Journal of Cancer Research and Practice

journal homepage: www.ejcrp.org

Review Article

A Review on the Recent Advancement in "Tumour Spheroids-on-a-Chip"

M. Ibrahim Khot[1]*, Mark A. Levenstein[2], Nikil Kapur[2], David G. Jayne[1]

[1]School of Medicine, Leeds Institute of Medical Research at St. James's, St. James's University Hospital, University of Leeds, Leeds, UK
[2]School of Mechanical Engineering, Institute of Thermofluids, University of Leeds, Leeds, UK

Abstract

Introduction: Three-dimensional (3D) cell cultures are identified as more accurate and representative models of human cancers than conventional two-dimensional monolayer cell cultures. However, currently established 3D culturing techniques are technically challenging, time- and resource-consuming, and performed using traditional laboratory tissue culturing equipment. In recent years, microfluidics has been introduced into biomedical research, allowing cells and tissues to be cultured in microfabricated devices. The current challenge is to adapt existing 3D cell culturing techniques to microfluidic devices, allowing for the fabrication of low-cost, rapid evaluation devices to facilitate biomedical research and clinical application. The aim of this review was to evaluate microfluidics and 3D cell culture research with particular relevance to oncological research. **Methods:** Journal articles were acquired from different scientific databases and were identified using specific keywords. **Three-Dimensional Cell Culturing Microfluidic Concepts:** Various 3D cell culturing microfluidic devices have been designed, based on existing 3D cell culturing methods. This includes non-cell adherent-based devices, matrix-embedding, hanging drop, and droplet-based culturing methods. These platforms facilitate the culturing, treatment, and analysis of 3D spheroids, while simultaneously scaling down traditional experimental requirements. **Limitations and Future Perspectives:** Beyond superficial analysis, a major drawback in the current scope of 3D cell culturing microfluidic devices is the inability to extract spheroids for examining histology. Polydimethylsiloxane is the preferred material to fabricate devices but may need revision for commercializing microfluidic platforms in the future. Integrating 3D bioprinting and organoid cultures could potentially improve the quality of 3D models in microfluidic devices. **Conclusion:** 3D spheroids are an effective representation of *in vivo* cancers and microfluidics has streamlined the culture, treatment, and analysis of 3D models. Considerable improvements have been made in combining the two entities, but further work is required to manufacture 3D cell culturing microfluidic devices on a commercial scale.

Keywords: Cancer, lab-on-a-chip, microfluidics, spheroids, three-dimensional cell culturing

Introduction

The search for new and better *in vitro* models of cancer is an on-going process, which aims to enhance our current

Address for correspondence: Dr. M. Ibrahim Khot, Section of Translational Anaesthesia and Surgical Sciences, St. James's University Hospital, University of Leeds, Wellcome Trust Brenner Building, Level 9, Beckett Street, LS9 7TF, Leeds, UK.
E-mail: m.i.khot@leeds.ac.uk

Received: 12-Sep-2018 **Revised:** 11-Dec-2018 **Accepted:** 12-Dec-2018

Access this article online

Quick Response Code:

Website: www.ejcrp.org

DOI: 10.4103/JCRP.JCRP_23_18

How to cite this article: Khot MI, Levenstein MA, Kapur N, Jayne DG. A review on the recent advancement in "tumour spheroids-on-a-chip". J Cancer Res Pract 2019;6:55-63.

understanding of basic cancer biology without relying on *in vivo* animal models. Conventionally, monolayered two-dimensional (2D) cell cultures have offered a robust, simple, and convenient route for conducting investigations with reliable test reproducibility within highly controlled laboratory environments. However, the lack of tissue architecture and inability to capture the complex heterogeneity within a tumor, and its microenvironment limits the ability to translate research findings from 2D models to a clinical setting. Ultimately, the ineffective translation from *in vitro* 2D cell models to human clinical trials has led to the failure of many anti-cancer clinical studies.[1-4]

Multicellular three-dimensional (3D) tumor spheroids are recognized as superior models for the preclinical evaluation of anti-cancer therapies[5,6] due to their better and more advanced representation of tumors *in vivo*. The organization of cells within the 3D spheroidal structure means that they are in direct contact with each other and the secreted extracellular matrix (ECM), allowing them to utilize alternative cell growth and survival signaling mechanisms not readily observed in 2D monolayers [Figure 1a and b].[7] Similar to *in vivo* cancers, 3D spheroids exhibit differential rates of cell proliferation throughout the spheroid, and physiologically relevant gradients of oxygen, nutrients, and waste products are also observed.[8,9]

Many techniques have been used for culturing 3D spheroids in laboratories,[5] with the main limitations being the practical difficulties in using traditional cell culturing equipment to produce fragile spheroids which are also difficult to manipulate.[10] Effectively, the routine use of 3D spheroids is time-consuming, costly and requires patience and experience.

Microfluidics involves the flow of fluid (on micro- and nanoscales) on custom manufactured platforms to perform experiments. Recently, microfabricated devices with capillary-like channels have become attractive platforms in assisting researchers to overcome the current technical issues associated with 3D spheroids and scaling up investigations for clinical translation. Custom-designed microfluidic devices, also known as "Lab-on-a-chip," enable biological evaluations to be undertaken within a controlled environment [Figure 1c].[11,12] The advantages of microfluidics are that tailored experiments can be conducted with ease, and the dependency on conventional tissue culturing equipment is reduced, thereby saving costs and downscaling the consumption of laboratory consumables. Combining 3D cell culturing and microfluidics is an exciting area of development, which has been made possible by improvements in the quality of microfluidic devices, that are currently being applied to biological systems [Table 1].

The aim of this review was to highlight the recent developments in microfluidics and 3D tumor cell culture systems with particular relevance to oncological research and clinical translation.

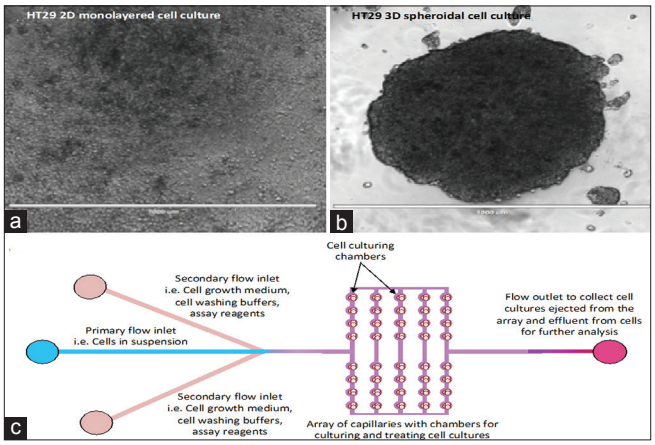

Figure 1: (a) HT29 colorectal cancer two-dimensional monolayered cell culture was prepared by seeding 10000 cells into a conventional 6-well plate and cultured for 3 days. (b) HT29 colorectal cancer three-dimensional spheroidal cell culture was prepared by seeding 10,000 cells into an ultra-low attachment 96-well plate and cultured for 3 days. (c) Simple schematic diagram of a microfluidic chip highlighting the ports on the chip for introducing and extracting fluids, capillaries through which the fluid flows, and cell culturing chambers

Methods

Embase, Medline, PubMed, and Google Scholar databases were searched using the keywords: "3D cell culture," "Cancer," "Lab-on-a-chip," "Microfluid flow," "Microfluidics," "Spheroids," "Tumor," "Tumour."

Three-Dimensional Cell Culturing Microfluidic Concepts

Non-cell adherent-based systems

A popular method for generating 3D spheroids is to culture cells on non-cell adherent surfaces. This is achieved by precoating tissue culture plastics with substrates that prevent cell adherence [Figure 2a].[13] Cells then attach to each other,

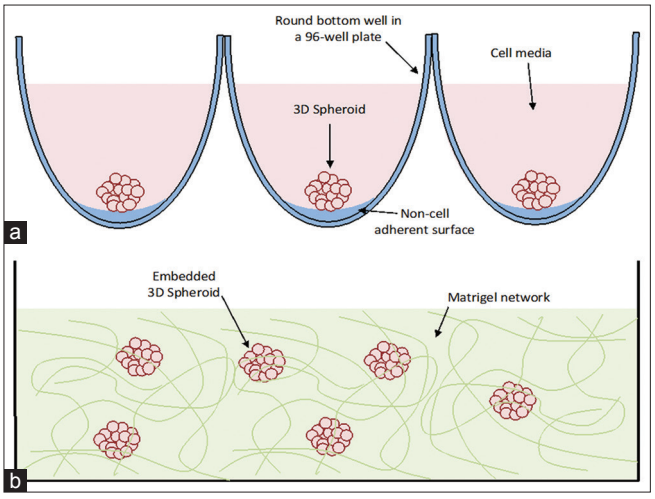

Figure 2: Multicellular three-dimensional spheroids can be produced by seeding cells into (a) 96-well tissue culturing plates with noncell adherent surfaces and (b) embedding cells into matrix or Matrigel to physiologically and structurally support spheroid growth

Table 1: A summary of the different methods for producing tumor spheroids-based microfluidic platforms as discussed in this review highlighting their advantages and limitations

3D cell culturing microfluidic system	References	Advantages	Limitations
Noncell adherent	[14-22]	Simple and convenient microfluidic setup for spheroid culture Produce uniform -sized spheroids Culture large numbers of spheroids on a single chip Spheroids can be cultured for long-term through flow Spheroids can be superficially analyzed Spheroids are easily retrievable Suitable for high-throughput applications Various non-adherent coating options available	Predetermined sizes of wells means the sizes of spheroids is not adjustable Spheroids are unanchored and are subjected to shear stress through flow
Matrix-embedded spheroids	[24,25,27,29-31]	Matrix hydrogel provides support and mimics a natural tumor microenvironment for spheroids Stromal cells, i.e., fibroblasts, mesenchymal stem cells maybe co-embedded with spheroids Suitable for studying chemotaxis and metastatic behavior of spheroids as well as the transport and delivery of anti-cancer therapeutics through hydrogel in real time	Spheroids have to be grown externally before being embedded into matrix Retrieving embedded spheroids for further analyses can be challenging
Hanging drop	[33-35]	Simple and easy method for producing spheroids Spheroids are easily accessible Capillary and surface tension forces permit the seeding of cells and the transport of nutrients/drugs Size of spheroid can be adjusted by altering the concentration of cell suspension	Exchanging cell media is challenging if it is not precisely controlled Spheroidal structures are easily compromised if disturbed
Droplet-based spheroids	[38-44]	Rapid generation of spheroids through cell-loaded alginate beads Alginate beads provide structural support to spheroids as well as providing space to proliferate and migrate Double-emulsion droplets avoid the need for gelation of alginate	Complex microfluidic system compared to other techniques External bath of calcium ions is required for gelation Compartmentalization of spheroids in beads can hinder long-term culture by limiting the availability of nutrients from cell growth medium to the cells and can also lead to the accumulation of waste products, further limiting spheroid growth Production of alginate encapsulated spheroids and subsequent anticancer analysis are not carried out in a single microfluidic unit
Other 3D cell culturing microfluidic setup	[45]	Microfluidic model is compatible with conventional 96-well format-based applications	Spheroids are grown externally before being introduced into microfluidic platform Perfusion of fluids and positioning of spheroids is done manually and is gravity driven which limits the precision in controlling the flow
	[46]	Spheroids are trapped into wells with cell media perfusing over them allowing for long-term culture Supernatant from cells is easily obtainable for analysis	Spheroids are grown externally before being introduced into microfluidic platform
	[47]	Pressure regulated inflation/deflation of cell trapping barrier allows for the long-term culture and extraction of spheroids from platform	Unique microfluidic design which may be difficult to mass produce
	[48]	Microfluidic platform that models pharmacokinetic and pharmacodynamics anticancer drugs	Spheroids are grown externally before being introduced into microfluidic platform

3D: Three-dimensional

forming cellular aggregates and growing into 3D spheroidal structures. Patra *et al.* described a simple microfluidic device that allowed spheroids to be cultured and harvested, in which cells in suspension were flowed into the device through an inlet port where they were captured into Synperonic F-108 coated wells, forming uniform-sized spheroids.[14] The authors were able to exchange cell media and extract spheroids from wells by manipulating the rate of fluid flow through the device. In a follow-up experiment, the authors used the same concept and designed a microfluidic platform that could accommodate the growth of 5000 uniform spheroids. The device allowed anti-cancer drug testing to be performed by perfusing chemotherapeutics through the device and extracting spheroids to analyze cytotoxicity using conventional techniques.[15] Similarly, Ziółkowska *et al.* designed a microfluidic array of microwells, where a syringe-pump driver pushed fluid through the inlet and outlet ports allowing the long-term culture of spheroids in poly (vinyl alcohol) (PVA)-coated microwells. The authors successfully cultured 3D cancer cell spheroids in the flow device and were able to evaluate the effects of an anti-cancer drug on the spheroids, using live/dead cell viability dyes and fluorescent microscopy.[16] Zuchowska *et al.* also constructed a similar microfluidic flow chip that was composed of two layers of the organosilicon polymer polydimethylsiloxane (PDMS) and filled with PVA prior to the addition of cells. A549 lung cancer cell spheroids were exposed to aminolevulinic acid-mediated photodynamic therapy, and spheroid viability was assessed using live/dead cell viability dyes and fluorescent microscopy.[17] Sun *et al.* and Chen *et al.* both described similar microwell array microfluidic systems and highlighted the convenience and simplicity of using their systems to culture 3D spheroids on non-cell adherent surfaces.[18,19]

Lee *et al.* described a dual microfluidic flow system involving two interconnected tissue-culturing chips. The concept was to investigate paracrine interactions between different cell types through indirect cell–cell contact. Hepatic stellate cells were cultured on the first chip, which was connected to a second chip culturing 3D hepatocyte spheroids in microwells coated with bovine serum albumin. The flow of cell media from the first chip to the second occurred due to differences in osmotic pressure that the authors were able to simulate, replicating the interstitial flow of fluid *in vivo*.[20] Okuyama *et al.* also described an albumin-covered microfluidic device in which they were able to coculture 3D hepatocyte spheroids and fibroblasts on the same chip. By simply exploiting the adhesive property of the albumin-coated surface of the fluid flow device through electrostatic adsorption, they were able to culture 3D spheroids in non-cell adhesive microwells, and upon switching the surface from non-cell adhesion to cell adhesion, they were able to culture fibroblasts.[21] These 3D spheroid culturing microfluidic examples were primarily devised and operated in custom-designed prototypes. However, Kwapiszewska *et al.* described a microfluidic chip based on a conventional 384-well plate. In this system, suspended cells were flowed into PVA-coated interconnected wells, resulting in the production of uniformly sized spheroids. The authors were then able to culture 3D cancer cell spheroids, treat them with an anti-cancer drug, and assess cytotoxic effect using live/dead cell viability dyes and spectrofluorometry.[22]

Culturing cells on non-cell adherent surfaces is an appealing method for producing multicellular tumor spheroids, and similar techniques can be applied to microfluidic devices allowing tumor spheroids to be cultured, treated, and analyzed conveniently in an all-encompassing flow system. Superficial techniques such as conventional microscopy and analyzing the supernatant/fluid in the effluent that flows out from the microfluidic device are the most common methods used for analyzing the 3D spheroids. The reason for this is that the non-cell adherent-based microfluidic flow devices that have been reported are fabricated as closed systems, which restricts the user from accessing the spheroids and performing more invasive methods of analysis.

Matrix-embedded spheroid-based systems

Another popular technique for producing 3D spheroids and tumor-like structures is by culturing cancer cells in matrix- and Matrigel-like substances [Figure 2b]. The resulting Matrigel-embedded spheroid is an attractive *in vitro* tool for anti-cancer research as it simulates an *in vivo* tumor (3D spheroid), and its corresponding microenvironment (Matrigel). Other advantages of this technique include the delivery of essential nutrients and growth factors through the Matrigel network, as well as structural support provided by the Matrigel for the 3D spheroid to grow.[23] Due to the practicality and user-friendliness offered by this method, it is favored by many researchers for investigating 3D spheroid cultures and has been used extensively to produce microfluidic platforms.

Shin *et al*. described a simple fluid flow system for evaluating the efficacy of anti-cancer therapeutics using Matrigel-embedded 3D spheroids. Externally grown spheroids suspended in Matrigel solution were seeded into the central channel of the microfluidic device. Upon polymerization of the Matrigel, cell culture medium was passed through side channels that ran parallel to a central channel and could perfuse through the Matrigel to the 3D spheroids. The continuous flow through the side channels allowed the authors to treat embedded spheroids in the central channel with the anti-cancer drug doxorubicin and assess spheroid viability.[24] Similarly, Ayuso *et al.* described a microfluidic setup whereby externally grown spheroids were embedded into collagen and cultured in the central chamber of the device. The authors used the setup to study chemotaxis by flowing chemoattractants through lateral side channels and observing the migration of cells from the spheroids through the collagen.[25]

Coculturing different types of cells, such as cancer cells, fibroblasts, and endothelial cells, is believed to recreate a better representation of the cancer environment.[26] Jeong *et al.* described a microfluidic setup where HT-29 colorectal cancer cells and fibroblasts were cultured in collagen in independent

chambers with cell media being perfused between them. The authors showed that collagen-embedded HT-29 spheroids cocultured with fibroblasts were more resistant to anti-cancer treatment compared to spheroids cultured alone, highlighting the crucial role played by cancer-associated fibroblasts in conferring resistance to anti-cancer treatment.[27,28] Liu *et al.* described a similar microfluidic setup where Matrigel-embedded salivary gland carcinoma spheroids and fibroblasts were cocultured in independent chambers and illustrated the invasion of cancer cells from the spheroids into the Matrigel.[29] Matrix and Matrigel embedding is an effective means of coculturing spheroids with other types of cells because it facilitates signaling between different types of cells, comparable to *in vivo* tumors and their stromal network.

The incorporation of a tumor network and microenvironment into an *in vitro* model is believed to be a better predictor of *in vivo* behavior. In addition to providing support through intercellular communication, the physiological elements of the tumor microenvironment play a role in aiding the progression of cancer. To highlight this, Albanese *et al.* designed a single-channel flow device culturing MDA-MB-435 (melanoma) cell line 3D spheroids in ECM. Unlike the other microfluidic devices in which spheroids in static environments were subjected to predictable uniform flow conditions dictated by the geometrical design of the flow platform, the authors designed a network of interstitial spaces resembling the tumor microenvironment on the microfluidic device. They were then able to show the effects of variable fluid flow and the influence of interstitial fluid pressure in the delivery of nanoparticles to the 3D spheroids through the ECM network.[30] Prabhakarpandian *et al.* reported a more complex flow fluid platform to model the tumor microenvironment. Their concept involved engineering a device with a synthetic tumor microvascular network by designing irregular capillary-like structures with gaps in the flow device. Cells in a Matrigel solution were passed into a compartment in the middle of the capillary network, and upon Matrigel gelation, a network of interstitial spaces was created around the 3D spheroids. Endothelial cells were flowed into and occupied the capillary-like network. This device successfully recreated the irregular microvasculature and circulatory flow of *in vivo* tumors, mimicking the leakiness of tumor vessels due to the precisely designed gaps in the capillary structure, and simulating interstitial fluid pressures created by the internal spaces within the Matrigel.[31]

The major advantage of combining microfluidics with matrix/Matrigel-embedded 3D spheroids is that collagen, or other Matrigel-like substances can be easily flowed precisely into a defined cell-culturing compartment of a custom-designed fluid flow device and allowed to gel. Once this has been achieved, designing a flow channel adjacent to the embedded 3D spheroids for perfusing cell media, buffers, and cytotoxics in solution can be designed with relative ease. However, a drawback in the matrix/Matrigel microfluidic models described here is that many rely on 3D spheroids to be generated externally before being introduced into the microfluidic system. Unlike the non-cell adherent microfluidic concepts, cells in suspension cannot be flowed into the Matrigel, as this will not result in the production of 3D spheroids once the Matrigel has polymerized.

Hanging drop-based microfluidics

The hanging drop method for producing spheroids has been highlighted for its simplicity, reproducibility in generating uniform spheroids, and the lack of specialized equipment and culturing conditions required [Figure 3a].[32] Frey *et al.* described a simple fluid flow model based on the hanging drop technique, in which individual 3D spheroid-containing hanging drops were interconnected via a capillary-like flow channel, allowing the exchange of cell media and treatment of spheroids to occur without manually intervening with the droplets.[33] Aijian and Garrell described another flow system based on the hanging drop method involving two plates, where cell suspension was added through a hole in the top plate and drawn through the capillary-like gap between the plates. Droplets were then formed in holes in the bottom plate through capillary forces.[34]

Bender *et al.* improved on the aforementioned hanging drop flow systems by initially culturing spheroids in holes in the bottom plate of a two-plate setup, similar to the one described by Bender *et al.* Twenty-four hours after spheroids had formed in the droplets, the cell medium was replaced by collagen-containing solution and hanging drops were gelled. This allowed the authors to prevent the 3D spheroids from easily being disrupted and losing their 3D structures. Furthermore, the collagen matrix allowed the authors to study the migration of cells from human fibroblast cell line spheroids through the collagen. This was achieved by directing the flow of fluid from droplets containing HT-29 spheroids to the collagen droplets containing fibroblast spheroids, demonstrating the invasive nature of advanced cancers.[35]

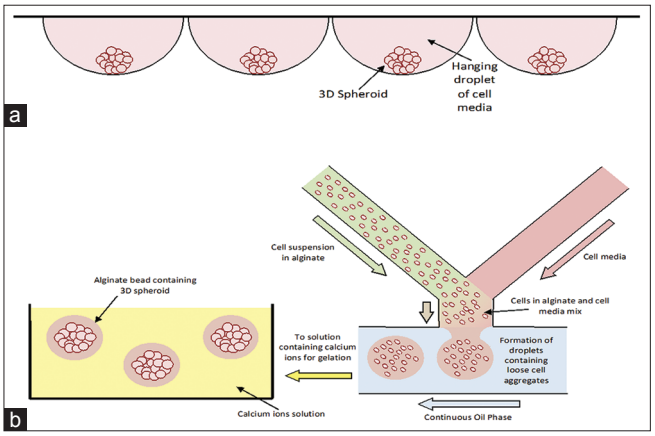

Figure 3: Multicellular three-dimensional spheroids can be produced by seeding cells into (a) hanging drops of cell suspension in media. (b) droplet-based techniques involve mixing cell suspension in alginate with cell media in an immiscible oil phase to form alginate beads containing cells and cell media. Alginate is then polymerized (gelation) in a solution containing calcium ions

The hanging drop technique is a convenient method that is routinely used for producing multicellular 3D spheroids. However, a major limitation of the hanging drop method is instability of the droplets, meaning a small disruption to the droplet can compromise the structural integrity of the 3D spheroids. While creating an array of spheroids in droplets from cells in suspension through capillary flow sounds attractive, in practice, the technique can be quite complex. In comparison to the "non-cell adherent" and "Matrigel-embedding" methods for producing 3D spheroids, the hanging-drop method is not as widely adopted or used. This is due to the technical difficulties in keeping the 3D spheroid-containing fluid droplets stable over a prolonged period of time during experiments. Certainly, this has also translated into hanging-drop-based microfluidic systems.

Droplet-based microfluidics

Droplet-based, two-phase flow systems utilize the immiscibility of aqueous alginate and oil phases to create fluid flow systems for microscale evaluations of 3D spheroids. Spheroids encapsulated within the aqueous alginate phase are driven through a continuously flowing oil phase before coming into contact with calcium ions to polymerize and gel the alginate [Figure 3b].[36] Alginate has been extensively used in biomedical applications due to its low toxicity, biocompatibility, low cost and simple gelation in the presence of divalent cations.[37] In addition, gelled alginate provides structural support to the 3D spheroids, and the porous structure of alginate facilities the exchange of nutrients and waste products.

Wang and Wang described a droplet-based microfluidic concept involving an aqueous cell suspension flowing through an alginate mixture, before making contact with a perpendicular flow of mineral oil, that forced the immiscible continuous flow of cells in alginate to bud off into droplets. The droplets were then placed into a solution containing calcium ions, causing the copolymers of alginate to gel via cross-linkage, effectively creating a bead-like structure with the encapsulated spheroid.[38] Yu *et al.* described a system which took this setup one step further. This involved the flow of cell suspension in alginate through an immiscible solvent, forming cell-encapsulating droplets. The droplets were collected externally in a calcium ion-containing solution to allow the alginate to gel. The gel-beaded cells were then loaded into another microflow setup, containing U-shaped micro sieves that captured one bead per sieve. Through continuous perfusion of fluid, the growth of 3D spheroids was continued, and they were then treated with an anti-cancer drug, also through fluid flow. Subsequent dose-dependent response analysis of doxorubicin-treated LCC6/Her-2 breast cancer cell line spheroids showed increased resistance to treatment in multicellular spheroids compared to their monolayered counterparts.[39] Furthermore, the authors then improved upon their flow system by redesigning a more robust droplet generation and evaluation system. Specifically, droplets were generated by flowing a primary alginate and cell suspension mixture through a secondary alginate and calcium carbonate mixture flow and then a tertiary mineral oil and acetic acid mixture flow. Conceptually, the alginate and calcium carbonate flow had a high or neutral pH. Once the primary and secondary flow came into contact with the acid in the tertiary continuous oil phase, calcium ions were released due to the lowered pH, which in turn initiated the gelling of alginate and eliminating the need for an external bathing solution of calcium ions. This allowed the authors to coat the 3D spheroids containing alginate beads with outer alginate shells, preventing the 3D spheroids from outgrowing the alginate beads, and also facilitating the 3D spheroids to be cultured for a longer period of time.[40]

McMillan *et al.* also described a flow system that used the droplet method for culturing 3D spheroids. Cell suspension in alginate was flowed into a continuously flowing oil phase, forming cells containing alginate droplets in the oil. The droplets were then plugged into T-junction chambers that were designed in a microfluidic device. Perfusion of calcium chloride through the device caused gelation of the alginate droplets in the chambers, and continuous flow of cell media supported the growth of 3D spheroids. The usability of the flow device was demonstrated by culturing human glioblastoma cell line spheroids and treating them with anti-cancer drugs and radiation. Reduction in the dimensions of the 3D spheroids was used to determine the response to treatment.[41] Sabhachandani *et al.* described a similar flow setup with simultaneous perpendicular flows of cells in alginate and mineral oil-generated droplets. After the droplets had docked in a flow array in a microfluidic device, the third flow of calcium chloride solution was used to induce gelation. In cocultures of MCF-7 breast cancer cell line spheroids and fibroblast cells. An increase in cell resistance to anti-cancer treatment was demonstrated in cocultured models compared to MCF-7 monocultured spheroids.[42]

Other examples of droplet-based microfluidics include a microfluidic model described by Yoon *et al.* The authors were able to infuse iron oxide nanoparticles into 3D spheroid-encapsulated alginate beads, and an external magnetic force was then used to pull the alginate beads from the flowing oil phase into a cell medium phase.[43] Chan *et al.* built upon existing droplet systems with double-emulsion droplet encapsulated spheroids. Upon generation of alginate droplets in a flowing oil phase, the droplets crossed through a perpendicular flow of cell culture medium. The subsequent layer of oil around the alginate-containing spheroids confined the droplets, eliminating the need for calcium ion-induced gelation, and also acting as a selectively permeable barrier for the 3D spheroids.[44]

The droplet-based method is effective for generating 3D spheroids and is quite similar to the matrix-embedding technique. The immiscibility of the flowing oil and aqueous phases allows for the easy integration of this technique into fluid flow systems. Once spheroids have formed within the alginate or Matrigel structures, long-term culture of spheroids is possible through the perfusion of nutrients and growth factors from the flowing cell media through the porous beads. Although the drop-based technique is not a

well-established method for producing 3D spheroids, as with the "non-cell-adherent," "matrix/Matrigel-embedding," and "hanging drop" methods, the use of different phases of fluids to create 3D spheroids can be seamlessly integrated into microfluidic applications.

Other spheroid-based microfluidic systems

The primary focus of engineering " tumor-on-a-chip " models is to take existing 3D spheroid culturing techniques and apply a flow element, which facilitates the growth, treatment, and analysis of 3D spheroids. Kim *et al.* described a simple flow setup based upon a conventional 96-well tissue culturing plate. The advantage of this system was that commercially available equipment designed to be used with 96-well plates, such as microplate readers and multichannel pipettes, could be used, negating the need to purchase or manufacture specialized equipment. Externally grown spheroids were seeded into a modified 96-well plate with interconnected wells, and gravity-driven flow of fluid from well to well occurred by simply tilting the device by hand.[45] In another example, Ruppen *et al.* designed a simple unidirectional flow system in which externally grown spheroids were flowed into the device and trapped into side channels. Flowing in drug-enriched solution, collecting the supernatant from the output, and measuring caspase activity as a measure of cytotoxicity was achievable with relative ease, allowing the effects of chemotherapeutics on 3D spheroid growth to be assessed.[46]

The entrapment of spheroids into micro-chambers built into devices is effective as a means of facilitating treatment and analyzing 3D spheroids in a single unit. However, retrieval of spheroids for further analysis can be challenging. Jin *et al.* addressed this in a microfluidic device, in which cell suspension was flowed in and cells were trapped into a pressure inflated membranous barrier. 3D spheroids were then formed and treated. Posttreatment, the cell-trapping barrier was deflated, and the spheroids could be retrieved and collected from the outlet end of the microfluidic device.[47]

Pharmacokinetic and pharmacodynamic models are vital in drug development to measure, predict, and optimize the therapeutic effect of the drug. Toley *et al.* designed a T-shaped microfluidic device, in which externally grown spheroids were cultured in the lower arm of the device, and through mathematical modeling, drug delivery and clearance in a typical *in vivo* tumor microenvironment were mimicked through fluid flow in the upper lateral arm. The usability of this flow system was demonstrated by evaluating the differences in pharmacokinetics of doxorubicin and a liposomal formulation of doxorubicin, which illustrated the differences in drug retention within the vicinity of the spheroid, its impact on spheroid growth, and clearance from the flow system.[48]

Limitations and Future Perspectives

Most of the 3D cell culturing microfluidic flow systems described in this review were designed as enclosed structures relying on cell suspensions being passed through the device to form cellular aggregates and 3D spheroids. Microscopic imaging, fluorescence, and colorimetric-based assays are the preferred techniques for analysis because they do not involve the manual handling of the 3D spheroids. Unlike superficial analytical methods, further invasive and in-depth analysis of 3D spheroids would require the physical extraction of 3D spheroids from the microfluidic platforms, i.e., extracting spheroids and subjecting them to formalin-fixed paraffin embedding for histological sectioning and immunohistochemistry.[49]

3D bioprinting is a technique that could address the issue of retrieving cultured 3D spheroids from microfluidic chips. Bioprinting involves the precise layered positioning of cells and biological material with accurate spatial control, to fabricate 3D structures resembling spheroid-like micro-tissues.[50,51] Successful applications of bioprinting include the production of 3D *in vitro* "mini-livers" from hepatocyte-like cells,[52] aortic valve conduits,[53] and breast cancer spheroids.[54] The advantage of bioprinting cells is that the process is relatively user-friendly and convenient for an automated large-scale production of microfluidic chips. An adequately designed microfluidic device which has been set up for bioprinting could potentially accommodate 3D cell models and spheroids to be printed directly into the flow device. The cell models could then be cultured and treated via microfluidic flow and also retrieved easily from their culturing chambers.

Until recently, 3D cell culturing has relied on the manipulation of commercial cancer cell lines to produce 3D spheroid structures. Organoids is the term given to 3D organotypic cultures which are derived from primary tissues, embryonic stem cells, and induced pluripotent stem cells. Organoids are a huge development in the 3D modeling of cancers, as unlike 3D cell spheroids, organoids exhibit similar organ functionality, architecture, gene expression and response to treatment as the tissue of origin.[55] The use of organoids for biomedical and clinical research is still in its infancy, however, the integration of organoids into microfluidics shows great promise in developing platforms for personalized medicine.

The microfluidic flow systems discussed here, and other similarly designed devices, are primarily fabricated using PDMS.[14-16,18-22,24,27,29-31,33,34,38-48] PDMS is an inexpensive, easy to manipulate, optically transparent, and biocompatible material that has become one of the most popular materials for creating microfluidic flow devices for biological applications.[56] However, as reviewed by Levenstein *et al.* and Mukhopadhyay *et al*. PDMS can easily absorb organic solvents and small molecules which can be a hindrance for biological research, especially in experiments that rely on growth factors and vital proteins to support the growth of cells. The use of PDMS devices could ultimately influence the outcomes of experiments and impact results. In addition, the logistics in fabricating and appropriately treating PDMS-based devices can be technically challenging for nonspecialist and inexperienced users.[57,58] For the purpose of designing one-off experimental flow devices, PDMS has thus far proven to be sufficient.

However, alternative materials may need to be investigated in order to take these devices from research prototypes to commercial products.

The setup of most microfluidic devices involves a custom-designed fluid flow platform attached to external fluid handling systems such as syringe-pumps and pressure systems. Although the path and flow of fluid are preset, setting up and alternating between bulky reagent reservoirs still requires manual input and is a matter that has yet to be addressed.[59] Safavieh and Juncker described a flow system combining microfluidics and electrical components built into a microfluidic chip that could facilitate the autonomous delivery of multiple preloaded chemicals according to a preprogrammed sequence, flow rate, and time.[60] This integration of electronics and microfluidics to create digitally automated systems is a unique example, however, it could advance the field further.

Conclusions

3D spheroids are more representative models of *in vivo* cancers compared to conventional 2D cell cultures. Although 3D spheroids are attractive for biomedical research, established 3D spheroid-culturing techniques are manually intensive and can be technically challenging. Microfluidics provides a way for conventional cell culturing to be miniaturized, while substantially reducing manual efforts and costs and could pave the way to transform how laboratory-based experiments are performed. Significant improvements have been made over the past decade, which have taken simple fluid flow mechanisms from the engineering industry to complex small-scale setups for scientific research. However, there is still a long way to go before microfluidics flow systems meet industry standards and can be used commercially for clinical applications.

Financial support and sponsorship

This review was supported by the National Institute for Health Research (NIHR) infrastructure at Leeds. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

Conflicts of interest

There are no conflicts of interest.

References

1. Salahudeen AA, Kuo CJ. Toward recreating colon cancer in human organoids. Nat Med 2015;21:215-6.
2. Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, *et al.* Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biol 2012;10:29.
3. Hickman JA, Graeser R, de Hoogt R, Vidic S, Brito C, Gutekunst M, *et al.* Three-dimensional models of cancer for pharmacology and cancer cell biology: Capturing tumor complexity *in vitro*/*ex vivo*. Biotechnol J 2014;9:1115-28.
4. Mak IW, Evaniew N, Ghert M. Lost in translation: Animal models and clinical trials in cancer treatment. Am J Transl Res 2014;6:114-8.
5. Breslin S, O'Driscoll L. Three-dimensional cell culture: The missing link in drug discovery. Drug Discov Today 2013;18:240-9.
6. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: Advantages and applications. J Cell Physiol 2015;230:16-26.
7. Pickl M, Ries CH. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. Oncogene 2009;28:461-8.
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;12:207-18.
9. Ludwig K, Tse ES, Wang JY. Colon cancer cells adopt an invasive phenotype without mesenchymal transition in 3-D but not 2-D culture upon combined stimulation with EGF and crypt growth factors. BMC Cancer 2013;13:221.
10. Joshi P, Lee MY. High content imaging (HCI) on miniaturized three-dimensional (3D) cell cultures. Biosensors (Basel) 2015;5:768-90.
11. Young EW, Beebe DJ. NIH public access. Chem Soc Rev 2010;39:1036-48.
12. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. Nature 2014;507:181-9.
13. Cesarz Z, Tamama K. Spheroid culture of mesenchymal stem cells. Stem Cells Int 2016;2016:9176357.
14. Patra B, Chen YH, Peng CC, Lin SC, Lee CH, Tung YC. A microfluidic device for uniform-sized cell spheroids formation, culture, harvesting and flow cytometry analysis. Biomicrofluidics 2013;7:54114.
15. Patra B, Peng CC, Liao WH, Lee CH, Tung YC. Drug testing and flow cytometry analysis on a large number of uniform sized tumor spheroids using a microfluidic device. Sci Rep 2016;6:21061.
16. Ziółkowska K, Stelmachowska A, Kwapiszewski R, Chudy M, Dybko A, Brzózka Z. Long-term three-dimensional cell culture and anticancer drug activity evaluation in a microfluidic chip. Biosens Bioelectron 2013;40:68-74.
17. Zuchowska A, Jastrzebska E, Chudy M, Dybko A, Brzozka Z. 3D lung spheroid cultures for evaluation of photodynamic therapy (PDT) procedures in microfluidic lab-on-a-chip system. Anal Chim Acta 2017;990:110-20.
18. Sun D, Lu J, Chen Z, Yu Y, Li Y. A novel three-dimensional microfluidic platform for on chip multicellular tumor spheroid formation and culture. Microfluid Nanofluidics 2014;17:831-42.
19. Chen YC, Lou X, Zhang Z, Ingram P, Yoon E. High-throughput cancer cell sphere formation for characterizing the efficacy of photo dynamic therapy in 3D cell cultures. Sci Rep 2015;5:12175.
20. Lee SA, No da Y, Kang E, Ju J, Kim DS, Lee SH. Spheroid-based three-dimensional liver-on-a-chip to investigate hepatocyte-hepatic stellate cell interactions and flow effects. Lab Chip 2013;13:3529-37.
21. Okuyama T, Yamazoe H, Mochizuki N, Khademhosseini A, Suzuki H, Fukuda J. Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device. J Biosci Bioeng 2010;110:572-6.
22. Kwapiszewska K, Michalczuk A, Rybka M, Kwapiszewski R, Brzózka Z. A microfluidic-based platform for tumour spheroid culture, monitoring and drug screening. Lab Chip 2014;14:2096-104.
23. Härmä V, Virtanen J, Mäkelä R, Happonen A, Mpindi JP, Knuuttila M, *et al.* A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One 2010;5:e10431.
24. Shin CS, Kwak B, Han B, Park K. Development of an *in vitro* 3D tumor model to study therapeutic efficiency of an anticancer drug. Mol Pharm 2013;10:2167-75.
25. Ayuso JM, Basheer HA, Monge R, Sánchez-Álvarez P, Doblaré M, Shnyder SD, *et al.* Study of the chemotactic response of multicellular spheroids in a microfluidic device. PLoS One 2015;10:e0139515.
26. Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating *in vivo* environment. J Steroid Biochem Mol Biol 2012;131:68-75.
27. Jeong SY, Lee JH, Shin Y, Chung S, Kuh HJ. Co-culture of tumor spheroids and fibroblasts in a collagen matrix-incorporated microfluidic chip mimics reciprocal activation in solid tumor microenvironment. PLoS One 2016;11:e0159013.
28. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-associated fibroblasts: Their characteristics and their roles in tumor growth. Cancers (Basel) 2015;7:2443-58.
29. Liu T, Lin B, Qin J. Carcinoma-associated fibroblasts promoted tumor

spheroid invasion on a microfluidic 3D co-culture device. Lab Chip 2010;10:1671-7.
30. Albanese A, Lam AK, Sykes EA, Rocheleau JV, Chan WC. Tumour-on-a-chip provides an optical window into nanoparticle tissue transport. Nat Commun 2013;4:2718.
31. Prabhakarpandian B, Shen MC, Nichols JB, Garson CJ, Mills IR, Matar MM, *et al.* Synthetic tumor networks for screening drug delivery systems. J Control Release 2015;201:49-55.
32. Foty R. A simple hanging drop cell culture protocol for generation of 3D spheroids. J Vis Exp 2011. pii: 2720.
33. Frey O, Misun PM, Fluri DA, Hengstler JG, Hierlemann A. Reconfigurable microfluidic hanging drop network for multi-tissue interaction and analysis. Nat Commun 2014;5:4250.
34. Aijian AP, Garrell RL. Digital microfluidics for automated hanging drop cell spheroid culture. J Lab Autom 2015;20:283-95.
35. Bender BF, Aijian AP, Garrell RL. Digital microfluidics for spheroid-based invasion assays. Lab Chip 2016;16:1505-13.
36. Gu H, Duits MH, Mugele F. Droplets formation and merging in two-phase flow microfluidics. Int J Mol Sci 2011;12:2572-97.
37. Lee KY, Mooney DJ. Alginate: Properties and biomedical applications. Prog Polym Sci 2012;37:106-26.
38. Wang Y, Wang J. Mixed hydrogel bead-based tumor spheroid formation and anticancer drug testing. Analyst 2014;139:2449-58.
39. Yu L, Chen MC, Cheung KC. Droplet-based microfluidic system for multicellular tumor spheroid formation and anticancer drug testing. Lab Chip 2010;10:2424-32.
40. Yu L, Ni C, Grist SM, Bayly C, Cheung KC. Alginate core-shell beads for simplified three-dimensional tumor spheroid culture and drug screening. Biomed Microdevices 2015;17:33.
41. McMillan KS, Boyd M, Zagnoni M. Transitioning from multi-phase to single-phase microfluidics for long-term culture and treatment of multicellular spheroids. Lab Chip 2016;16:3548-57.
42. Sabhachandani P, Motwani V, Cohen N, Sarkar S, Torchilin V, Konry T. Generation and functional assessment of 3D multicellular spheroids in droplet based microfluidics platform. Lab Chip 2016;16:497-505.
43. Yoon S, Kim JA, Lee SH, Kim M, Park TH. Droplet-based microfluidic system to form and separate multicellular spheroids using magnetic nanoparticles. Lab Chip 2013;13:1522-8.
44. Chan HF, Zhang Y, Ho YP, Chiu YL, Jung Y, Leong KW. Rapid formation of multicellular spheroids in double-emulsion droplets with controllable microenvironment. Sci Rep 2013;3:3462.
45. Kim JY, Fluri DA, Kelm JM, Hierlemann A, Frey O. 96-well format-based microfluidic platform for parallel interconnection of multiple multicellular spheroids. J Lab Autom 2015;20:274-82.
46. Ruppen J, Cortes-Dericks L, Marconi E, Karoubi G, Schmid RA, Peng R, *et al.* A microfluidic platform for chemoresistive testing of multicellular pleural cancer spheroids. Lab Chip 2014;14:1198-205.
47. Jin HJ, Cho YH, Gu JM, Kim J, Oh YS. A multicellular spheroid formation and extraction chip using removable cell trapping barriers. Lab Chip 2011;11:115-9.
48. Toley BJ, Tropeano Lovatt ZG, Harrington JL, Forbes NS. Microfluidic technique to measure intratumoral transport and calculate drug efficacy shows that binding is essential for doxorubicin and release hampers doxil. Integr Biol (Camb) 2013;5:1184-96.
49. Clayton NP, Burwell A, Jensen H, Williams BF, Brown QD, Ovwigho P, *et al.* Preparation of three-dimensional (3-D) human liver (HepaRG) cultures for histochemical and immunohistochemical staining and light microscopic evaluation. Toxicol Pathol 2018;46:653-9.
50. Murphy SV, Atala A. 3D bioprinting of tissues and organs. Nat Biotechnol 2014;32:773-85.
51. Faulkner-Jones A, Greenhough S, King JA, Gardner J, Courtney A, Shu W. Development of a valve-based cell printer for the formation of human embryonic stem cell spheroid aggregates. Biofabrication 2013;5:015013.
52. Faulkner-Jones A, Fyfe C, Cornelissen DJ, Gardner J, King J, Courtney A, *et al.* Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D. Biofabrication 2015;7:044102.
53. Duan B, Hockaday LA, Kang KH, Butcher JT. 3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. J Biomed Mater Res A 2013;101:1255-64.
54. Ling K, Huang G, Liu J, Zhang X, Ma Y, Lu T, *et al.* Bioprinting-based high-throughput fabrication of three-dimensional mcf-7 human breast cancer cellular spheroids. Engineering 2015;1:269-74.
55. Fatehullah A, Tan SH, Barker N. Organoids as an *in vitro* model of human development and disease. Nat Cell Biol 2016;18:246-54.
56. Li X, Wu N, Rojanasakul Y, Liu Y. Selective stamp bonding of PDMS microfluidic devices to polymer substrates for biological applications. Sens Actuators Phys 2013;193:186-92.
57. Levenstein MA, Bawazer LA, Mc Nally CS, Marchant WJ, Gong X, Meldrum FC, *et al.* A reproducible approach to the assembly of microcapillaries for double emulsion production. Microfluid Nanofluidics 2016;20:1-11.
58. Mukhopadhyay R. When PDMS isn't the best. What are its weaknesses, and which other polymers can researchers add to their toolboxes? Anal Chem 2007;79:3248-53.
59. Korir G, Prakash M. Punch card programmable microfluidics. PLoS One 2015;10:e0115993.
60. Safavieh R, Juncker D. Capillarics: Pre-programmed, self-powered microfluidic circuits built from capillary elements. Lab Chip 2013;13:4180-9.