gradients of oxygen, lactate, glucose, ATP and drug penetration are subsequently established in spheroids. However, this leads to a lack of oxygen and nutrition in the core, resulting in necrosis. In co-culture of hepatocytes and endothelial cells, endothelial cells move into spheroids and form endothelial networks.

Primaria dishes possess a positively charged surface that prevents cells from adhering, thereby forming spheroids. This device cannot control spheroid size, but the method for culturing cells and spheroids is the same as for two-dimensional culture, and this simplifies the process of forming spheroids. The hanging drop method is used to form embryos and has been adapted to spheroid formation. As hanging drop culture involves culturing and aggregating cells in a drop of culture medium suspended from the lid of a culture dish, cells settle out and aggregate due to the microgravity acting in each drop. This method can control spheroid size based on cell density and drop size, and does not require specific systems or devices; however, the formation of spheroids depends on technician skill, which reduces reproducibility.

Scaffold devices can create three-dimensional spheroids by filtering and adhering cells in polymers, such as PGS (poly (glycerol-sebacate) (46) (1) and PUF (33) or biomacromolecules, such as agarose (28), matrigel (47) and collagen (48). These devices form and maintain numerous three-dimensional spheroids perfused with fresh medium by connection with a perfusion system, and allow culture conditions to be readily changed. Finatsu *et al.* developed a hybrid artificial liver using a PUF-based scaffold and applied it to clinical practice (33). Although spheroid size cannot be controlled, scaffolds are ideal when used as components in artificial organs that need large-scale production and culture of spheroids. Scaffold-based devices using biomacromolecules take advantage of cell migration and adhesion to form three-dimensional spheroids. These devices cannot readily control spheroid size, and the relationship between cells and substrates needs to be considered, although most biomacromolecules are commercial products and are easily obtained. Furthermore, hydrogels have been used to supply oxygen and nutrition to cells in spheroids without causing necrosis or creating vessels. Thus, since the 1970s, mass production models have been used for artificial organs and research that requires mass production of spheroids (32).

Mass production approaches have difficulties in controlling spheroid size, and culture conditions are difficult to change. Therefore, these devices are not suitable for drug screening applications or fundamental biological studies, which require spheroids of designated sizes with good uniformity and adjustable culture conditions.

Since the late 1990s, there have been numerous three-dimensional spheroid formation “micro” devices resulting from the rapid advancement of micro/nano fabrication technology and understanding of microfluidics (49-55). Spheroid formation devices might need to satisfy five criteria: (1) form cell aggregates with good size controllability; (2) allow formed spheroids to be cultured for long periods (i.e., longer than one day) and permit their morphologies to be observed at any time; (3) allow reagents that dye or chemically stimulate cells to be supplied into the system; (4) have space for spheroids to grow; and (5) form spheroids with a high throughput. The spheroid formation “micro” devices are expected to have the potential to overcome these criteria.

In this paper, we review the state of three-dimensional spheroid formation methods, particularly, highlighting the emergence of micro/nano technology-based devices. We also discuss the future perspectives of spheroid research.

3. CELL SPECIES

As stated in the Introduction, hepatocytes have frequently been used in spheroid research. In addition to hepatic spheroids, spheroids composed of cancer cells are able to illustrate the hypoxic conditions in tumor tissue and have been proposed as a model for *in vivo* translational research (Figure 1).

For clinical applications, such as artificial organs, primary liver cells are used. Experiments have revealed variations in the characteristics and morphology of the cells forming spheroids, including upregulation of beta 1 integrin and E cadherin, lactate accumulation and oxygen gradient, ATP distribution, cell proliferation and gene changes (56).

Almost all organs consist of multiple cellular species. In addition, spheroids do not have vessels to supply oxygen and nutrients to the cells inside, which prevents the spheroids from growing and can even result cell necrosis. Therefore, hetero-spheroids consisting of different cell species are considered to more closely imitate human tissues than mono-spheroids composed of only one cell species, and may be useful as optimal models in research into tissue engineering, invasion and metastasis (57). Several methods for forming hetero-spheroids have

Table 2. Micro/nano devices for spheroid/tissue formation

Category		Method	Advantages	Disadvantages	References
Micro manipulation	Microstructure	Micro-well	Good control of spheroid size	Requires special equipment	(53) (54)
			Simple to perform		
			Co-culture fo spheroids and monolayer		
			Simple structure		
	Microfluidics	Micro-container	Good control of spheroid size	High shear force	(71) (72)
			Constant supply of nutrition	Scale up difficult	
			Rapid cell aggregation		
		Self-assembly structure	Good control of spheroid size	Scale up difficult	(99)
			Rapid cell aggregation		
			Formation of small spheroids		
		Semi-porous membrane model	Good control of spheroid size	Only formation of specific cell types	(100) (101)
			Simple structure	Scale up difficult	
			Simple to perform		
		Micro-rotation flow	Good control of spheroid size	High shear force	(49)
			Rapid cell aggregation	Dependent on skill	
			Simple structure	Scale up difficult	
			Constant supply of nutrition		
	Gel beads	Hydrogel beads	Good control of spheroid size	Requires special equipment	(76)
	(Alginate, Gelatin, PEG, Matrigel, Collagen)		Rapid cell aggregation	Dependent on skill	
			Formation of large spheroids		
		DEP	Rapid cell aggregation	Potential negative effects of external force	(89)
		Magnetic force	Rapid cell aggregation	Potential negative effects of external force	(51)
		Ultrasonic force	Rapid cell aggregation	Potential negative effects of external force	(88)
	Nano/micro-modefied surface	Micro-contact printing	Good control of spheroid size	Only allows formation of specific cell types	
			Co-culture of different cell species		
		PIPAAm	Inexpensive	Time consuming	(90)
			Simple to perform	Dependent on skill	
			Allows formation of tissue via build-up		
			Constant supply of nutrition		
			Co-culture of different cell species		
	Others	Paper culture plate	Inexpensive	Time consuming	(67)
			Build-up on culture plates	Depend on skill	
			Observation of each layer		
		Eudragit	Simple to perform	Only allows formation of specific cell types	(94)
		Avidin-biotin binding	Simple to perform	Cannnot control spheroid size or number	(95)
			Rapid cell aggregation		

been reported (Figure 2) (38) (58) (59), and the arrangement of different cells was found to be critical in determining spheroid functions. Co-cultures composed of HepG2 spheroids and 3T3 monolayers formed by micro-patterning are reported to have higher hepatic functions than random co-culture of HepG2 and 3T3 cells (mixing spheroids) (60). Randomly mixed spheroids consisting of fibroblasts and endothelial cells exhibit internal endothelial networks (61) (62). On the other hand, co-culture of hepatic spheroids coated by endothelial monolayers did not result in endothelial networks, and gave high albumin synthesis when compared with randomly mixed spheroids of hepatocytes and endothelial cells. Co-culture models are required for the desired spheroid formation depending on the optimal state of the cell species (62).

Spheroids consisting of stem cells are suitable as models for both regenerative medicine, and for more precise drug screening. Formation of spheroids of bone marrow stromal stem cells (BMSSC) and embryo stem (ES) cells (63) (64) have been reported. Induced pluripotent stem (iPS) cells are expected to be able to imitate congenital disorders in patients and assist in the development of tailored medication. Development of devices that can efficiently form stem cell spheroids is thus vitally important.

4. MICRO/NANO SPHEROID FORMATION DEVICES

Micro/nano spheroid formation devices that have microfluidic components, including channels, chambers, pumps, valves, electrodes, etc., manufactured by mechanical and chemical microfabrication techniques, such as micromachining and photolithography, can manipulate cells on the micro scale and create spheroids and tissues in a more controlled manner by exploiting mechanical, electrical and magnetic forces (Figure 3) (51). The application of micro/nano devices to chemistry and biology, termed as microTAS (micro total analysis

Figure 2. Hetero-spheroids composed of endothelial cells and hepatocytes. (a), (b) and (c) show hetero-hepatic spheroids containing 0%, 5% and 20% endothelial cells, respectively.

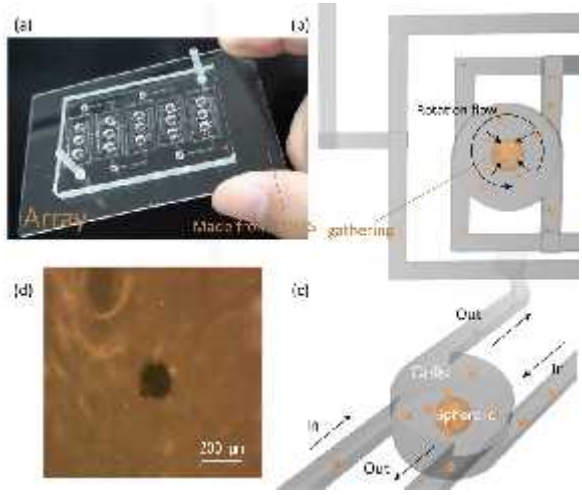

Figure 3. Conceptual images of micro devices for forming three-dimensional hetero-spheroids using micro-rotational flow. (a) Image of fabricated device. (b), (c) Schematic diagrams of spheroid formation array. (d) Hepatocytes are assembled by micro-rotation flow and form spheroids in the chamber centers.

systems), has been increasing rapidly since the late 1990s (Table 2).

PDMS (poly dimethylsiloxane) are most widely used material for microTAS. PDMS is transparent, which is beneficial in measurement of cell functions *in situ*, is resistant against reagents and heat, and highly oxygen permeable (65). Degassed liquid PDMS is poured over the mold, which is often micro manufactured by photolithography, to form channels and wells at microscale in the device (66), and the PDMS structures are cured and then peeled from the molds. MicroTAS devices complete when the PDMS structures are bonded to glass substrates after oxygen plasma treatment to activate PDMS surface. Oxygen can be constantly supplied to cells just by placing an oxygen chamber outside of the cell culture chamber owing to the oxygen permeability of PDMS (65). Other materials have been explored for high performance as well as cost effective micro TAS devices. Recently, Derda *et al.* developed a cell culture method based on paper (67) (68). The culture plate is formed by printing patterned wax on paper using a commercially available printer. This approach reduces the cost of substrates and will lead to further advancement of spheroid/tissue formation devices in industrial applications.

Devices using nano/micro mechanical or chemical fabrication surfaces can aggregate cells to form spheroids and tissue. Micro-contact printing methods are often used to form three-dimensional spheroids (69). In this method, cells can be aggregated on the patterned surface using a PDMS stamp, which offers advantages in controlling spheroid sizes and constructing spheroid-spheroid (69) and spheroid-monolayer co-cultures consisting of several cell species (70).

Spheroids of several hundred micrometers are compatible with micro/nano technology. Micro-well devices contain wells of around 100 micrometers to confine the space where cells aggregate and form spheroids, thereby precisely determining spheroid size. These micro-wells are manufactured by micro molding (53) (54) and photolithography (44). These devices can control spheroid sizes within a standard deviation of 15% and have good usability (54). Micro contact printing of collagen on the well center has also been reported to be capable of controlling spheroid sizes as precisely as approximately 5% (54). Such surface treatments as extracellular matrix coating enable co-culture of hepatic spheroids and fibroblast monolayers (70).

Various microfluidics-based three-dimensional spheroid-forming devices have been demonstrated, which utilize micro-containers (71) (72), semi-porous membranes (73) (74), and micro-rotation flow (75). The devices are connected with perfusion systems to constantly supply oxygen and nutrition for long-term culture and can induce cell aggregation to form spheroids within a few minutes. Micro-containers, such as micro pillars (71) and U-shaped containers (72) trap cells while the cell suspension is perfused. These micro-container type devices have the capacity for good size control and high-speed spheroid formation. Microfluidic devices that sandwiched semi-porous membranes with two layers of microchannels and captured cells at the pores were demonstrated (73) (74). Cells flow in the top channel, while media perfuse continuously in the bottom channel. Such devices are suitable for forming multilayered spheroids or spheroid co-cultures, and satisfy three of the fiv\e desired criteria: (1) good size control; (2) long-term culture and *in situ* observation; (3) *in situ* stimulation. However, these systems may not be able to provide the space for spheroid growth and have low productivity compared to mass production models. Spheroid formation devices using micro-rotation flow can form three-dimensional spheroids and control their sizes based on chamber characteristics and cell density (Figure 4) (75). Perfusion medium is introduced into a microchamber, in which a micro-rotational flow is generated. Cells are attracted to the center by hydrodynamic forces and they aggregate to form spheroids. The cell densities in the media can be varied in order to control spheroid size, giving a standard deviation of spheroid size of about 18%. However, this device microfluidically maintains spheroids in the chamber, which provides space for spheroid growth. As these microfluidic models are less productive than mass production models, increasing their productivity will make them more attractive and useful.

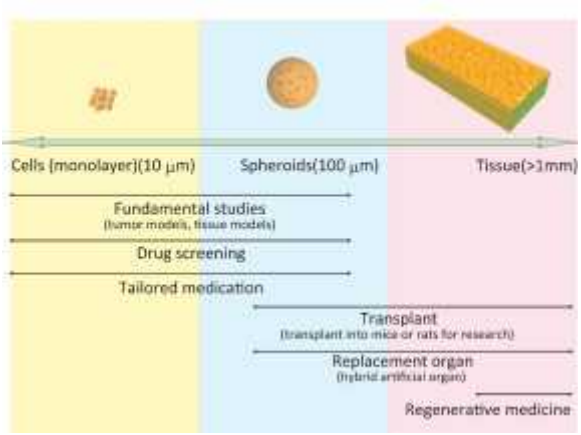

Figure 4. Applications based on size and state of the cells. Single cells and monolayers can be used in fundamental studies and drug screening. In the future, mono- and hetero-spheroids will be used for efficient drug screening and the realization of tailored medicine. In addition, large spheroids and/or artificial tissue, such as multilayered cell sheets, will be the basal technologies of transplant models for *in vivo* research, replacement organs and regenerative medicine.

Spheroid formation devices using hydrogel beads composed of alginate (76), gelatin (77), PEG (poly (ethylene glycol) (78), Matrigel (79) and collagen (80) (81) have also been developed. Hydrogel beads with extremely good size uniformity can be created using microfluidic devices (82). The microfluidic devices can also encapsulate cells in the hydro gel beads, which allows mass production of size-controlled spheroids. This method was experimentally verified to be effective in hetero-spheroid formation (81). Hydrogels can also be used as adhesion agents for binding different cell species. Such hydrogel coating methods have allowed the formation of endothelial monolayers on the outside of previously formed spheroids. Endothelial cell-coated hepatic spheroids exhibit higher metabolic functions when mixed spheroids composed of hepatocytes and endothelial cells (62). Hydrogel micropellet devices have also been demonstrated for mass production of spheroids, and can graft pellets to animals and allow the investigation of binding of grafted tissue to the animal body (83) (84) (85). These spheroid formation devices also result in network or tube structures of endothelial cells within the spheroids (61), but such structures are not functional vessels and are unable to deliver oxygen and nutrition to the cells in the spheroid (86) (87).

External forces including ultrasonic (88) and DEP (89) can manipulate cells to form three-dimensional spheroids. Microfabrication technology can form electrodes and/or magnetic components on the micro scale, which enables cell manipulation in microfluidic devices. However, these external forces may lead to cell injury, and undesirable culture conditions, such as media composed of sucrose and glucose, with have poor electrical conductivity, are sometimes required. The influences on cell viability need to be assessed further.

Tissue formation methods using thermoresponsive polymers, poly (N-isopropylacrylamide) (PIPAAm) (90) (91), can collect sheets of cells that contain extracellular matrix at their base. Several sheets can then be built up, and tissue at the centimeter scale has been prepared in this manner (92). Tissue composed of sheets of oral mucosal epithelium has been transplanted into an actual patient, resulting in a cure for opacification of the cornea (92). This cell sheet method will be adopted not only in therapies for the cornea, but also in therapy for gum disease (93). This cell sheet method may be one of the most promising technologies in clinical regenerative medicine.

5. USEFUL TECHNOLOGIES

Several chemicals are known to augment cell adhesion, which facilitates three-dimensional spheroid formation. Eudragit, a copolymer, promotes binding of cells in spheroids, which increases yield (94). Avidin-biotin complex promotes adhesion between different cell species and allows rapid formation of hetero-cell aggregates (95).

Cell aggregation was achieved in minutes using microtechnology (49) (95). However, we have to note that cell aggregates become spheroids a few days after the cell aggregation, which can be verified by increase of biological functions and it is difficult to predict when the formed cell aggregates become spheroids precisely. However, methods using avidin-biotin as an adhesive agent can form three-dimensional hetero-spheroids within a few minutes.

In order to measure cell functions in spheroids, general biological measurement techniques such as immunofluorescence (96), green fluorescent protein (90), immunostaining of frozen sections (97), Western blotting (98) and RT-PCR (96) are used. However, it is difficult to perform precise or automated measurements of biological function, as it is difficult to the remove media, which may be diluted with a solvent, in which spheroids are cultured (99). To overcome these issues, devices that cannot only culture cells, but also perform automated measurements, and chips that allow PCR to be performed from single cells are currently being developed (99) (100) (101).

6. SCALE-UP OF SPHEROID AND CREATION OF TISSUE *IN VITRO*

Scale-up of spheroids is demanded to create highly functional artificial organs for first-aid in immediate therapies or accidents (33). For this purpose, it is crucial to create sheet-shaped tissue and functional vessels in spheroid and tissue to supply oxygen and nutrition to the cells inside and to overcome the size limitation.

Currently, sheet-shaped tissue is made with piling cell sheet collected from dish coated by PIPAAm or paper containing extracellular matrix gel (102) (67). Multilayerd cell sheet isolated from PIPAAm dish enhanced neovascularization after grafting engineered tissue *in vitro* to the body and improved cardiac function of ischemic hearts in patients *in vivo*. Paper-based multilayered cell sheet enabled us to observe cells movement and function

on each layer easily and verify the role of each cell in the large tissue (67). However, there is the size limitation of spheroids and engineered-tissue. For example, cells in the center of paper-multilayered tissue became dead because of lacking of oxygen and nutrition. HIF-1 (Hypoxia Inducible Factor-1) transcriptional activity, which is barometer showing hypoxia (103), increased in the center of the spheroid and tissue. Therefore, it is imperative to supply nutrition and oxygen to the center of the tissue to create large tissue and maintain cell viability until grafted engineered-tissue connects the organ and neovascularization occurs.

One of potent solution for size-limitation is creating vessel in the engineered-tissue *in vitro* (104). Formation of vessels is divided into two phase: (1) endothelial cells move in the spheroids and form networks and (2) vessels transport oxygen and nutrition. Endothelial cells movement and network formation are observed in a few papers, which increase biological function (61) (62) (80). However, the network was not verified to become functional vessels. To form functional vessels and then, to create large spheroids/tissues will be the next challenge. Another approach to create large tissues is to use spheroids as "parts". Spheroids consisting of different cells are formed in advance and then assembled to form large tissues. Gluing spheroids while constructing networks among them will be a great challenge. This approach may reduce the culture time which is currently longer than 1 week for large tissues, such as multilayered cell sheets, to acquire tissue-specific functions.

7. SUMMARY AND FUTURE PROSPECTS

In current spheroid formation technologies, not all the cells introduced into the device form spheroids. The ratio of the cells used for spheroids to those not used, or efficiency, need to be enhanced, particularly when cells are hard to proliferate, such as primary cells and stem cells. New devices to efficiently form spheroids as well as mechanisms to collect unused cells during perfusion are to be developed.

There is arising a greater demand for devices that can aggregate and form spheroids composed of stem cells in order to allow more precise investigation of human tissues and embryos. The formed stem cell spheroid would maintain biological function specific to the donor. Development of spheroid forming devices capable of inducing ES and iPS cells is mandatory, when device materials, surface conditions, flow velocities, must be optimized.

In industry, it is necessary to reduce manufacturing and material costs. At present, material costs are being reduced using polymers such as PDMS and paper. However, spheroid and tissue formation processes are still performed by hand. Therefore, (semi-)automation of spheroid and tissue formation is necessary to decrease manufacturing costs, increase throughput and guarantee reproducibility. Standardization of protocols including assessment of cell functions will also be mandatory, while numerous gene and protein analyses are currently performed.

Creating 3 dimensional spheroids under zero gravity environment such as space will be an interesting challenge. This zero gravity method may offer stress-free culture to cells and spheroids, and leads to more efficient creation of spheroids and realization of spheroids which have higher biological functions than those created using current three dimensional spheroid creation methods. Currently, there are a few devices which try to decrease gravity loading on cells and spheroids such as NASA rotary cell culture system (43). However, they cannot get rid of gravity force to cells and spheroids completely and load centrifugal force on cells and spheroids instead of decreasing gravity.

Ultrafast control of cell aggregation using micro-robots will be another innovative approach. Sufficient high throughput and precise cell manipulation will contribute ultrafast spheroid formation. This way enables spheroid formation with arbitrary shapes and sizes.

This review has highlighted past and current three-dimensional spheroid/tissue formation devices as new experimental platforms that possess both the biomimetics of *in vivo* models and the reproducibility of *in vitro* models. Although this field has a history of more than six decades, with the help of emerging micro/nano technologies, innovative and practical technologies will continue to be developed and applied to numerous applications beneficial for human life.

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