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Opportunities and Challenges for use of Tumor Spheroids as Models to Test Drug Delivery and Efficacy

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Abstract

Multicellular spheroids are three dimensional *in vitro* microscale tissue analogs. The current article examines the suitability of spheroids as an *in vitro* platform for testing drug delivery systems. Spheroids model critical physiologic parameters present *in vivo*, including complex multicellular architecture, barriers to mass transport, and extracellular matrix deposition. Relative to two-dimensional cultures, spheroids also provide better target cells for drug testing and are appropriate *in vitro* model for studies of drug penetration. Key challenges associated with creation of uniformly sized spheroids, spheroids with small number of cells and co-culture spheroids are emphasized in the article. Moreover, the assay techniques required for the characterization of drug delivery and efficacy in spheroids and the challenges associated with such studies are discussed. Examples for the use of spheroids in drug delivery and testing are also emphasized. With these challenges and the possible solutions, multicellular spheroids are becoming an increasingly useful *in vitro* tool for drug screening and delivery to pathological tissues and organs.

Keywords

Spheroids; Drug delivery; Drug screening; High throughput; Tissue engineering; Imaging

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Introduction

Drug delivery

Drug delivery is the process of administering an active pharmaceutical ingredient (API) *in vivo* to achieve a therapeutic effect in the patient [1–3]. Inside the body, the API reaches the target of interest after crossing many biological barriers such as other organs, the extracellular matrix, cells and intracellular compartments. During this process, the API may become inactivated or trapped at non-target sites as well as produce undesirable effects on non-target organs and tissues. Thus, efficient delivery of API to target tissues is critical to reduce unintended toxicity to other organs, while delivering the drug in a cost-effective manner. The challenge is that this complex physiological process is affected by many factors such as the nature of the API itself, carrier material used, targeting moieties attached if any, and additional external mechanism if any (such as changes in temperature, pH, magnetic field, ultrasound etc.) [2, 4]. Two common approaches to specifically target drug-loaded carrier systems to required pathological sites in the body are 1) *active*, involving cell-specific delivery of the API based on attachment of specific ligands to the surface of the pharmaceutical carriers to recognize and bind pathological cells (for example: antibody mediated targeting); and 2) *passive*, mediated by the enhanced permeability and retention (EPR) effect). EPR effect is based on the longevity of the APIs and its carriers in the blood, due to their extravasation and their accumulation in pathological sites with compromised vasculature (such as tumors, which have leaky blood vessels). This delivery method works very well for tumors, inflammation, and infarcts [3].

There are several requirements for the safe and effective delivery of therapeutic agents to target tissues and organs for human use. Before clinical trials and FDA approval, the drugs and delivery mechanism need to be rigorously tested to determine effectiveness, toxicity and safety. The first step in this process begins with *in vitro* testing of the drugs and their delivery methods. Typically such testing of delivery mechanisms against target cells is undertaken in 2-D, multi-well plate-based cell culture formats [5]. Results from such platforms, however, often are very different from the scenario in the body. From the drug delivery perspective, this is readily understood from the viewpoint of lack of appropriate physiological barriers arranged in a reasonable geometry. Therefore, *in vitro* models of tissues of interest that are more physiological than such conventional 2-D culture are needed for better prediction of drug effects and delivery mechanisms. This review focuses on one such platform: 3-D multicellular spheroids.

Spheroids as appropriate models for drug delivery

Platforms for 3-D cell culture, such as scaffolds, hydrogels, and microfluidics can provide enhanced models for testing of drug delivery, toxicity, and metabolism compared to conventional 2-D cultures due to their more physiological cell-cell contact geometry, mass transport, and mechanical properties [6]. Spheroids, which are microscale, spherical cell clusters formed by self-assembly, are one of the most common and versatile methods of culturing cells in 3-D [7]. Spheroids with radii of 200 micrometers and larger will have zones of proliferating cells on the outside and quiescent cells on the inside due to nutrient and oxygen transport limitations. Significantly larger spheroids can also harbor necrotic cells at the center as can be observed in some cancers *in vivo*. Spheroids have been used as models for evaluations of drug sensitivity and resistance, and they are typically more resistant to chemo- and radiotherapies compared to cells cultured as 2-D monolayers [8]. These models also allow analyses of different growth constraints such as oxygen tension and nutrient consumption, radiation effects, and angiogenesis [9].

Spheroids present a more physiological platform for drug delivery testing due to the following reasons (Figure 1):

1. Spheroids model the 3-D architecture of tissues, including multicellular arrangement and extracellular matrix deposition, found *in vivo*. Such arrangements, which are absent in conventional culture formats, reproduce how drug delivery might occur *in vivo*.
2. Being closely packed 3-D structures; spheroids have sizeable cell-cell interactions, including tight junctions, comparable to those in *in vivo* tissues. These cell-cell contacts and ensuing communication have been found to influence response of cells to drugs [10, 11].
3. Spheroids have diffusional limits to mass transport of drugs, nutrients and other factors, similar to *in vivo* tissues. Due to their mimicry of the physiological barriers to drug delivery *in vivo*, spheroid can serve as an improved assay format for testing efficacy.
4. Co-culture spheroids are formed with two or more cell types in varying ratios representing intercellular signaling and architecture seen *in vivo*. These co-culture spheroids can help decipher how multiple cell types found in tissues *in vivo* might impact drug delivery.
5. Rare cells such as cancer stem cells or primary stem cells may be incorporated and maintained in spheroids, which can facilitate targeting these specific cells with drugs. It is often difficult to maintain small numbers of such cells in conventional culture formats and to decode how these cells respond to the drug and delivery mechanism.
6. Spheroids are 3D models of *in vivo* solid tumors. Larger spheroids develop central necrosis and regions of hypoxia present in many cancers, which is critical for testing anti-cancer therapeutics. Cellular microenvironments such as hypoxia, which have been identified as one cause of drug resistance [12–14], can be modeled and created within spheroids for accurate testing of drug efficacy.

Spheroids are advantageous *in vitro* models for recreating some of the mass transport limitations that the API and carrier system are likely to encounter *in vivo*. One valuable characteristic of spheroids is their diffusion limit of about 150–200 μm to many molecules, particularly oxygen [15]. As a result of these mass transport limitations, spheroids generally display gradients of oxygen, nutrient distribution, metabolic waste accumulation, and proliferation profile inside them. Spheroids with diameters larger than 400–500 μm commonly follow a concentrically layered structure consisting of a necrotic core surrounded by a viable layer of quiescent cells and an outer rim of proliferating cells [15, 16]. The peripheral cells closely reflect the *in vivo* environment of actively proliferating tumor cells next to capillaries, while the distant inner cells stay quiescent or die through necrosis and apoptosis [17]. Many groups have characterized the oxygen profile inside various types of spheroids, and the typical oxygen tensions inside spheroids are of the characteristic profile shown in Figure 1 [18–22]. Spheroids thus are excellent models for generating 3-D cultures with such inherent pathophysiological gradients inside. For example, spheroids have been especially valuable in the study of therapeutic problems related to 3-D metabolic and proliferative gradients. Such applications of spheroids have increased understanding of the response of hypoxic tumor cells to chemotherapy and radiation therapy, as well as intricate 3-D cell-cell and cell-matrix interactions [17]. The combined effect from the pathophysiological gradients and complex 3-D cell-cell and cell-matrix interactions often lead to altered RNA and protein expression in spheroids, thus affecting the treatment

responses from therapeutic agents. Therefore, they can serve as excellent models for testing drug delivery systems.

Cancer stem cells (CSCs), which are deemed to be responsible for relapse of cancers after treatment, have been cultured successfully as spheroids. Compared with conventional culture, spheroid cultures maintain key properties of stem cells, including gene expression profiles, colony-forming and/or tumorigenic activity, differentiation potential, cytokine secretion, and resistance to chemotherapy [23–36]. As an example, sphere-forming primary human colon tumor cells maintain CD133 expression (a marker of colon CSC), and can generate and expand spheroids under serum-free culture conditions, initiate xenograft tumors, and exhibit resistance to chemotherapy-induced apoptosis, whereas cells in 2D conditions fail to do so [25]. Similarly, along with enhanced resistance to standard anticancer agents, cells within ovarian cancer spheroids display increased self-renewal potential, higher invasiveness, and migration potential relative to parental cells [35]. These examples illustrate that spheroids represent a physiological model for these rare cells, and present a great opportunity for therapeutic intervention targeting these cells with different drug delivery approaches.

Mathematical models predicting metabolic, transport and cellular phenomena in spheroids

Mathematical models are invaluable tools for understanding cellular and transport phenomena within spheroids and predicting metabolic responses to drug delivery. Along with experimental studies, mathematical models can provide pertinent perspectives for understanding mechanisms of drug delivery and effectiveness in spheroids. Due to their symmetry, spheroids can be easily defined mathematically in radial dimensions. As shown in Figure 1B, gradients and concentrations of nutrients, oxygen, lactate and glucose, and overall growth rate of cells within a spheroid can be predicted using mass transport and reaction diffusion equations. Mathematical models can also help predict the extent and location of quiescent cells in multicellular spheroids, leading to the finding that the oxygen transport has a greater effect than glucose transport on the distribution of quiescent cells within spheroids [37].

Mathematical models have also been developed that can simulate how the ligand-targeted drugs are distributed within spheroids as well as predict biological responses of the cells to such therapeutic treatments, providing an important tool to understand the kinetics and the dynamics of mechanisms responsible for cellular phenotypes in the spheroids [37–40]. For example, mathematical models have helped understand that immunotoxins are generally less effective against spheroids than monolayer cells at equivalent conditions due to heterogeneous receptor distribution and barrier to penetration into the spheroid [39]. They also help predict the poor drug delivery and low rates of cell proliferation that occur in spheroids in part due to hypoxia in necrotic core regions, consequently explaining the limited efficacy of therapies that target proliferating cells [41]. Mathematical models are also applicable to understanding the dynamics of nanoparticle penetration into multicellular spheroids [42].

The utility of mathematical models is demonstrated specifically in the example below, where treatment of a spheroid with a drug is described with the following equations [38]:

$$\frac{\partial n}{\partial t} + \frac{1}{r^2} \frac{\partial(r^2 v n)}{\partial r} = [k_m(c) - k_d(c) - KG(k_m(c))f(w)]n, \quad (1)$$

$$\frac{\partial c}{\partial t} + \frac{1}{r^2} \frac{\partial(r^2 v c)}{\partial r} = \frac{D}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial c}{\partial r} \right) - \beta k_m(c) n, \quad (2)$$

$$\frac{1}{r^2} \frac{\partial(r^2 v)}{\partial r} = [V_L k_m(c) - (V_L - V_D) \{k_d(c) + KG(k_m(c))f(w)\}] n, \quad (3)$$

$$\frac{\partial w}{\partial t} + \frac{1}{r^2} \frac{\partial(r^2 v w)}{\partial r} = \frac{D_w}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial w}{\partial r} \right) - \frac{K}{\omega} G(k_m(c)) f(w) n, \quad (4)$$

where the dependent variables n , c , v and w are the live cell density (cells/unit volume), nutrient concentration, velocity and drug concentration, respectively. Eq. (1) declares that the rate of change of n is dependent on the difference between the birth ($k_m(c)$) and death rates, where death is either natural, at a rate of $k_d(c)$, or due to the drug, at a rate $KG(k_m(c))f(w)$; the constant K is the maximum possible rate of drug induced cell death and $f(w)$ and $G(k_m(c))$ are positive functions described below. Functions k_m and k_d are based on Michaelis–Menten kinetics:

$$k_m(c) = A \left(\frac{c^{m_1}}{c_c^{m_1} + c^{m_1}} \right), \quad k_d(c) = B \left(1 - \sigma \frac{c^{m_2}}{c_d^{m_2} + c^{m_2}} \right),$$

where A , B , c_c , c_d , m_1 , m_2 and σ are positive constants. Eq. (2) arises from Fick's law describing the diffusion of the nutrients. Eq. (3) states that the spheroid is made up of living cells (volume fraction $V_L n$) and necrotic material (volume fraction $1 - V_L n$), and the rate of volume change is given by the difference in volume generated via birth ($V_L k_m(c)n$) from that lost by death ($(V_L - V_D)[k_d(c) + KG(k_m(c))f(w)]n$), where V_L and V_D are the inverse densities (volume/cell) of an alive and dead cell. Eq. (4) states that the drug may diffuse (by Fick's law) and is degraded only when it acts upon a living cell, giving a maximum degradation rate of K/ω , while neglecting any other process of drug breakdown. The dimensionless constant, ω , is a measure of the drug effectiveness, with increasing ω implying that less drug is consumed during the cell killing process.

Numerical analysis of these equations led to the conclusion that drug penetration is the most crucial factor in determining drug effectiveness in spheroids, and one of the main reasons for the difference in drug effectiveness between 2-D cultures and multicellular spheroids. Further, it was observed that an earlier exposure to a drug could generate an outer necrotic rim of cells, which provides an additional barrier to drug access to the remaining cells in the spheroid. Thus, the cells at the core can still survive despite further treatment with high doses of drug. In the future, mathematical models will continue to serve as important tools for understanding drug penetration, transport processes within a spheroid, and responses of cells within the spheroid to drug treatment.

Challenges for using spheroids as drug delivery and efficacy testing models

Although the advantages of multicellular spheroids have been widely recognized [9], challenges involved in the tedious procedures required for spheroid culture are still holding back the biological community from adapting the well-validated spheroid tissue models for studying drug delivery more widely. Here, we focus on the following challenges associated with the use of spheroid models for drug delivery and *in vivo* efficacy: (1) forming and maintaining spheroids of uniform size; (2) forming spheroids with small numbers of cells;

(3) making tissue-like spheroids with multiple cell types; and (4) analyzing tissue-like spheroids (including quantitative assays incompatible with spheroid platforms) and making them compatible with readouts associated with drug delivery and efficacy testing. Each of the challenges and their possible solutions are outlined in the following sections.

Challenge 1: Forming and maintaining spheroids of uniform size

Formation of spheroids occurs spontaneously, in environments where cell-cell interactions dominate over cell-substrate interactions (Figure 2). Conventional methods for spheroid generation include hanging drops, culture of cells on non-adherent surfaces, spinner flask cultures, and NASA rotary cell culture systems [9, 15, 43–48]. The *hanging drop* technique typically employs cell suspension droplets hanging on the underside of the lid of a tissue culture dish due to surface tension. It utilizes gravity to induce aggregation of cells into a single cluster, which then form into a spheroid. The hanging drop method, although simple and allows for defined size spheroid control, is extremely labor intensive. Maintaining spheroids in hanging drops and regular change of medium are time consuming.

Another technique for making spheroids involves culture of cells on non-adherent surfaces, which essentially prevents cells from attaching to a substrate, thus inducing cells to aggregate with themselves to form spheroids. Culture of cells in large bioreactors such as spinner flasks and NASA rotary cell vessels produces spheroids via continuous spinning of cell suspension to keep cells from settling down. Such systems are suitable for mass production and long-term culture but are limited by non-uniform spheroids and the need for specialized equipment. Size uniformity is critical for achieving reproducible experimental results since size affects cell behavior and function as well as drug penetration and transport.

More advanced spheroid formation methods developed recently often utilize micro- and nano-technologies. Such techniques generally provide great improvements over spheroid size-control, cellular composition, and throughput. Various groups have developed microfluidic devices to trap clusters of cells into micro-chambers, or posts [49–51] to control spheroid sizes and simplify liquid handling procedures (Figure 2). Others have created arrays of microwells to increase throughput and generate defined-size spheroids [44, 45, 52]. Through forced-aggregation techniques and micro-textured surfaces, scalable uniform size-specified embryoid bodies were generated from human embryonic stem cells, which can differentiate to clinically relevant cell types [45]. In addition, some groups have developed surface-modified substrates, porous 3D scaffolds, or nano-imprinted scaffolds to induce spheroid formation [27, 53–57]. Using photolithography, microwell arrays have been generated from poly(ethylene glycol) (PEG)-based hydrogels. These gel arrays are effective in confining single cells and aggregates, and in guiding their reorganization into spheroids [58]. However, many of these sophisticated platforms are complicated and thus require specially trained users to fabricate and operate the devices. Or they may allow efficient spheroid formation but not allow for convenient prolonged culture and drug testing where media exchange or reagent additions are required. In addition, many of these devices suffer from material compatibility issues with many hydrophobic compounds such as anti-cancer therapeutic drugs, which limits the applicability of these high-throughput devices to drug screening and testing.

Recently, our group has developed a 384-format hanging drop array plate as a user-friendly solution to spheroid formation and culture [59–61]. This versatile platform is based on the conventional hanging drop method, allows for high-throughput drug screening and testing, is amenable to high-throughput multiplexing (e.g. liquid handling robots and plate readers), but greatly simplifies the tedious liquid handling processes as well as increased robustness of droplet stability to allow long-term spheroid culture (Figure 2G). The hanging drop plate comprises of access holes that can accommodate a 15–20 μ l drop of cell suspension in

culture medium confined by the diameter of the plateau on the bottom surface, which results in consistent geometry of the hanging drop without spreading out, and leads to more robust and stable culturing conditions not possible on conventional flat hanging drop substrates. The cells slowly aggregate in the bottom center of the hanging droplet, and eventually form into a tight spheroid. The access holes on the top of the plate allow direct manipulation of the droplets, greatly simplifying the initial droplet formation and subsequent media exchange procedures by eliminating the tedious hanging drop culture dish inversion required in the conventional hanging drop method.

Using this platform we have successfully formed spheroids (mono-, co- and tri-cultures) with varying initial cell seeding density, ranging from 50 cells to 15,000 cells with different cell lines and primary cells [59–61]. The spheroids created on this platform have been analyzed for their cell-based assay capabilities by testing their sensitivity to anticancer drugs with distinctly different activity profiles: a conventional anticancer drug 5-fluorouracil (5-FU) that inhibits cellular proliferation, and a hypoxia-triggered cytotoxin tirapazamine (TPZ) that causes DNA damage at low oxygen tensions [59]. Compared to 2D cultures, 3D spheroids of epithelial carcinoma cell line (A431.H9) were more resistant to 10 μ M 5-FU treatment, suggesting the presence of quiescent cells in the spheroids. However, 3D spheroids were susceptible to 10 M TPZ treatment compared to 2D controls, possibly because active oxygen consumption by cells and limits in diffusive oxygen transport creates a hypoxic core similar to *in vivo* tumors. When treated with these drugs together, there was an additive effect on the cell death in 3D spheroids, since 5-FU targets proliferating cells in the peripheral layers of spheroids and TPZ kills cells in the hypoxic core of spheroids [59].

Unfortunately, the first generation of the 384 hanging drop array plate was susceptible to small mechanical shocks and could not reliably maintain hanging drops for longer than 14 days. Stabilization of liquid droplets was enhanced in the second generation of the 384 well plate, which was optimized with micro-rings to stabilize droplets against mechanical perturbations and prevent surface fouling. This system enables long-term cell spheroid culture for more than 22 days within the droplet array [60, 61]. With enhanced droplet stability, the second-generation hanging drop array plates provide new opportunities for high-throughput preparation of microscale 3D cell constructs for drug screening and cell analysis. A systematic analysis of the advantages and disadvantages of different spheroid forming techniques is listed in Table 1.

Challenge 2: Forming spheroids with small number of cells

Efficient formation of spheroids requires segregated groups of cells maintained within close proximity to each other to aggregate together, which becomes especially challenging when the number of available cells is small, as in the case of primary stem cells and CSCs. Many groups focusing on cancer stem cell research have formed spheroids from single cells by monoclonal growth [23, 62–68]. This method has been particularly successful for certain cancer cell lines as many transformed cells are anchorage independent, thus do not require a stiff substrate to survive. Such cultures have been shown to enrich the stem cell subpopulation within bulk tumor cells. Nevertheless, manipulating, forming, growing, and maintaining spheroid from single cells can be very challenging. First of all, it is very difficult to titrate and manipulate cell suspensions into single cell levels, especially from small numbers of CSCs that may have already undergone stress through isolation and sorting. In addition, for cells that are still anchorage-dependent, lack of cell-cell interaction often slows down proliferation and may even lead to cell death. Therefore, such experiments have not only been challenging, but also very time-consuming. To overcome these challenges, the addition of Matrigel or other extracellular matrix (ECM) proteins (such as collagen and fibronectin) or materials such as methylcellulose to cultures have been shown to enhance spheroid assembly by providing cell-matrix support [69–74]. Alternatively, small

numbers of CSCs may be co-cultured with other supporting cell types as spheroids to achieve monoclonal expansion of the tumor cell within a supportive 3-D microenvironment.

Challenge 3: Making tissue-like spheroids with multiple cell types

Tissues *in vivo* typically integrate interactions between the surrounding multiple cell types: such as, epithelial cells, fibroblasts, mesenchymal stem cells (MSCs), endothelial cells, and immune cells, and the abundant extracellular matrix proteins, immobilized protein factors, proteoglycans, mineralized tissue, soluble protein factors, and small molecule signals [75]. Response of cells to drug delivery systems is dependent on these interactions; indeed, drugs effective against specific cells in one site may not work against the “same” cells in a different organ/tissue [76, 77]. In order to faithfully develop effective drug delivery systems, some of the *in vivo* microenvironmental interactions need to be captured *in vitro* in 3-D models of tissue, such as spheroids. We and others have established that spheroids can integrate multiple cell types, ECM and other molecules. With our 384 hanging drop system, we have successfully formed spheroids (mono-, co- and tri-cultures) with a varying initial cell seeding density, ranging from 50 cells to 15,000 cells with different cell lines and primary cells [59–61]. Additionally, the 384 hanging drop array plate is also capable of spheroid transfer and retrieval for Janus spheroid formation, sequential addition of cells for concentric layer patterning of different cell types, and culture of a wide variety of cell types [60, 61]. Mouse mammary tumors were recapitulated recently in spheroids with tumor and endothelial cells and it was found that the initial primary or micrometastatic stages of solid tumor progression could be more accurately modeled in co-culture spheroids than conventional 2-D cultures [78]. When implanted *in vivo*, the co-culture tumor-endothelial spheroids enhanced angiogenesis and metastasis, compared to tumor cell only spheroids, emphasizing the importance of crosstalk between the cells comprising the tumor microenvironment.

Microscale technologies have also been applied to spheroid co-cultures. Co-culture spheroid arrays have been developed using micropatterning techniques [52, 79]. Hepatocyte spheroids co-cultured with endothelial cells or fibroblasts maintain their liver-specific functions for at least 1 month in such formats. Growing spheroids inside PDMS microfluidic bioreactors exposes them to uniform and non-uniform flow. The culture of HepG2 cells was demonstrated in such microbioreactors, and cell functionality (albumin production, glucose consumption) was evaluated [80]. Similarly, breast tumor cells were encapsulated within alginate, and gelled *in situ* within the microchannels of a microfluidic device. The cell aggregates were treated with various concentrations of doxorubicin. Drug effects on cell viability and proliferation were measured [81].

Interactions of cancer cells with the cellular and acellular components of their stroma control the growth and malignant behavior of invasive cancer [82] and should be included in at least rudimentary form in tumor models used in screening anti-cancer drug candidates. This requirement has been addressed by generating tumor histoids (TH) which are formed through the spontaneous interaction of tumor and stromal cells to form spheroidal TH that have a microscopic architecture closely resembling that of true tumor microlesions [83, 84]. TH generated in low shear, rotating suspension culture (original method) varied in size and tumor cell content (Figure 3). Although they could be analyzed and sorted by COPASTM flow cytometry (which can accommodate samples as large as 100 – 1,500 μm) to deliver relatively uniform TH into multiwell plates, the additional manipulation was a drawback. TH production is currently being adapted to the 384 hanging drop format referred to above to address this problem.

Challenge 4: Characterization of drug testing and efficacy in spheroid platforms

One of the major reasons spheroids have not entered mainstream drug screening process is due to the lack of simple, controlled techniques and protocols for rapid, standardized assay of cellular responses *in situ*, prediction of *in vivo* activity, as well as poor integration with high throughput systems widespread in industrial labs. However, new developments in spheroid technology are challenging these dogmas. Analyses must integrate quantitative population based information (cell number, position), subcellular functional readouts (viability, cell cycle, and signaling), along with more qualitative information (microscopy and histology). As spheroid models become widespread in biological studies, the feasibility of conducting standard biological assays with cells grown on such platforms must be carefully considered. Harvesting the spheroids from the culture platform such as microfluidic devices and hanging drops poses a challenge. Spheroids are often lost during medium exchange. Compatibility with standard biological assays such as RT-PCR, western blots, flow cytometry, are also a challenge when working with spheroid models. Here we discuss amenability of current 3-D spheroid systems for drug delivery systems.

Screening techniques currently applied to monolayer cultures in multiwell plate formats, such as various assays for cytotoxicity, proliferation, drug binding, apoptosis, and ATP level, can be adapted to spheroids. Measurement of the growth or shrinkage of spheroids in response to the drug can be accomplished by standard phase-contrast microscopy and image analysis. Moreover, confocal microscopy techniques can be developed for measuring drug penetration into individual spheroids [94–97], which could potentially be automated into a high throughput approach. Important assays such as drug binding, uptake and penetration can be accomplished using spheroids in a moderate to high throughput manner as well.

Standard biological assays such as fluorescence activated cell sorting (FACS) can also be applied to spheroids. For example, the differential cytotoxicity in the cells near the hypoxic core of the spheroid relative to the well-oxygenated peripheral cells, was measured by FACS after selectively recovering cells from various depths within the spheroids [98].

Due to the need for a large number of cells within a test sample, molecular biological assays such as western blotting and RT-PCR are difficult to perform on lysates obtained from spheroids. However, microscale versions of western blot have now been developed [99–101] which are capable of handling samples with low cell numbers. Quantitative RT-PCR assay still remains a challenge for the RNA harvested from spheroids. However, if lysates can be collected from at least 2000 cells per sample (by pooling duplicates), RNA can be purified using RNA purification kits designed for rare cell samples, and subsequently be used to make cDNA for qRT-PCR gene expression analysis. Alternatively, for low cell number spheroids, kits that convert cells directly to cDNA bypassing the RNA purification step (Ambion Inc. Cells-to-cDNA™ II Kit), can be utilized for qRT-PCR analysis of mRNA levels. Although the minimum number of cells required for flow cytometric analysis (at least 10,000) is higher than what can be typically isolated from a single spheroid, samples can be pooled and aggregates disassociated for analysis of cells post culture. Microscale flow cytometers that do not require such a large number of cells in a sample can also be utilized [102]. Pooling of samples would necessitate a large number of samples, which are resource and time consuming to maintain. Moreover, all of these techniques add unwanted complexity to the spheroids culture and maintenance, and may not be easily integrated into cell biology labs that have little expertise in microscale technologies.

Quantitative measurements of cell type specific phenotypes are often difficult to assess in co-culture spheroids. For example, one of the methods for quantifying proliferation rates of each cell type would require labeling each cell type via cell tracker stains, which tend to bleed over time, or tagging the cells with fluorescently expressed proteins such as GFP,

which require additional transfection steps before spheroid formation. Another method for quantifying proliferation rates of each cell type could involve flow cytometric analysis of harvested spheroids at a given time point, stained with markers that identify each cell type, and would require a large sample size of spheroids in order to obtain at least 10,000 cells that are needed for flow cytometric analysis.

Emerging micro- and nanotechnologies have enabled fabrication of many different spheroids-on-a-chip devices that substantially increase throughput for preparing spheroid cultures. However, hydrophobic materials, such as poly(dimethylsiloxane) (PDMS), used in many of these devices bind hydrophobic compounds, making the devices incompatible with library screening and drug testing applications. Even if material is compatible with anti-cancer therapeutic compounds, chip formats are usually not compatible with HTS instruments (robots/plate readers). Therefore, there is a need for incorporating assay readouts and analyses within the devices.

Many commercially available cellular assays are designed for 2-D monolayer cultures, so caution must be taken when applied to 3-D spheroids. Such assays need to be validated in spheroid platforms with appropriate controls and generation of standard curves. We recently evaluated robustness of the 384 hanging drop plate array in terms of assay performance by calculating Z- factors for fluorescence- and colorimetric-based assays, to allow for practical 3-D cell-based high-throughput screening and enable broader use of the 384 hanging drop plate [61]. Z-factor, an assay performance measurement that provides an easy and useful summary of assay quality and robustness [103–105], was found to be well above 0.5 for both the fluorescence- and colorimetric-based assays, indicating that such assays performed in the 384 hanging drop plate would have excellent accuracy and robustness in terms of readout reliability [60]. When using assay readout reagents, care must be taken that there is sufficient reagent penetration to provide readouts from a representative number of cells within the spheroid. Reagent penetration is in and of itself a sort of “drug delivery” process that depends on reagent type, cell types, as well as spheroid size. We and others have demonstrated that high throughput assays such as alamarBlue can be standardized for high throughput spheroid assays in general and the 384-hanging drop array platform in particular.

Bioluminescence assays with luciferase enzymes have emerged as a powerful alternative to fluorescence and colorimetric readouts for high throughput screening in cell-based formats. Advantages of bioluminescence relative to fluorescence include enhanced sensitivity due to lower background, greater dynamic range of quantitative signal, and reduced susceptibility to interference by colored compounds [106]. Bioluminescent reporters have been used successfully to identify promising therapeutic agents, including compounds that perturb tumor-stromal signaling [107]. Benefits and proven efficacy of bioluminescence for compound screening and drug testing in two dimensional cell cultures support use of luciferase-based readouts for high throughput screening and validation studies in spheroids [108].

Bioluminescence reporters for drug testing in spheroids can be designed to quantify basic parameters such as cell viability, or more advanced assays can be developed to measure protein-protein interactions, cell signaling, and/or promoter activity. Beetle luciferases, including firefly and click beetle luciferases, are ATP-dependent enzymes that provide robust quantification of cell viability [109]. Bioluminescence from these enzymes is directly proportional to numbers of living cells over 4 orders of magnitude, enabling facile analysis of drug cytotoxicity in intact spheroids (Figure 4). To measure effects of drugs on specific molecular pathways, investigators have established reporters and biosensors based on split luciferase complementation [110]. While not yet used for drug testing in spheroids, split luciferase complementation assays for key drug targets, such as ligand binding to seven-

transmembrane receptors and activation of the proto-oncogene AKT, have been validated in 2-D cultures [111, 112]. Integration of such reporter systems into spheroid models will advance high throughput screening for molecularly-targeted drugs. In addition, availability of luciferase enzymes with distinct substrates and/or emission spectra will allow readouts of multiple drug targets in individual spheroids, increasing efficiency of a single screening assay.

Comparison of drug delivery and efficacy between multicellular spheroids and *in vivo* models

In vitro models using multicellular spheroids provide an important link between monolayer cell cultures and animal experiments for effective drug delivery testing. Key similarities between outcomes of drug targeting in spheroid and animal models are discussed below:

Drugs can be delivered to cells within spheroids by several techniques such as liposomes, nanoparticles, and nonviral gene delivery [1, 113, 114]. Encapsulation of certain drugs in lipid vesicles (liposomes) has been shown to result in reduced toxic effects [115]. Parameters such as liposomal surface charge, mean diameter, lipid bilayer fluidity, lipid bilayer composition, duration of liposome-spheroid interaction, mean liposome size, steric stabilization of liposomes and fusogenicity impact spheroid penetration [95, 116, 117]. Non-viral gene transfer to spheroids was optimized for 22 kDa linear and 25 kDa branched polyethyleneimine (PEI) molecules [118]. Confocal imaging of fluorescent PEI indicated that the cationic complexes could only penetrate the outer 3–5 proliferating cell layers of a spheroid, sparing the deeper quiescent cells, which could be targeted via electroporation. Although the efficacy of electroporation in quiescent tissue was improved, gene expression was still confined to the outer regions of the spheroid, suggesting that expression of transgenes is better in dividing cells, and that limited access to central regions of the spheroid remain a significant barrier to gene delivery.

Extracellular matrix in tumors restrict nanoparticle penetration for cancer imaging and therapy. To overcome this barrier, effects of nanoparticle size and collagenase treatment on penetration of carboxylated polystyrene nanoparticles were systematically assessed in a multicellular spheroid model [119]. It was found that penetration of nanoparticles into the spheroid core was limited to nanoparticles smaller than 100 nm. However, collagenase-coated, 100 nm nanoparticles demonstrated a 4-fold increase in the number of particles delivered to the spheroid core compared with control nanoparticles, revealing that nanoparticle delivery to tumors may be substantially improved by incorporation of extracellular matrix-modulating enzymes in the delivery formulation.

The rate of drug diffusion through the tissue is determined by physicochemical properties such as molecular weight, shape, charge and aqueous solubility. Penetration of a drug into a tissue is also dependent on its consumption, which removes the amount of free drug, thus inhibiting further permeation. Spheroids can serve as excellent model systems to study permeation of drugs and their carriers through organs and tissues. Low permeability of delivery systems across the blood-brain barrier (BBB), drug resistance, and poor penetration into tumor tissue limit treatment strategies in glial tumors. Dhanikula et al. studied permeability of methotrexate (MTX)-loaded polyether-copolyester (PEPE) dendrimers across an *in vitro* BBB model and their distribution into avascular human glioma tumor spheroids to evaluate their potential as drug carriers for gliomas [120]. Glucosylated dendrimers were endocytosed in significantly higher amounts than nonglucosylated dendrimers. Moreover, glucosylation enhanced cumulative permeation of dendrimers across the BBB and avascular tumor spheroids and increased the amount of MTX delivered to tumor cells. These results established that glucosamine can be used as an effective carrier not only for targeting glial tumors but also for enhancing permeability across BBB. Thus,

glucosylated PEPE dendrimers can serve as potential delivery system for the treatment of gliomas.

To assess *in vivo* tumor targeting and therapeutic efficacy, neuroblastoma spheroids and nude mice xenografted with human neuroblastoma cells were treated with radioisotope iodine-131-metaiodobenzylguanidine ([¹³¹I] MIBG) without any added carrier [121]. The drug was uptaken successfully in the tumor *in vivo*, where it reduced tumor burden, and prevented regrowth of spheroid *in vitro*, suggesting that the spheroid results correlate well with those in the animal model. However, the large spheroids were more vulnerable to the treatment than smaller spheroids, indicating that the *in vivo* treatment may spare smaller micrometastases [122]. Similarly, cytotoxic activity of the galactoside-specific lectin from mistletoe towards anaplastic glioma was investigated in a spheroid model and *in vivo* in rats intracerebrally implanted with F98 glioma cells [123]. Dose dependent cytotoxicity was observed in spheroids as well as *in vivo*. These studies indicate that spheroids are a relevant model of the *in vivo* tumors, and screening of cancer therapeutics in spheroid models can lead to selection of promising drug candidates and decrease time between drug discovery and clinical trials.

Drugs (such as anthracyclines) accumulate preferentially in cells at the periphery of spheroids, resulting in reduced rates and concentrations of drug delivered to cells in the interior. However, drugs are retained more effectively in intact spheroids than in dispersed cells, resulting in more cytotoxicity *in situ* in spheroids [124]. Doses of drugs can also have varying effects on tumor spheroids. While low concentrations (<100 μ M) of gamma-linolenic acid (GLA), an essential fatty acid, increased both apoptosis and proliferation with a net increase in glioma tumor growth and invasion, higher doses significantly impaired cell growth. The proliferative effects of low-dose GLA could be a hazard in the clinical treatment of malignant glioma; however, because of the low toxicity of GLA against normal cells, local delivery of millimolar doses of GLA could significantly reduce tumor size [125].

Similar to *in vivo* tissues, spheroids are generally more resistant than 2-D cultures to a given dose of many drugs [126–131]. Reasons for this difference involve factors including drug penetration, cell–cell contact, cell-cycle distribution and varying microenvironment (such as pH, extracellular matrix) within spheroids [12, 132–138]. For example, intercellular contacts promote cell survival through activation of signaling pathways such as PI3K/Akt, NF- κ B and Stat3. Moreover, the reductions in rate of cell division within the spheroid impairs the effects of many cancer drugs since most anti-cancer drugs exert selective toxicity on dividing cells. Further, drug binding to ECM not only slows down the movement of drugs towards target cells, but also changes the number of available drug molecules. Drug penetration can also change due to the modifications in its charge.

Not surprisingly, similar to the *in vivo* tissues, penetration of a drug into a spheroid is the rate-limiting step for drug delivery, and it has been shown that some drugs may penetrate at non-negligible quantities only to a depth of a few cells despite prolonged exposure [94, 95, 97, 137–139]. Some drug molecules (non-conjugated or conjugated with targeting agents such as radionuclides) can easily penetrate through spheroids while others cannot. Durand et al. treated V79 (lung fibroblast) spheroids with doxorubicin, bleomycin, 5-fluorouracil, carmustine, cisplatin, chlorambucil, and mitomycin. Drug penetration within a spheroid is also a function of the drug molecule structure. As an example, doxorubicin penetrates poorly into V79 spheroids as compared with other tested drugs tested [140]. Lack of drug penetration can be due to avid binding to surface cells [141] and/or barriers to drug transport due to extracellular matrix, cell-cell junctions, and cell membranes [94, 97, 116, 137]. Due to pH gradients within the spheroids, cells in the acidic core region can be protected from weak acid drugs such as mitoxantrones and anthracyclines, at the same time potentiating the

effect of weak base drugs such as chlorambucil and mitomycin C [128, 142, 143]. Due to mass transport limitations and a 3-D structure similar to *in vivo* tissues, drug penetration limitations occurring *in vivo* can be studied in spheroids.

Testing in multicellular spheroids is increasingly regarded as an essential step in drug development [16, 43, 134, 144, 145]. As discussed in this article, a variety of biomedical researchers have applied multicellular spheroid models for assessing various therapeutic treatments, some of which include: chemotherapy including target-specific approaches, experimental radiotherapy followed by photodynamic treatment, cell- and antibody-based immunotherapy, gene therapy and combinatorial therapies [43, 126, 127, 132, 133, 145–158]. Together, these experiments demonstrate that spheroid-based assays have improved predictive power for *in vivo* therapeutic efficacy. These examples also reveal that structure-function relationships from interactions of different delivery systems with 3-D spheroids can lead to construction of improved delivery systems for drug penetration and targeting into tissues. The spherical shape is also easy to analyze and model mathematically, providing benefits as a screening tool for a variety of therapeutics.

Conclusions

Development of uniform, high throughput, multi-cell-type spheroids present an opportunity to screen and select drugs and drug delivery systems *in vitro* in a format that more closely resembles conditions in patients. Spheroids have similarities to *in vivo* tissues in terms of complexity of cell types (spheroids can be made with many cell types), key cell-cell interactions, extracellular matrix deposition by cells, and chemical gradients. These features of spheroids limit drug diffusion to an extent comparable to organs and tissues *in vivo*. Spheroids can support growth of rare cell types such as cancer stem cells, providing a model for testing drugs targeted against specific cell populations and types that are otherwise difficult to perform conveniently. Despite challenges that have limited broader use for the past half century, advances including new technologies for spheroid formation, culture, and analysis that are highlighted in this review suggest that use of spheroids are ripe for becoming a mainstream *in vitro* tool for testing drug delivery and efficacy, providing readouts that better predict results in patients than standard 2D cultures.

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Figure 1. Spheroids are appropriate models for testing drug delivery systems *in vitro*.

A) *In vivo*, concentration of drug, oxygen and nutrients decreases on increasing distance from blood vessels. The effectiveness of drug and carrier system *in vivo* is a function of drug dose, potency, kinetics, molecular weight, charge, solubility in water and lipids, diffusion, barriers in the microenvironment, binding, metabolism, and sequestration. **B)** Spheroids are 3-D microscale tissues which exhibit an inherent gradient of nutrients, oxygen and metabolites within themselves, which leads to a central necrotic core region surrounded by quiescent viable cells and an outer layer of actively proliferating cells. Due to similarities between *in vivo* tissues and spheroids as well as mass transport limitations, spheroids can serve as high throughput screening platforms for drug and carrier effectiveness. Scale bar is 200 μ m.

Figure 2. Current state-of-the-art methods of generating uniformly sized spheroids

A) A spheroid co-culture system utilizing chitosan hydrogel microarray molded with PDMS. The uniformity of spheroids generated was controlled by the patterned chitosan. Reproduced from [52] by the kind permission of Elsevier Press. **B)** Schematic of arrays of v-bottom PDMS microwells to generate uniformly-sized spheroids in a high-throughput fashion. Reproduced from [45] under the Creative Commons Attribution License. **C)** A microfluidic device with micro-posts to trap single cell suspensions into uniform clusters of cells that will then self-aggregate into spheroids. Reproduced from [49] by the kind permission of Elsevier Press. **D)** A microfluidic device with micro-chambers to trap single cell suspensions and induce spheroid formation. The size of the spheroids is controlled by the chamber dimensions. Reproduced from [51] by the kind permission of Springer Science. **E)** Cartoon of a v-bottom microwell-based device to generate spheroids of the same size. Reproduced from [44] by the kind permission of Elsevier Press. **F)** Schematic showing a two-layer PDMS-based microfluidic device for the generation of uniformly-sized embryoid bodies. After introducing cells into the microchannel (with surfaces blocked to prevent cell adhesion) as a uniform monolayer, cells spontaneously aggregate and break up into uniformly-sized embryoid bodies. Reproduced from [50] by permission of The Royal Society of Chemistry. **G)** 384 hanging drop array plate and a cartoon of the spheroid formation process. The size of the spheroid is controlled by the number of cells seeded into each hanging drop. Reproduced from [59] by permission of The Royal Society of Chemistry.

Figure 3. Tissue and tumor histoids are generated through the spontaneous interaction of multiple cell types

A) Histological section of breast cancer histoid (BCH) containing BT20 cancer cells and, generated in low shear, rotating suspension culture and immunostained to demonstrate cytokeratin in cancer cells. Photographed using 20x objective; inset shows size variability. Suspension of intact, viable pancreatic tumor histoids containing fibroblasts and pancreatic cancer cells (MIAPaCa) were generated in rotating suspension cultures, and imaged under **B)** 10x and **C)** 20x objectives. Pancreatic cancer cells were transfected to express green fluorescent ZsGreen 1, a variant of the widely used GFP. These histoids can be sorted according to size and fluorescent intensity (using COPASTM flow cytometer) to obtain a relatively uniform population.

Figure 4. Bioluminescence quantification of doxorubicin cytotoxicity in MDA-MB-231 human breast cancer cells

A) Image of luciferase activity in intact spheroids, and **B)** quantification of bioluminescence after 48 hours of treatment. Luminescence data were normalized to pre-treatment values for each spheroid. Loss of bioluminescence directly measures relative cytotoxicity. Error bars denote mean values + SEM.

Table 1

Comparison of different spheroid forming approaches

Method	Advantages	Disadvantages
Hanging Drops [9, 15, 22, 43, 46, 47]	Simple No specialized equipment required Defined spheroid size control Uniform spheroid size Co-culture with defined cellular composition control	Tedious handling Time consuming Not stable Long-term culture difficult Low throughput Not efficient
Non-adherent Surfaces [15, 43]	Simple No specialized equipment required High-throughput (*Multiwell plate-format only) Compatible with HTS instruments (*Multiwell plate-format only)	Not efficient Non-uniform spheroid size No control of spheroid size Tedious media exchange Defined co-culture cellular composition difficult
Spinner Flask [15, 43]	Simple Mass production of spheroids Long-term culture	Special equipment required No individual compartment for each sample No spheroid uniformity control (composition and size) High shear force for cells
NASA Rotary Cell Vessel [7, 15, 43]	Simple Mass production of spheroids Long-term culture	Special equipment No individual compartment for each sample No spheroid uniformity control (composition and size)
Micro/Nano Technology: Microfluidic Devices and Spheroids on a Chip [49–51, 55, 85]	Improved efficiency Uniform spheroid size control Uniform cellular composition High-throughput Simplified handling procedures Analysis may be integrated into the device	Complicated set-up and device fabrication Customized devices may require trained users to operate Devices not mass producible for research community Material compatibility issue with drugs (hydrophobic compounds) Compatibility with HTS instruments Spheroid retrieval for further analysis difficult Long-term culture issue Lack of isolated compartment for each sample
Micro/Nano Technology: Microwell Arrays [44, 45, 52, 86]	Improved efficiency High-throughput production Uniform spheroid size control Simplified handling	Long-term culture issue Sample retrieval difficult Fabrication of device require trained users Devices not mass producible for research community Material compatibility issue with drugs (hydrophobic compounds) Compatibility with HTS instruments Long-term culture issue
Micro/Nano Technology: Scaffold Technology [57, 87–92]	High-throughput production Spheroids immobilized on scaffolds so easy handling Long term culture Mass production of scaffolds possible May be compatible with multiwell plates May be compatible with HTS instruments Uniform spheroid size	Samples retrieval for further analysis difficult Scaffold material biocompatibility and biodegradability issues
Multiwell Hanging Drop Plates [59–61, 93]	Material compatibility with drugs High-throughput Compatible with HTS instruments Individual compartment for each sample for HTS Long term culture Robust assay performance User-friendly Mass production of device possible Defined spheroid size control Uniform spheroid size Co-culture with defined cellular composition control	Media exchange tedious without HTS instrument Hard to work with small number of cells