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Review

An Overview on Spheroid and Organoid Models in Applied Studies

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Abstract: From its inception, cell culture has been a great scientific tool for researchers in many diverse fields. The advancement from monolayer 2D cultures into three-dimensional cellular systems enabled a better experimental tool, as the 3D culture mimics in vivo environments more closely. Cells are aggregated in clusters, allowing for more cell-to-cell interactions, cell migration, and differences in nutrient and oxygen availability. Spheroids and organoids are most commonly used and have proven themselves as models for a large number of analytical purposes. The simplicity of spheroid production is often a good starting point. Because organoids are more complex, they can provide better and more complete data, but they can be difficult to grow and maintain. With increasing concern about the applicability of data obtained from animal studies and questions regarding animal welfare, these can replace a large proportion of these models and provide accurate and rapid results. In this overview, aimed at someone looking for an introductory summary of the requirements and possibilities of different 3D culture approaches, we give the basic information on various uses of spheroids and organoids in different fields of science. Assays based on spheroids and organoids can be adapted for a range of applications, and their use will continue to grow.

Keywords: cell culture; cytotoxicity; disease modelling; drug discovery; personalised medicine; regenerative medicine

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1. Introduction

The development of pharmaceuticals is a time-consuming and expensive process. The average cost of developing a new drug to market is more than 1.2 billion dollars, and it takes about 12 years [1,2]. More than half of all failures in phase II and III clinical trials today are due to lack of efficacy. Safety concerns, such as an inadequate therapeutic index, account for about one-third of failures. New tools that enable more accurate drug discovery are crucial, as the dropout rate in this area is still very high [3]. Many adverse effects observed in initial animal studies were not detected in clinical trials. In addition, the high dropout rates in drug research suggest that incorrect preclinical testing procedures and in vitro models—which do not provide adequate data for predicting therapeutic efficacy and safety concerns—may be the main cause of drug failure. To obtain more accurate data, one of the main areas expected to increase the success rate of the drug development process could be the application of new technologies in preclinical testing and in vitro models [1,2].

Several current and developing technologies have been used to more accurately predict drug selection problems in clinical trials, such as two-dimensional (2D) tissue culture and three-dimensional (3D) culture models. First, in vitro cell cultures composed

of cells attached to a flat surface and forming a monolayer. In these conditions, all cells were equally exposed to the media and to any tested compound. Since the cells were organised in a monolayer, this approach to culturing was subsequently defined as 2D cell culture. Two-dimensional cell cultures have their limitations, as they cannot accurately replicate the conditions of cell development and differentiation in vivo due to differences in cell shape, biochemical properties, cellular environment, and morphology compared to the original tissue. Three-dimensional cultures allow cells to build three-dimensional structures that are more similar to the in vivo environment than 2D cultures, where cells are confined to a single layer. In three-dimensional models, there is more cell-to-cell contact. Cells aggregate due to external conditions during culturing (gravitational force, spin, supportive scaffolding, magnetic force, etc.). This spatial arrangement enables complex cell–cell and cell–matrix interactions that are essential for a range of cellular functions, such as differentiation, proliferation, and response to stimuli. Therefore, tremendous efforts have been made over the last decade to create in vitro cell culture models that are more realistic and can mimic in vivo conditions. Monolayer cell culture has been replaced by three-dimensional culture models, which include spheroid and organoid cultures [3–5]. This review aims to explore the potential of 3D cell culture models, particularly spheroids and organoids, to revolutionise drug discovery and development.

2. Basic Concepts of 3D Culture

Three-dimensional cell cultures represent a revolutionary approach to cultivating cells outside the body, allowing them to multiply and function in a three-dimensional space that is much closer to the conditions in our bodies. In contrast to monolayer cultures, 3D cell models increase physiological relevance, improve reproducibility, and thus enable more consistent results. Specific 3D culture models, such as spheroids and organoids, have been used in cell culture to implement a variety of culture techniques [5,6]. The differences between spheroids and organoids are obvious, although the nomenclature is sometimes misunderstood. Spheroids consist of simple cell clusters in a scaffold-free environment, whereas organoids have the ability to self-differentiate and exhibit properties and functions similar to those of human organs. Spheroids are valuable tools for the study of basic cellular processes, drug screening, and tumour biology. Organoids, on the other hand, are more complex structures that can mimic the organisation and function of tissues at the organ level. This makes them ideal for the investigation of disease mechanisms and the development of personalised medicine [6]. There are various considerations to be made when choosing the appropriate 3D option for your research (Table 1) [7,8].

Table 1. Contrasting and comparing the key features and consideration for spheroids and organoids.

	Spheroids	**Organoids**
Cells of origin	cell lines, primary cell lines, single cancer cells, cell mixtures, tissue	cell lines, primary cell lines, single cancer cells, cell mixtures, tissue
Structure	multi-layered structure	reflects specific tissue characteristics
Generation	easy, aggregation methods	difficult, requires specific culture conditions and signalling molecules
Culturing	difficult to maintain long term	long term culture and cryopreservation
Vascularization	absent or limited	can be induced but often insufficient
Reproducibility	high	moderate
Translational value	limited	high
Cost	relatively low	high
Applications	drug testing, toxicity, tumour modelling	disease modelling, regenerative medicine, personalised medicine

3. Spheroid as a 3D Cell Culture Model

Sutherland and his colleagues originally introduced spheroids in the early 1970s. Since then, several spheroid models and techniques for their generation have been developed [9]. Multicellular spheroids (MCTSs), three-dimensional spherical cell aggregates, have thus become one of the most popular and versatile methods for culturing cells in 3D [10]. In spheroids, cells are densely packed together. As a result, MCTS cells maintain intricate connections between them and the extracellular matrix (ECM) through strong communication. The downside is that many variables, including cell type, cultivation methods, medium volume and composition, and cell density, are related to the production of MCTSs. These elements lead to unpredictability in the production of MCTSs, making reproducible spheroid formation difficult [11].

The formation of spheroids can be divided into three phases—aggregation, compaction, and growth. For cell aggregates to form, transmembrane receptors (integrins) are required, which facilitate cell–cell and cell–extracellular matrix adhesion [12]. Dispersed cells initially form loose cell aggregates due to the binding between long-chain ECM fibres (extracellular matrix) with multiple integrin RGD (arginine–glycine–aspartic acid) motifs on the cell surface [12,13]. These interactions can lead to compaction, in which the spheroids become more densely packed and take on a round shape. As the spheroids continue to grow, they change their internal structure and organisation. These changes may include cell proliferation, differentiation, and the development of gradients in oxygen and nutrient availability. In addition, the growth of 3D spheroids can also lead to the formation of complex cellular structures and more closely mimic the microenvironment in vivo [14,15].

Aggregation causes the cells within the spheroid to be in close contact, reducing oxygen permeability in their central region. Thus, the inner spheroid structure consists of outer proliferating, intermediate senescent, and quiescent cells and an inner apoptotic and necrotic core [14,16]. The excessive oxygen consumption of the cells, the low oxygen permeability, and the limited nutrient transport cause hypoxia and necrosis in the core of the spheroid [17].

The process of spheroid formation involves several methods, each with unique advantages and applications. From the use of simple hanging drop techniques to advanced bioprinting systems, the methods used to produce spheroids are diverse and can be tailored to meet specific research needs. These methods are crucial for the production of spheroids that closely mimic the physiological conditions of human tissue. For example, MCTSs can be cultured only with carcinoma cells or in combination with other cell types, such as immune, fibroblast, or endothelial cells [11,18]. These introductory statements form the basis for a deeper exploration of the specific methods used to form 3D spheroids (Figure 1) [5,12,13,19–21]. The hanging drop method is the fastest and simplest, but due to the limitations of the drop size, the resulting spheroids can be irregular in size. Scaffolds provide structure and can be used to grow larger aggregates but are difficult to manipulate. Choice of the method or combination of methods depends on the needs of the assay.

Regardless of the method of choice, achieving consistency and size is one of the main obstacles in spheroid culture. The size and uniformity of spheroids are influenced by a variety of factors. Initial cell seeding density plays a crucial role, with higher densities generally leading to larger spheroids. Culture conditions, such as media composition, oxygen tension, and nutrient availability, can significantly impact spheroid growth and morphology. Additionally, different cell types exhibit varying propensities to form spheroids and may have distinct growth rates. As spheroids grow larger, their core regions can become hypoxic due to limited oxygen and nutrient diffusion, leading to cell death and necrosis. To address the issue of necrotic core formation in larger spheroids, researchers have developed several strategies. Rotating bioreactors provide continuous agitation and nutrient exchange,

promoting spheroid growth and viability by ensuring adequate oxygen and nutrient supply throughout the culture. Perfusion bioreactors allow for continuous perfusion of fresh media, further enhancing oxygen and nutrient delivery to the spheroids. Additionally, engineering cells to be more tolerant to hypoxia, such as by genetically modifying them, can improve spheroid viability [22,23].

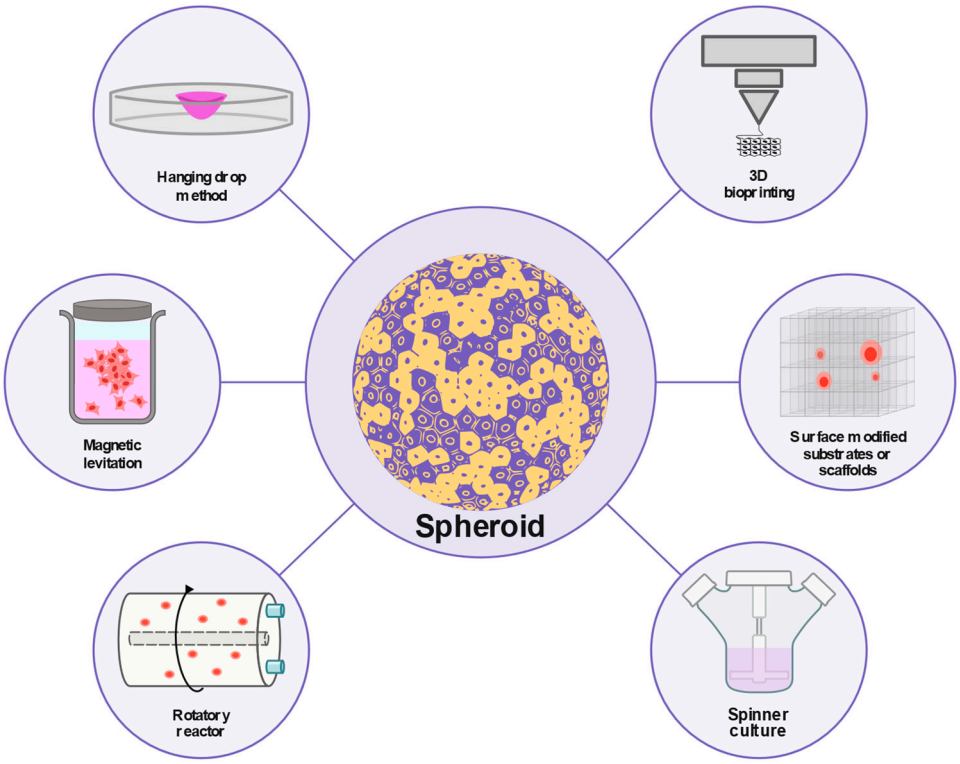


Figure 1. Methods of spheroid formation. Spheroids are formed through the aggregation of cells facilitated by the growth environment or applied force. They can be scaffold-free (hanging drop method, magnetic levitation) or utilise scaffolds (surface-modified substrates, 3D bioprinting). Rotary reactors and spinner cultures are used for spheroids cultivated in larger quantities and stress conditions.

3.1. Classification of Spheroid Models

Spheroid models are mainly classified according to their cellular origin. Therefore, we divide the spheroid models into the four most commonly used groups: multicellular tumour spheroids, tissue-derived tumour spheroids, tumour-derived tumour spheroids, and organotypic multicellular spheroids. Each of the above spheroid models requires a specific protocol for fabrication. This includes the selection of scaffold-based or scaffold-free approaches, the choice of an appropriate medium to support cell growth, the different duration of the growth phase in a spheroid formation, and the cell density [24]. Currently, there are a variety of methods for spheroid formation, and some have been developed to produce spheroids in large quantities to enable reproducible results in drug screening assays [25].

Multicellular tumour spheroids (MCTSs) are 3D structures composed of single or multiple cell types that can be formed using either a scaffold-based or scaffold-free approach [26]. In scaffold-based systems, the cells grow in a 3D extracellular matrix or in hydrogel-based microwell plates [11,27]. This approach allows for better cell adhesion by creating an in vitro environment that resembles the original tumour. Biomaterials used as scaffolds must fulfil certain criteria for biocompatibility, biodegradability, and biosafety [28]. For the development of 3D MCTSs, the biomaterials can be natural or made of synthetic materials. Natural polymers such as collagen, gelatine, or alginate have, on average, more biocompatible properties than synthetic materials, but their use in the clinical setting raises

questions regarding the possible transmission of diseases. Synthetic scaffolds such as chitosan and polycaprolactone are more suitable for the production of MCTSs with uniform size and shape. Angiogenesis, chemotaxis, and self-renewal can all be enhanced by cultivating on chitosan films. Particulate factors are being used for spheroid cultures to regulate the microenvironment of the cell culture. Nonetheless, while culturing, particles inside spheroids have the ability to regulate internal conditions. As a result, cell viability and proliferation increase. However, these scaffolds lack any bioactive properties [29–31]. In contrast to scaffold-based MCTSs, which consist of loosely dispersed cells with a uniform distribution of fibronectin throughout the spheroid, scaffold-free MCTSs consist of compactly distributed cells with fibronectin expression restricted to the peripheral edge. The correct approach depends on several factors, including the specific requirements of the intended application and the characteristics of the target MCTSs. For example, inflammatory breast cancer cells that produce ECM proteins and self-assemble into highly organised three-dimensional tissue-like structures can be cultured using scaffold-free approaches [11].

In tissue-derived tumour spheres (TDTS), the cells are derived from the enzymatic degradation of the original tumour tissue. Therefore, this model consists exclusively of tumour cells and makes it possible to maintain interactions between these cells. The interactions between tumour cells are particularly robust and are not disrupted by enzymatic dissociation, whereas the interactions between stromal and tumour cells are disrupted [24]. Thus, TDTS become excellent, smaller versions of non-vascularised tumour regions [15]. Recently, much attention has been paid to various TDTS models, including colospheres [32], spontaneously forming in vitro xenograft cancer spheroids termed MARY-X spheroids [33], and CTOS (cancer tissue-originated spheroid) [32]. Colospheres have a different structure and mucus production than MCTSs, which are formed by a cancer cell line derived from the same parent tumour. After 1 week of cultivation, the proportion of proliferating cells in colospheres is similar to that observed in vivo. In contrast, MCTSs generated from the cancer cell line show a high proliferative capacity, which is probably due to the selection pressure induced by the culture. However, even after 15 days in culture, the colospheres show minimal volume growth. This could be due to the fact that active cell proliferation is compensated by significant cell death and/or detachment. In most CTOS, size growth slows down after about 14 days in culture. Proliferating cells in vivo tend to accumulate mainly at the outer edge of the developing CTOS [24,32].

Tumourspheres, or tumour-derived spheroids, are models produced from cell suspensions after disruption of the original tumour tissue. Cancer stem cells (CSCs) are the source of these tumourspheres, and an isolated CSC should generate a spheroid by clonal expansion [34]. Therefore, the accumulation of CSCs or cells with stem cell-like characteristics is the main feature of tumour-derived spheroids. There is increasing evidence for the presence of cancer stem cells in human tumours. Confirmation of the existence of CSCs could potentially reveal new therapeutic targets that offer improved efficacy in the treatment of cancer. Zhao and colleagues conducted a study in which they used sphere-forming culturing to enrich lung CSCs and then evaluated their stem-like properties. The results showed that the lung tumourspheres exhibited increased proliferation, clonality, invasion, and cisplatin resistance. The levels of CD133 and breast cancer resistance protein were significantly higher in the tumourspheres. These findings suggest that culturing cancer cells in the form of spheres enriches CSCs and exhibits stem cell properties. In addition, the increased expression levels of CD133 and ABCG2 in tumourspheres may represent therapeutic targets for lung cancer [35].

Organotypic multicellular spheroids (OMSs) are generated by slicing tumour tissue in a non-adherent environment [36]. This results in ex vivo rounded and non-dissociated tumour tissue fragments in the form of spheroids [37]. The extracellular matrix, the vascular

components, and the cell–cell interactions of the original tumour tissue are completely present in the OMS model [38]. OMSs differ significantly from TDTS, as the stromal component is preserved in OMSs [24].

3.2. Application of Spheroids

3.2.1. Cancer Research and Response to Anti-Cancer Drugs

Traditional animal models, while valuable, have limitations in predicting human responses to drugs. Their use is often time-consuming, costly, and ethically questionable. Additionally, interspecies differences can hinder accurate predictions of clinical efficacy [39]. Over the past 30 years, experimental 3D models have been developed and studied. These models have outperformed 2D cultures in simulating the in vivo environment and are therefore more suitable for the study of cell biology and physiology. MCTSs, as cell aggregates, are commonly used to study tumour response and susceptibility to various treatments such as chemotherapy, combination therapies (e.g., small molecule inhibitors or chemotherapeutics), targeted drug therapy, and drug delivery systems. They are a suitable tool for drug penetration studies, as multicellular 3D models of drug absorption and diffusion closely mimic these processes. Numerous screening tools, including electrochemical detection (ECD), fluorescence-activated cell sorting (FACS), immunofluorescence, absorption assays, and optical coherence tomography (OCT), have been developed to evaluate drug screening platforms. Generation of spheroids, incubation with drugs, assessment of spheroid integrity and growth kinetics (growth delay and regrowth), and measurement of cell survival are steps involved in the drug screening process. In general, MCTSs are more resistant to therapy than cells in 2D culture and can mimic the drug resistance of solid tumours [40].

MCTSs are often used to study the response of tumours to chemotherapeutic agents. Recent studies measuring F18 fludeoxyglucose (FDG) in spheroids of breast cancer cell lines show that positron emission tomography (PET) radiotracers and image analysis in MCTSs provide a good model for assessing the relationship between tumour volume and metabolic tracer uptake before and after chemotherapy, offering potential for screening and selection of PET tracers for early assessment of response to treatment [20,41].

Non-invasive and targeted drug delivery strategies are essential to increase the concentration of chemotherapeutic agents in the tumour while minimising their undesirable side effects. One such approach, microbubble-guided ultrasound (MB-guided US), has emerged as a novel method for non-invasive, targeted drug delivery. In their study, Roy et al. (2023) investigated the efficacy of this ultrasound method in the delivery of irinotecan, doxorubicin, and bleomycin to colorectal cancer spheroids. Bleomycin administered in this way significantly reduced the growth of colorectal cancer (CRC) spheroid growth compared to spheroids treated with bleomycin alone. However, the use of this US technique did not improve the efficacy of doxorubicin and irinotecan on CRC spheroids [42,43].

CSCs have been discovered in various types of cancer for many years. These particular subsets of cancer cells are involved in tumourigenesis, development of resistance to therapy, spread to other parts of the body (metastasis), and formation of blood vessels for the tumour. Effectively targeting CSCs is critical for successful cancer therapy. The tumoursphere assay could also serve as a reliable platform for the further development of anti-CSCs agents and for the advancement of CSCs-targeted immunotherapy. Zhou and colleagues (2009) showed that 8-quinolinol selectively suppressed the ability of MCF7 breast cancer cells to form spheres. Furthermore, in combination with paclitaxel, it showed improved therapeutic efficacy in an MCF7 mouse model and reduced the risk of recurrence [44].

3.2.2. Toxicology

Concerns about the potential health risks of new chemicals and products, such as pharmaceuticals, cosmetics, and food additives, have led to increased scrutiny of their safety [45]. Traditional animal testing, while informative, is time-consuming, costly, and ethically controversial. Moreover, it often fails to accurately predict human responses. A thorough analysis of drug screening tests has shown that 57% of the toxicological information from rodent research does not match the results of human studies [46]. Furthermore, hepatotoxicity remains a major challenge in the field of drug development. Conventional preclinical liver toxicity studies using 2D cell lines and xenotransplanted mice have yielded unsatisfactory results [47]. As a result, numerous 3D spheroid culture models are available for screening hepatotoxicity. However, quantitative assessment of the response of spheroids in situ remains a major challenge for current 3D liver toxicity platforms. In their research, Hong et al. (2021) created a HepG2 liver spheroid model using 3D printing for quantitative assessment and thorough monitoring of drug-induced hepatotoxicity. These spheroids were used to determine EC_{50} values and to evaluate various hepatotoxic effects such as apoptosis, mitochondrial permeability transition (MPT), and cytosolic calcium concentration. Three-dimensional HepG2 liver spheroids showed greater resistance to nefazodone-induced MPT compared to 2D HepG2 cells, suggesting that the gelatine/alginate hydrogel culture system provides better protection against hepatotoxic drugs. Drug sensitivity of HepG2 liver spheroids was also observed regardless of their size. These findings emphasise the potential of the current 3D cell printing-based platform for embedded HepG2 liver spheroids to evaluate and understand drug-induced liver injury [48]. Gori and colleagues (2020) used 3D-bioprinted liver tissue and developed these models using HepG2/C3A cells and pluronic/alginate hydrogels, which exhibited strong viability and metabolic activity specific to the liver. The study also demonstrated increased sensitivity of the 3D-printed tissues to acetaminophen-induced hepatotoxicity compared to traditional 2D cell cultures, suggesting that these models could serve as a viable alternative for in vitro animal studies of drug-induced liver injury [49].

3.2.3. Application in Regenerative Medicine

The in vitro generation of tissues with a high cell density similar to that of original tissues and organs and compatible in size with the damaged sites has been the greatest challenge in tissue engineering over the last decade. This has triggered the development of "bottom-up" tissue engineering, in which tissues are produced by assembling "building blocks" into higher-order 3D structures. Spheroids are an excellent option for the building blocks in bottom-up tissue engineering, as they can replicate the key morphogenetic events in tissue formation, including fusion [50,51].

Cell-based therapies are being explored as a way to deliver cells to patients that enhance wound closure or produce the many signals required for coordinated repair. Spheroids support tissue repair in two ways: directly, by providing functionally differentiated cells for tissue development, or indirectly, by secreting potent trophic factors that support tissue formation and attract the necessary host cells for regeneration. Spheroids have already been used to develop cartilage [51], skin [52], heart [53], liver [54], brain [55], and many other tissues or tissue mimics. The ability of stem cell-derived spheroids to develop along chondrogenic, osteogenic, adipogenic, and neurogenic lineages justifies their use in tissue engineering. Cell-based therapies show great promise for repairing blood vessels and improving blood circulation in a variety of injuries and diseases. Due to their proangiogenic and immunoregulatory properties, mesenchymal stem cells (MSCs) are widely used in the treatment of critical limb ischaemia. More importantly, MSC spheroids release more angiogenic growth factors and cytokines by triggering hypoxia-induced

paracrine secretory signalling [56,57]. Spheroids have recently attracted much attention because the hypoxia gradient they contain is very similar to the environment of native cartilage and promotes the differentiation of MSCs and adipose-derived stem/stromal cells (ASCs) along the chondrogenic pathway [51].

Due to the short half-life of the active agents (recombinant factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF)), most current therapies for non-healing skin wounds that attempt to stimulate neovascularisation and epithelialisation are ineffective [58]. Thanks to their potent secretome, MSCs can dramatically improve granulation tissue development, angiogenesis, and inflammation. In the study by Zeng et al. (2022), the porous poly(N-isopropylacrylamide)-based hydrogel is used to generate a large number of ASCs spheroids with adjustable size to achieve a reversible "gel-sol" transition that can be injected into the wound site. The formed ASCs spheroids were fixed to the wound site by the re-gelled hydrogel, which facilitated the work of stem cell treatment. The in vivo wound healing study showed that the ASCs spheroids promoted the polarisation of macrophages and successfully suppressed the local inflammatory response of the wound, which promoted wound healing [52]. Rapid wound healing, scar-free healing, and effective regeneration of skin appendages, such as hair follicles and sweat glands, are the future challenges for stem cell-based regeneration of skin tissue [59].

4. Organoids as a 3D Cell Culture Model

Organoid technology has revolutionised biomedical research. The term "organoid" was introduced in the 1940s and has evolved from describing simple cell aggregates to complex 3D tissue cultures that mimic the structure and function of human organs [60]. They consist of clusters of organ-specific cells derived from pluripotent stem cells (PSCs)—embryonic (ESCs) and induced (iPSCs)—or organ-specific adult stem cells (aSCs). These stem cell-derived miniature organs provide a powerful tool for studying human development, disease modelling, and drug discovery [61,62]. By replicating the intricate details of native tissues, organoids provide invaluable insights into protein and gene expression, as well as metabolic processes. Additionally, they can be used for high-throughput drug screening and personalised medicine [63].

Adult stem cells (aSCs) can be cultured in vitro to generate heterotypic 3D organoid structures. These structures require the isolation of tissue-specific stem cells and implantation into an ECM with appropriate growth factors [64]. Organoids produced from aSCs are epithelial in origin and do not naturally contain mesenchymal or immunologic components unless such components are subsequently added [65]. Organoids can be serially passaged, maintaining their genetic and epigenetic stability.

Three-dimensional organ models can also be produced from pluripotent stem cells (ESCs or iPSCs), which can be used to culture tissues where there are either no easily culturable stem cells or which are difficult to access for stem cell collection (such as the brain) [64]. PSCs can be differentiated into various organoids, which mimic the structure and function of adult organs, by manipulating their growth conditions and cellular interactions [62]. Thus, the protocol for organoid development begins with the growth of PSCs, the differentiation of PSCs with an essential step involving the specification of the germ layer—endoderm, mesoderm, or ectoderm. Thereafter, induction and maturation depend on intrinsic or extrinsic biochemical signals (culturing PSCs with specific growth factors) and cell–cell interactions that control the further fate of the cell. To closely resemble their counterpart in vivo, certain PSC-derived organoids may contain cells from different germ layers [65,66].

4.1. Application of Organoids

4.1.1. Drug Discovery and Disease Modelling

The rapid growth of organoid systems has developed into an important medical technology with a wide range of potential applications. By growing a patient's tumour tissue in vitro in a 3D culture, it is possible to produce patient-derived organoids (PDOs). PDOs have significant application value in medicine as they effectively represent the characteristics of tumour origin while preserving heterogeneity between patients. PDOs belong to the patient-derived xenograft (PDX) models, one of the most promising models for high-throughput drug screening [67]. Thanks to their ability to rapidly identify and validate drugs, this method could replace the use of laboratory animals. The organoid surface area, number of live cells, and volume-based growth rates are compared for different treatment scenarios to evaluate the efficacy of cancer drugs. In addition, it is critical to evaluate 3D morphological changes, especially sphericity and ellipticity characteristics, to assess treatment response [68]. PDOs biobanks provide a useful platform for cancer research in both clinical science and basic medicine. Finally, living cancer biobanks containing malignant cells and matched organoids with different genetic origins, subtypes, and individual characteristics will aid in the discovery of new treatments [69]. Interestingly, most of these samples come from patients with different types of cancer such as kidney [70], lung [71], colon [72], breast [73], and bladder [74,75]. Papaccio and colleagues (2019), for example, have developed a method for establishing a biobank of organoids from colorectal cancer patients that can be used to study molecular characteristics, test various treatments, and investigate the basic molecular mechanisms of cancer and treatment resistance. Most experiments involving the cultivation and manipulation of organoids can be completed in about four weeks, suggesting that PDOs could provide valuable insights for clinical treatment within a relatively short time frame [76].

4.1.2. Organoids as a Model for Human Development

Because we know very little about human development, most early experiments aimed at generating organoids with properties similar to human tissue relied on comparisons with mouse development [65]. hPSCs can be used in a variety of ways to generate organoids that resemble different organ or tissue types. Each system has both similarities and differences to its original counterpart [77]. Organoid models made from iPSCs can be used to study human development in two ways. The first way is to establish organ-specific progenitor cells from iPSCs by exposing them to a series of defined stimuli under coordinated conditions and a mixture of different media formulations. The organ-specific progenitor cells then self-assemble into organoids that represent the developing organ. The second pathway involves stimulating iPSCs to form cellular aggregates that resemble the early, preimplantation embryo. These units, often referred to as gastruloids or embryoids, pass through the embryonic stages independently. Embryoid bodies, which are often disorganised 3D aggregates of pluripotent or differentiated cells, have been used extensively as models of early development for some time [78,79].

4.1.3. Toxicology

In toxicology, organoids are mainly used to determine neurotoxicity and to understand the potentially harmful effects and concomitant mechanisms of toxicants on the human central nervous system (CNS), considering the susceptibility of the CNS to external influences [80]. External influences and changes in exogenous chemicals can affect the regular development or function of the brain and lead to dysfunction of the nervous system. Studies in animal models such as rabbits, rats, zebrafish, and mice have provided a wealth of knowledge on neurotoxic substances, but these are not always transferable to humans

due to differences between species. Most importantly, there are major differences between the CNS of animals and humans at the anatomical, cellular, and molecular levels. Therefore, it is essential to develop rapid and affordable in vitro approaches based on human donor cells to identify potential neurodevelopmental toxicants [81,82]. For studying the process of neurodevelopment and the pathogenesis of brain diseases, 3D human brain organoids derived from ESCs or iPSCs offer exceptional potential. The human brain is particularly vulnerable to toxic influences during early life and development, and there is growing concern that exposure to environmental chemicals may be contributing to the global rise in neurodevelopmental disorders. In order to find an efficient approach to assessing the safety of chemicals, Schwartz et al. (2015) have developed a 3D brain organoid model that can be used to examine drugs and chemicals for potential damage to the growing brain. The brain organoids they created contain microglia and blood vessels derived from human pluripotent stem cells (hPSCs). They cultivated stem cell-derived microglia, vascular cells, and neural progenitor cells on synthetic hydrogels. These progenitor cells fuse spontaneously to form 3D brain organoids that resemble the growing human brain in many respects. For neuronal constructs exposed to 60 toxic and non-toxic experimental substances, machine learning was used to create a predictive model from changes in global gene expression. Nine out of ten new substances were correctly categorised by the algorithm in blind tests. Combining these actively perfused devices with the vascularised 3D neuronal organoids described here could promote more advanced differentiation and growth, while accounting for blood–brain barrier function would help to investigate the delivery of therapeutic agents and increase the predictive accuracy of 3D brain models by placing toxic chemicals in a more physiologically relevant pathway [83].

A human predictive system is needed to explore the complex mechanisms underlying drug-induced liver injury (DILI) and to determine how the susceptibility of individual drugs affects patients. Preclinical identification of compounds at risk of causing DILI is still a major obstacle in drug development [84]. As they mimic various toxicologic symptoms, organoids that retain morphologic and functional complexity may be useful in assessing DILI risk. Both iPSCs and human liver tissue biopsies can be used to produce liver organoids that represent physiologic and patient-specific organotypic systems that mimic organ structure, cell composition, and function [85]. The use of various drugs, such as acetaminophen, methotrexate, fialuridine, and TAK-875, each of which induces liver injury via a different mechanism, demonstrates the value of human liver organoids (HLOs) in recapitulating various phenotypes of DILI. Steatosis, fibrosis, and immune system responses were among the observed toxicologically relevant phenotypes, most of which correlated with previous human data, demonstrating the applicability of HLOs for safety assessment. The main advantage of HLOs over a variety of in vitro cellular toxicity prediction systems using only hepatocytes is the presence of multiple functional cell types involved in DILI. Thus, fibrosis, immune responses, and cholestasis can be detected, all of which play an important role in DILI [86].

4.1.4. Applications in Genetic Engineering and Personalised Medicine

In addition to cancer research, there are already a number of examples of the use of organoid cultures for research into congenital or acquired human diseases. Cystic fibrosis (CF), which was modelled in the intestinal organoid system, was the first human disease to be studied in organoids [87]. Cystic fibrosis is caused by mutations in the CFTR gene that impair the permeability to chloride and bicarbonate. This leads to dysfunction in multiple organs due to abnormal fluid transport and mucus production. There are over 2100 known mutations of the CFTR gene. Two drugs that control the folding of the mutated protein (VX-809 as a CFTR corrector) and increase the activity of the chloride channel

(VX-770 as a CFTR potentiator) are currently available for CF patients with certain CFTR mutations. However, it is still difficult to assess the efficacy of these drugs or to find new drugs for patients with other rare mutations [65]. Due to their high validity and high throughput capacity, patient-derived intestinal organoids may provide an alternative to current preclinical models for CF research. In particular, these patient-derived organoids can express intronic and intergenic enhancers that control CFTR gene expression [88]. The applicability of organoid systems is greatly expanded by the combination of CRISPR-Cas9 genome editing. One of the monogenic diseases for which this model of genome editing has been used is CF. Current research focuses on the use of organoids for personalised medicine. The results obtained by Meier et al. (2022) suggest that the rapid development of patient-derived tumour organoids will enable in vitro drug testing so that patients with the rare subtype of primary liver cancer (hepatocellular carcinoma with neuroendocrine differentiation) can receive individualised treatment. The study provides the first proof of concept for the use of organoids in these rare primary liver tumours [89].

4.2. Ethical Considerations of Organoid Technology

Organoid research, while promising, raises significant ethical concerns. The use of human embryonic stem cells, the potential for creating sentient organoids, and the ethical implications of organoid transplantation are among the key issues. Additionally, ensuring informed consent and protecting the privacy of donors is crucial. A delicate balance must be struck between maximising the benefits and minimising the ethical risks of organoid technology [90].

Many ethical concerns are raised by the study and application of cerebral organoids. These range from more conventional research ethics concerns, such as informed consent, biobanking, and data protection, to clinical translation concerns, such as the lack of clear evidence supporting first-in-human trials or the possibility that organoid transplants could harm a patient's brain or change their personality. Additionally, there is a regulatory need to seek the agreement of the original cell donors. The creation of various organoids, such as brain or gonadal, may cause some reluctance and raise concerns about the level of knowledge and consent provided to the donors. It also calls into doubt the patentability and ownership of the resultant organoids, which can also be profitable. Although it is difficult to determine how, the genetic analysis of organoids also brings up major issues regarding the privacy of health data, especially genetic data, which must be considered at the permission stage [91,92].

There is a lot of promise for biomedical research with ESC-derived organoids. However, their derivation from human embryos raises ethical concerns about the moral status of early human life. The use of ESCs in organoid research involves complex ethical considerations related to the value of human life and the potential for misuse of this technology. Also, it is important to maintain realistic expectations. It is crucial to ensure that research findings are biologically relevant and can translate into meaningful medical applications [91].

5. Challenges and Future Prospects for Spheroids and Organoids

While in vitro culture systems inherently lack a general physiological background, the process of creating a three-dimensional living tissue mass suspended in culture media presents additional obstacles. So far, organoids are still micrometre-sized, and there is still a certain functional difference between them and normal tissues. The lack of a circulatory system is an important factor. Organoids cultured for several months can reach a diameter of up to 5 mm, although this problem becomes increasingly difficult as the size increases. The cells in the nucleus cannot be adequately supplied with nutrients and cannot easily excrete metabolic waste [70,93] (Table 1). The blood vessel network in native tissues controls

organ function, tissue homeostasis, and regeneration in addition to providing nutrients. In the same manner, vascularization is vital for organoid cultures because it makes it easier for nutrients to be transported, oxygen for delivery, and metabolic waste to be eliminated—all of which are essential for the survival of 3D structures. These problems are being addressed by the implementation of microfluidic systems in the development of 3D cell systems, which can then make them even better at mimicking tumour microenvironments. Microfluidics chips also have other benefits and research applications, like providing growth directionality, the ability to use sensors, and various options in co-culturing [94]. The development of self-assembly microfluidic devices allows us to perform antitumour tests directly in the growth platform after spheroid formation. This could save time and money by identifying more effective dosing regimens for anticancer drugs in preclinical in vitro studies [95].

There are significant advances being made in the area of 3D bioprinting applications for cell and tissue culturing. The basis for bio printable ink is various hydrogels. The hydrogels used are essential for simulating the ECM. They also facilitate nutrient diffusion and signalling while securing a sufficient amount of water for embedded cells. Bioprinting provides the grounds for scalability and reproducibility in 3D cell culture applications that cannot be achieved by traditional means. Ink optimisation is the major point for further development, but there are great advancements being made with the help of AI-based optimisation approaches [96].

As for organoids, a particularly successful multidisciplinary approach from the field of bioengineering is required to overcome the limitations associated with organoid lifespan and native tissue recapitulation. Microfluidics and advanced imaging techniques are just some of the approaches to increase the effectiveness of organoids in various applications [32]. The main organoid application development is focused on organ-on-a-chip (OoC) models, which are valuable tools that bridge the gap between simple cell cultures and complex animal models. While they can mimic many human organ systems, their physiological relevance is still being fully validated. To maximise the potential of OoCs, we need to address several challenges such as standardisation, data acquisition, and validation. There's a lack of standardised design, manufacturing, and operation protocols for OoCs, making them time-consuming and resource-intensive to set up and use. Considering a future challenge is the development of a universal culture medium that can mimic blood, providing essential nutrients and growth factors to support various cell types [97]. Most OoCs rely on endpoint measurements, limiting our ability to study dynamic biological processes. We need to compare data from OoCs with traditional in vitro and animal models to ensure their accuracy and reliability [98,99].

6. Conclusions

In this review, we have discussed the structure and biology of organoids and spheroids, highlighting their advantages and disadvantages as well as their potential therapeutic applications. Cell spheroids mimic the tumour microenvironment and cell–cell interactions more accurately than 2D cell cultures, making them a suitable tool for a number of applications from drug toxicity/efficacy evaluation to regenerative and personalised medicine. They are simple, fast, adaptable, and affordable enough to enable high-throughput screening. Organoids, on the other hand, are self-assembled tissues derived from stem cells (embryonic, induced, or organ-specific adult stem cells). Therefore, organoids represent an advance in the creation of 3D model systems that can represent multiple organic systems in miniature and better represent in vivo responses. The choice between the two must be made depending on the research objective and the capabilities of the user. The therapeutic potential of spheroids and organoids in clinical applications is further enhanced by the

use of biomaterials that mimic the biophysical properties of the original tissue. Further advancements in the development of bioprinting approaches and validation of complex OoC systems are necessary for a simpler and wider implementation of these methods in research and therapy.

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Abbreviations

The following abbreviations are used in this manuscript:

MCTSs	multicellular tumour spheroids
ECM	extracellular matrix
TDTS	tissue-derived tumour spheres
MARY-X	in vitro xenograft cancer spheroids
CTOS	cancer tissue-originated spheroid
CSCs	cancer stem cells
OMS	organotypic multicellular spheroids
MPT	mitochondrial permeability transition
ECD	electrochemical detection
FACS	fluorescence-activated cell sorting
OCD	optical coherence tomography
CRC	colorectal cancer
MSCs	mesenchymal stem cells
CLI	critical limb ischaemia
ASCs	adipose-derived stem/stromal cells
VEGF	vascular endothelial growth factor
PDGF	platelet-derived growth factor
bFGF	basic fibroblast growth factor
PSCs	pluripotent stem cells
ESCs	embrionic stem cells
iPSCs	induced pluripotent stem cells
aSCs	adult stem cells
CNS	central nervous system
hPSCs	human pluripotent stem cells
DILI	drug-induced liver injury
HLOs	human liver organoids
PDOs	patient-derived organoids
PDX	patient-derived xenograft
OoC	organ-on-a-chip

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