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*Review article*

# Advancements in 3D *in vitro* Cell Culture Systems: Enhancing Drug Pharmacokinetics and Toxicity Assessment in Pharmaceutical Development

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**ABSTRACT**
The development and screening of pharmaceuticals encounter significant predictive inaccuracies when transitioning from animal models to human trials, primarily due to interspecies differences in drug metabolism and effects. Traditional 2D and animal models, although fundamental in early drug development stages, often do not accurately reflect human physiological responses, leading to high attrition rates in clinical phases. This review highlights the emerging role of three dimensional (3D) *in vitro* models, including organoids and tissue chips, as more predictive and ethically favorable alternatives. These models mimic human physiological and pathophysiological conditions more closely, providing an enhanced platform for drug pharmacokinetics and toxicity assessment. Although there are some disadvantages, innovations in scaffold-based and scaffold-free 3D cultures, bioprinting techniques, and organ-on-chip technologies not only address the limitations of traditional models but also offer profound insights into complex tissue dynamics and drug behaviors. This paper discusses the significant advances in 3D *in vitro* technologies that promise to refine predictive accuracy, reduce reliance on animal testing, and streamline the pharmaceutical development pipeline.

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## Introduction

Pharmaceutical companies face a challenge in the development process, which requires significant investments of time and resources [1]. Despite considerable efforts, over 90% of drug candidates face rejection after phase I clinical trials [2], [3]. A key factor contributing to this rejection rate is the lack of alignment between the results obtained from preclinical animal studies and those observed in phase I clinical studies involving human subjects. The difference between the results obtained in preclinical animal studies and those observed in human trials is due to inherent variations in species-specific factors such as physiology and drug sensitivity [4]. Moreover, ethical concerns regarding animal studies have gained increasing attention. Therefore, the development of 3D *in vitro* models, such as organoids and tissue chips, is not only a promising approach to address the limited predictive accuracy associated with animal studies, but also to address ethical issues related to animal testing.

Pharmacokinetics studies the interaction between a pharmaceutical compound and the human body. This is achieved by investigating four main parameters (ADME); the entry into the body (absorption), the moving through the body (distribution), the changes undergoing in the body (metabolism), and the leaving of the body (excretion) of the drug compound [5], [6]. The outcomes of ADME investigations play a pivotal role in determining the effectiveness, safety and potential off-target effects of the pharmaceutical compound. The ADME parameters are crucial in the early drug development process.

In early drug development, traditional 2D *in vitro* and animal models remain crucial for assessing a drug's ADME, safety and effectiveness prior to clinical trials. These models aim to simulate human diseases and predict how humans will respond to the drug candidate. However, both traditional approaches showcase limitations. Traditional

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2D *in vitro* models exhibit limitations due to unnatural growth kinetics, cell attachment characteristics, and cell polarity. These methods fail to accurately represent essential cell-cell and cell-extracellular matrix (ECM) interactions, significantly impacting the drug development process [7]. While animal models offer improved reliability for evaluating human safety and efficacy compared to traditional 2D *in vitro* models, their predictive accuracy is continuously questioned. Species-specific differences often hinder their ability to fully replicate the biology and mechanisms of human diseases [8]. To address these shortcomings, *in vitro* 3D models have emerged as a promising alternative [9]. These models exhibit a remarkable capacity to recapitulate the complexities of solid tumors, including features like reduced oxygen availability leading to the formation of hypoxic regions within spheroids or organoids. Additionally, they effectively mimic the nutrient gradients and elevated glucose metabolism characteristic of tumors, offering significant advantages over traditional 2D culture models [10]. Furthermore, 3D *in vitro* models are demonstrating promising results in simulating drug disposition and pharmacokinetics, crucial factors for predicting drug safety and efficacy during early-stage drug development [11]. However, despite these promising advancements, further research is necessary to address remaining uncertainties associated with 3D *in vitro* models.

## Three-Dimensional (3D) *in vitro* Models

### Scaffold-based 3D models

Scaffold-based 3D models involve the strategic seeding of cells onto or within a prefabricated, 3D structure known as a scaffold. These scaffolds, typically composed of biocompatible biopolymers, are designed to mimic the natural extracellular matrix (ECM) which contains secretion of growth factors, cytokines or angiogenic factors that play a critical role in supporting various cellular functions [12]. By providing essential physical cues and facilitating cell attachment, migration, proliferation, and differentiation, scaffolds ensure optimal access to nutrients and oxygen while enabling efficient waste removal [13]. The pioneering work of Del Buono et al. established the foundation for scaffold-based 3D models by demonstrating the capability of colorectal adenocarcinoma cells to exhibit morphological differentiation within a collagen gel scaffold, marking a significant advancement from traditional models utilizing basement membrane extracts or normal rat mesenchymal cells [14]. Furthermore, the growth of prostate cancer cells was extensively higher in 3D forms because the upregulation of CXCR4 and CXCR7 signaling pathway which has cross-talk with tumor microenvironment [15]. Since this initial breakthrough, the field has witnessed continuous evolution driven by emerging technologies, leading to the development of increasingly intricate systems incorporating diverse scaffold types. Three primary categories of scaffolds are employed in this technique: membranes, matrices, and hydrogels. Moreover, scaffold-based 3D cell culture models can be classified as polymer-based, hydrogel, decellularized tissue scaffolds and microfluidics. Cell seeding strategies within scaffolds can also vary, with two prevalent approaches: seeding cells onto pre-fabricated, cell-free scaffolds or incorporating cells directly into the scaffold during its fabrication process with the usage of cell-laden bioinks [10].

3D scaffold-based models offer numerous advantages that make them invaluable tools in drug discovery. One of the primary benefits is their ability to closely mimic the *in vivo* cellular organization, providing a more accurate representation of human tissues compared to traditional 2D cultures. This 3D scaffold-based model supports cell growth, differentiation, and function in a way that closely resembles native conditions. These models also allow for the incorporation of co-culture systems, enabling the study of complex cell-cell and cell-matrix interactions. This is particularly useful for creating more physiologically relevant models, such as human-on-chip systems, which can simulate the interactions between different tissues and organs. Additionally, scaffold-based models offer a solution to one of the biggest problems in drug development assays with traditional 2D cultures, which is the control of the extracellular matrix (ECM) compounds. Earlier studies have found that ECM compounds and cell-ECM interactions play a crucial role in cellular response [7]. To overcome this limitation and mimic the biological environment and structure, the use of biologically derived (ECM-based) bioinks in the production of 3D printed ECM-mimicking scaffolds has shown great potential [23], [24]. The versatility of scaffold-based models is another significant advantage. They can be constructed from a wide range of materials, including natural and synthetic polymers, which can be tailored to possess specific mechanical, chemical, and biological properties. This customization allows for the creation of cell-, patient-, and disease-specific models, enhancing the relevance of experimental findings. Scaffold-based models are also more resilient to external factors, providing a stable environment for long-term studies [25]. Additionally, the ability to create complex tissue constructs supports the study of organ-level responses, offering insights into tissue-specific drug effects and toxicities. These models

facilitate advanced tissue engineering applications, enabling the development of functional tissues and organs. This capability is particularly important for regenerative medicine and the creation of organotypic models that can be used for disease modeling and drug testing. Furthermore, scaffold-based models are highly adaptable and can be integrated into high-throughput screening platforms. This integration allows for the simultaneous testing of multiple drug candidates, significantly accelerating the drug discovery process. Their ability to provide more predictive toxicology data also enhances drug safety assessments, reducing reliance on animal models. In the context of personalized medicine, scaffold-based models hold immense potential [26]. They can be derived from patient-specific cells, providing tailored disease models and treatment strategies. This personalization improves the accuracy of drug efficacy and toxicity predictions, leading to more effective and individualized therapies.

**Table 1** The advantages and disadvantages of the scaffold types

| Scaffold type | Characterization | Advantages | Disadvantages | References |
|---|---|---|---|---|
| Polymer-based | Mimics the ECM with sources of natural polymers such as collagen, gelatin, chitosan, silk fibroin, alginate. | Cells can easily adapt to the ECM-like environment, proliferate and differentiate. High biocompatibility and facilitate contribution to tissue regeneration during the transplantation phase. | Since they are natural ECM materials, they may vary depending on the time they are obtained. Mechanically sensitive and may cause an immune response during transplantation. | [16]–[18] |
| Hydrogel | Natural and synthetic molecules with extensively higher water contents. Poly(ethylene glycol) (PEG), oly(2-hydroxy ethyl methacrylate) are such samples of synthetic hydrogels. | Mimic the nature of most soft tissues. Facilitate the transition of nutrients and oxygen or organic waste with its elasticity feature. Biodegradable. | Low mechanical strength, lack of long-term stability. | [19], [20] |
| Decellularized tissue | Cellular components are removed and remained the native tissue structure with ECM and bioactive compounds. | Decellularized scaffolds are biologically identifiable which is advantageous for cell adhesion, proliferation, and survival. They have low immunogenicity. | Quantity of the decellularized tissues variable and hard to obtain. Because the residual DNA contents, the immune response can be triggered. Decellularization process consists of many steps which may disadvantage for preparation of successful material. | [21], [22] |

Despite the significant advancements in 3D scaffold-based models, several areas require further development to maximize their potential in drug discovery. One major limitation is the complexity and technical expertise required to fabricate and utilize these models [27]. Simplifying protocols and integrating automated systems could make scaffold-based models more user-friendly, reducing the reliance on highly specialized skills. Reproducibility remains a challenge, as variability in scaffold production and cell culture can lead to inconsistent results. Standardizing procedures and improving manufacturing processes are essential steps toward achieving reliable and reproducible outcomes across different laboratories. Another critical aspect is the compatibility of scaffold-based models with a wide range of cell lines and diseases. Developing versatile scaffold materials that can be

customized to replicate various tissue microenvironments would enhance their applicability [28]. This customization is particularly important for creating disease-specific models that accurately mimic pathological conditions. Cost-effectiveness is also a concern, as the high cost of materials and production can limit the widespread adoption of scaffold-based models. Identifying more affordable materials and scalable production methods could reduce costs and make these models more accessible to the research community. Improving the clinical relevance of scaffold-based models involves enhancing their physiological accuracy and stability for long-term studies. Advanced characterization and monitoring techniques are needed to better understand and optimize scaffold performance. Integrating sensors and developing high-resolution imaging and omics technologies can provide detailed insights into cellular responses and scaffold interactions in real-time. Finally, fostering interdisciplinary collaboration between cell biologists, engineers, material scientists, and clinicians is crucial for overcoming the multifaceted challenges associated with scaffold-based models [29]. By addressing these limitations, future advancements can make 3D scaffold-based models more user-friendly, reproducible, versatile, cost-effective, and clinically relevant, thereby enhancing their impact on drug discovery.

**Bioprinting-based scaffolds**

Advancements in medical imaging and structure design software have empowered the development of 3D bioprinting, a novel technology that enables the fabrication of complex tissue constructs. This technique utilizes bioinks, a composite material composed of hydrogels, cells, and biological molecules, to replicate the intricate geometry of various tissues as visualized through imaging techniques [30]. Notably, the first successful 3D printing using stereolithography was achieved by Charles W. Hull in 1986, laying the foundation for this transformative technology [31].

In the realm of tissue engineering, the development of bioinks that are robust and biocompatible is essential for successful 3D bioprinting. GelMA is a gelatin-based hydrogel modified with methacrylate groups to enhance its cross-linking capabilities and mechanical properties. This material is particularly noted for its ability to be finely tuned through photopolymerization, allowing for precise control over its physicochemical properties which is crucial for the integrity and functionality of bioprinted constructs. GelMA-based bioinks can incorporate various cell types and have been successfully used to bioprint complex tissue constructs such as skin and cartilage. These constructs exhibit significant intercellular communication and maintain specific tissue functions, which are vital for their integration and functionality post-implantation. Moreover, the versatility of GelMA bioinks is further demonstrated through the incorporation of different cell types within a single bioprinted construct, leading to the fabrication of tissues with distinct zones of extracellular matrix composition, mimicking the natural tissue heterogeneity. This adaptability highlights the potential of GelMA not only as a standalone bioink but also in combination with other materials, such as synthetic polymers and bioceramics, to enhance the mechanical strength and biological performance of the final constructs. Such developments are pivotal for advancing 3D bioprinting technologies towards more realistic, functional, and sustainable tissue-engineering solutions.

The tumor microenvironment plays a crucial role in drug behavior, highlighting the need for *in vitro* models that accurately mimic its characteristics. Naturally derived bioinks, possessing superior biocompatibility and ECM-like properties, offer significant advantages in this regard. These bioinks closely resemble the native extracellular matrix, facilitating the creation of tumor-specific architectures and fostering improved cell-material interactions. Bioinks encompass a diverse range, including natural materials sourced from living organisms, synthetic materials like engineered protein polymers, and hybrid combinations incorporating both elements [10]. Selection of the appropriate bioink type hinges on the specific research application and desired properties. Printability, characterized by optimal viscosity and structural integrity, remains a critical factor throughout the process, ensuring precise control and successful construct formation.

3D bioprinting can be performed through various techniques, each offering distinct advantages and limitations [10], [30]. Light-based or laser-assisted bioprinting employs focused light energy to precisely deposit bioink droplets, enabling high-resolution patterning. Extrusion-based bioprinting utilizes continuous extrusion of bioink filaments through a nozzle, offering efficient fabrication of larger structures. Inkjet bioprinting, also known as droplet-based bioprinting, leverages piezoelectric or thermal actuation to dispense bioink droplets in a controlled manner, facilitating the creation of complex cell arrangements.

In their innovative approach Kim et al. addressed the limitations of lung disease modeling and drug efficacy testing by developing a 3D Inkjet-Bioprinted Lung-on-a-Chip model [32]. This innovative approach utilizes a culture insert containing a micron-thick, three-layered human alveolar barrier model fabricated by drop-on-demand

piezoelectric inkjet bioprinting. These inserts are placed on a custom-made biochip that facilitates nutrient flow throughout the cultures. The three layers mimic the human lung structure, incorporating human lung microvascular

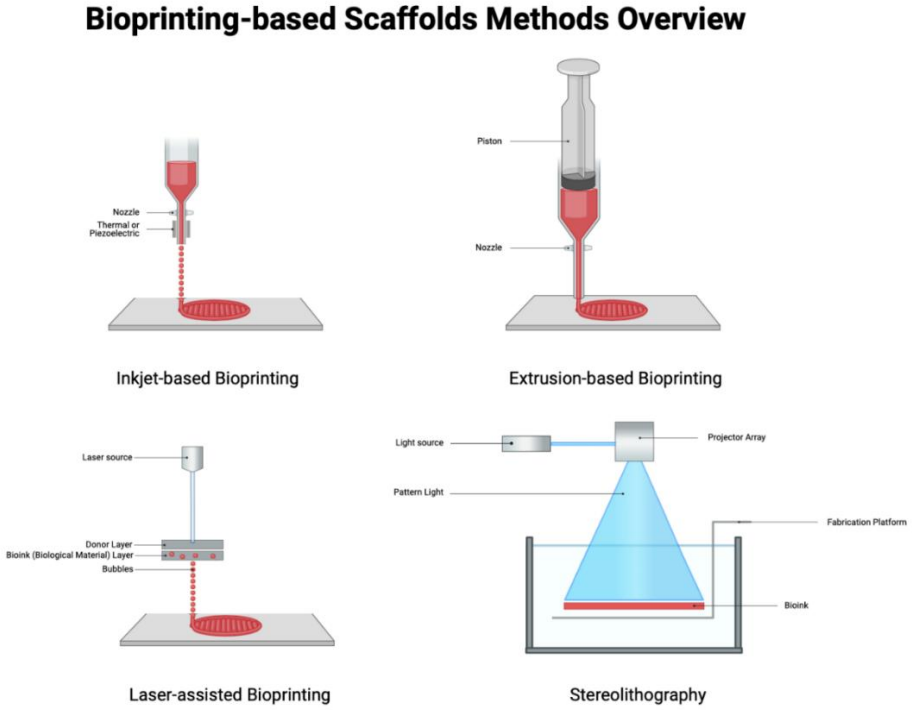


**Fig 1** Schematic diagram of different bioprinting techniques, providing a comprehensive overview of the four main bioprinting methods used to fabricate scaffolds for tissue engineering.

endothelial cells, a collagen solution with lung fibroblasts, and both type I and type II alveolar cells. After incubation and mounting on a lung-on-a-chip device, the tissues were analyzed for morphology, structure, and function. The results demonstrated that this model successfully recapitulates the key features of human lung tissue within a microfluidic device. The authors propose that this approach, combining inkjet bioprinting with microfluidics, holds promise for high-throughput analysis in drug discovery.

Bioprinting-based scaffolds offer numerous advantages that enhance their utility in drug discovery. They enable the creation of complex tissue constructs with precise architectural and functional properties through layer-by-layer deposition of cell-laden or acellular bioinks [30]. This precision allows for reproducible and detailed structures, reducing variability and improving research reliability. Bioprinting supports the incorporation of multiple cell types within a single construct, facilitating co-culture systems that better mimic *in vivo* tissues. The customizable properties of bioinks, including mechanical strength and bioactivity, allow for the development of models that closely replicate the extracellular matrix and support desired cellular behaviors [33]. A significant advantage of bioprinting is the ability to include vascularization within constructs, which is essential for nutrient delivery, waste removal, and long-term cell viability [27]. Bioprinting also facilitates the creation of patient-specific models using cells derived from individual patients, promoting personalized medicine by developing tailored therapies.

Bioprinting-based scaffold models, despite their advantages, face several limitations. One major challenge is the complexity and technical expertise required for their fabrication and use. Developing and optimizing bioinks and bioprinting protocols can be time-consuming and require specialized skills and equipment. Reproducibility is another issue, as the precision of bioprinting can lead to variability in the produced constructs, which may affect experimental consistency [33]. Ensuring uniformity across different bioprinted samples remains a significant hurdle. Cost is also a concern, with high expenses associated with the materials, equipment, and skilled labor needed for bioprinting. This limits the accessibility and scalability of the technology for many research laboratories.

**Organoid models**

Organoids are described as *in vitro* 3D structures grown from stem cells, that with the usage of organ-specific growth factors form a self-organized organ-like structure [34]. Organoid models are groups of cells, which can be derived from genetically modified stem cells or isolated cells from patient-derived tissues [9]. Organoids show physiological similarity to that of *in vivo* tissues and organs [35]. The first organoid was developed by Sato et al. in 2009 while studying the self-renewing epithelium of the small intestine. The renewal process is carried out by Lgr5+ stem cells in the crypt. Sato aimed to cultivate these stem cells in a 3D matrigel culture to observe their proliferation. Rather than observing mere proliferation, the stem cells unexpectedly formed a structural development that replicated the cell types and functions of the gut, creating a miniature version [36]. These engineered tissues mimic the structure and functionality of their natural counterparts, and they can be either transplanted to a specific location or maintained in culture for an extended duration [37]. Currently, multiple types of organoids have been successfully adapted to 3D culture systems.

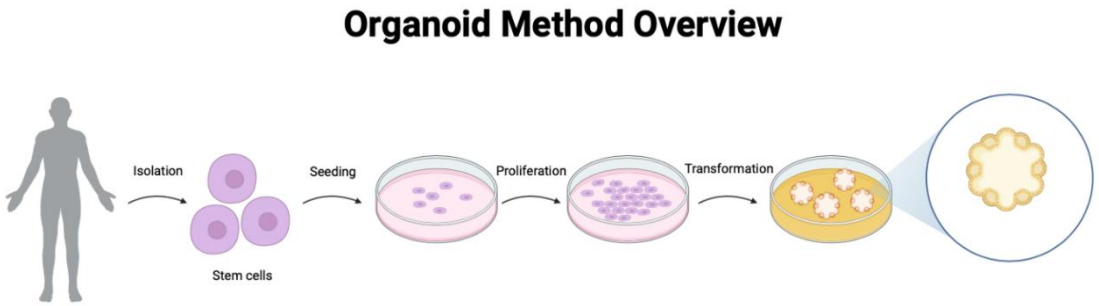


**Fig 2** Organoid method overview. The organoid methodology begins with the isolation of human-derived stem cells, sourced either from commercially available cryopreserved human stem cells or obtained from biopsy-derived human stem cells. Following isolation, these stem cells are cultured *in vitro*, undergoing controlled proliferation. Through the induction of organ-specific growth factors, the stem cells are directed to undergo organoid formation.

In their study Okada et al. investigated the passage of orally administered drugs through the gastrointestinal tract, a crucial step for drugs to reach the bloodstream [38]. To address this challenge, they explored an innovative strategy utilizing human intestinal organoids (HIOs). These organoids, derived from LGR5+ stem cells residing in the intestinal crypts, exhibit a remarkable capability for long-term preservation. Notably, HIOs express various pharmacokinetic-related enzymes and transporters, including key players like cytochrome P450 3A4 (CYP3A4), carboxylesterase 2 (CES2), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP). This elevated expression makes HIOs a promising resource for advancing pharmacokinetic investigations.

Organoid models offer significant advantages in drug discovery by closely mimicking human organ structure and function. These 3D models provide a more accurate representation of tissue-specific processes, enhancing experimental accuracy. Patient-derived cancer organoids show promising results for personalized medicine by reflecting individual tumor biology and predicting patient responses to therapy, facilitating the development of tailored treatments [39]. Organoids retain the gene expression and mutation profiles of the original tumors, making them robust and clinically relevant disease models. They address the limitations of traditional 2D cultures and animal studies, which often fail to closely replicate human disease conditions, offering better translatability of research findings [40]. Additionally, organoids are versatile, supporting applications in drug screening, toxicology, and regenerative medicine, and they maintain stability for long-term studies, making them invaluable tools in advancing medical research and therapeutic development.

Despite their advantages, organoid models face several limitations. One major challenge is assay reproducibility, which is critical for reliable and standardized drug screening [39]. Variability in organoid formation and response can lead to inconsistent results, although strategies like using reference organoids as internal controls can help mitigate this issue. Another significant limitation is the lack of vascularization in organoids, which restricts their growth, differentiation, and functional complexity [40]. This deficiency impacts their ability to accurately model drug delivery and metabolism. Cost and accessibility are also concerns, as the creation and maintenance of organoids can be expensive and require specialized equipment and expertise.

**Microfluidic 3D models**

In the 1990s, the advent of microfluidic devices revolutionized 3D cell culture systems. By controlling the flow of fluids through microchannels, these devices enable the creation of more intricate and flexible 3D environments. This advancement has been particularly influential in drug development and personalized medicine, where the precision and adaptability of microfluidic 3D models are invaluable. Their ability to mimic complex biological systems makes them a critical tool in these cutting-edge fields of biology [41]. Microfluidics technology extends its applications beyond cell culture models, finding significant utility in high-throughput drug screening, single-molecule analysis, and the development of advanced therapeutics [42].

**Organ-on-chip (OoC) models**

Organ-on-chip (OoC) models represent an interdisciplinary advancement at the intersection of biology and microtechnology. These scientific and technological systems serve as *in vitro* models designed to replicate the complexity of human physiology [43]. The chips used in these models are microfluidic devices constructed with microchannels, allowing for the precise control of solution volumes ranging from picolitres to millimeters per minute. OoC models provide a platform for recreating, *in vitro*, the complex array of mechanical, fluidic, spatial, and chemical stimuli that tissues experience *in vivo* [44]. OoC models can form intricated cellular microenvironments, which is key in drug assays to recreating *in vivo* conditions and results [42].

Evaluating ADME properties involves a complex process, starting from the drug's entry into the human body, passing in the bloodstream and its distribution to various organs. To replicate this intricate process in OoC models, researchers have developed multi organ human-on-chip (HoC) or body-on-chip (BoC), using various OoC models, which mimic the complexity of the human body's multi-organ systems [45], [46].

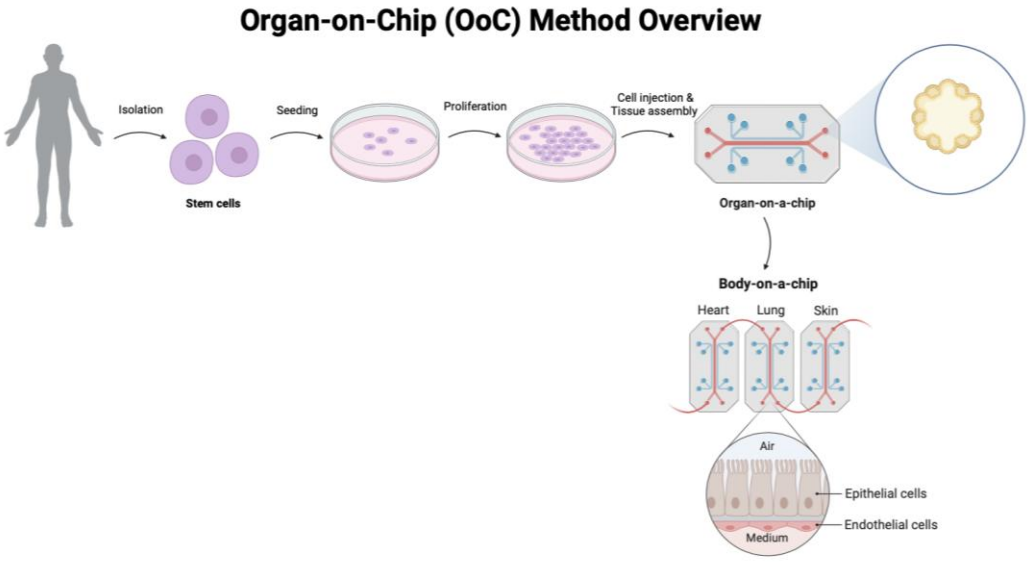


**Fig 3** Organ-on-chip (OoC) and body-on-chip (BoC) method overview. Both methodologies begin with the isolation of human-derived stem cells, sourced either from commercially available cryopreserved human stem cells or obtained from biopsy-derived human stem cells. Following isolation, these stem cells are cultured *in vitro*, undergoing controlled proliferation. Through the induction of organ-specific growth factors, the stem cells are directed to undergo organoid formation. Nutrients are brought to cells through microchannels. After having formed OoC's these can be brought together in a BoC to perform ADME and toxicity assessments.

In a study to analyze the mechanisms underlying drug transport, Tsamandouras et al. pioneered the development of the first multi-organ *in vitro* systems by combining gut and liver on a chip model [44]. This innovative approach enabled conducting complex pharmacokinetic assays like bioavailability, drug clearance, and interactions, which are challenging in traditional *in vitro* systems. However, limitations exist, such as the Caco-2/HT29-MTX cells not fully replicating *in vivo* metabolic functions, suggesting patient-derived cells as a potential solution. Additionally, the liver component tends to under-predict hepatic clearance, posing challenges for accurate drug screening.

Lee et al. developed a novel co-culture model using bovine lung-on-chip to evaluate the transport and efficacy of the antibiotic drug danofloxacin [47]. This model incorporated primary bovine bronchial epithelial cells and bovine pulmonary arterial endothelial cells. While the model successfully replicated *in vivo* plasma pharmacokinetics in the endothelial channel, it did not detect consistent levels of danofloxacin in the epithelial channels, likely due to a dilution effect during media sampling. This research represents a significant advancement in creating species-appropriate *in vitro* models, suggesting the potential of the bovine lung-on-chip as a viable alternative for *in vivo* studies in drug and pathogen research.

Microfluidic 3D models, offer several advantages in biomedical research and drug discovery. These models provide precise control over the cellular microenvironment, allowing for the replication of complex tissue and organ functions [43]. By integrating multiple cell types and tissues within a single device (HOC), they can mimic the interactions between different organs, providing a close resemblance to human physiology. One of the key strengths of microfluidic 3D models is their ability to simulate physiological fluid dynamics, including shear stress and nutrient gradients, which are crucial for maintaining cell function and viability [41], [48]. This feature enhances the relevance of these models for studying drug delivery, pharmacokinetics, and disease mechanisms. Microfluidic models also support real-time monitoring and high-resolution imaging, enabling detailed analysis of cellular responses and tissue development. Their compatibility with advanced sensing technologies allows for continuous observation of various parameters, such as pH, oxygen levels, and metabolic activity, improving the accuracy and depth of experimental data [49].

Despite their advantages, microfluidic 3D models, face several limitations. One major challenge is the complexity and technical expertise required for their design and fabrication. Developing and optimizing these systems can be time-consuming and require specialized skills and equipment, limiting their accessibility [27]. Reproducibility is another issue, as slight variations in the fabrication process or cell handling can lead to inconsistent results. Ensuring uniformity and standardization across different devices and experiments remains a significant hurdle. Cost is also a concern, with the high expenses associated with materials, fabrication, and maintenance. This limits the widespread adoption of microfluidic models, especially in resource-limited settings. Microfluidic models often struggle to fully replicate the complexity of living tissues and organs. While they can simulate certain physiological conditions, they may not capture all the intricate interactions and environmental factors present *in vivo*. Integration of multiple organ systems in a single chip, while promising, remains technically difficult and can lead to issues with scaling and inter-system communication [50]. This complexity can also complicate data interpretation and analysis.

**Scaffold-free 3D models**

In scaffold-free 3D models, unlike those incorporating structural scaffolds, cells are introduced into systems leveraging various forces to facilitate aggregation and spheroid formation. The concept of spheroids was initially introduced by Sutherland and colleagues in the 1970s [51]. Since then, diverse techniques have been developed to cultivate spheroid cultures. Spheroids arise through spontaneous aggregation driven by differentiated forces, where subsequent cell surface integrin interactions with the extracellular matrix (ECM) initiate the process. Following the initial cell-cell attachments, elevated E-cadherin expression enhances the establishment of robust intercellular bonds. The formation of spheroids is influenced by essential factors such as nutrient availability, oxygen levels, growth factors, and paracrine signals.

Scaffold-free 3D models provide several distinct advantages that significantly enhance their utility in drug discovery. One of the primary benefits is their ability to form more natural cell-cell interactions without the influence of an artificial scaffold [27]. This closely mimics *in vivo* conditions, providing a more physiologically relevant environment for studying cellular behaviors and responses. These models are particularly useful for creating uniform and reproducible spheroids, which can be easily scaled up for high-throughput screening applications. The simplicity of scaffold-free methods, such as forced floating, hanging drop, and magnetic levitation, makes them accessible and cost-effective, requiring less specialized equipment compared to scaffold-based techniques [52]. Scaffold-free models are also versatile, accommodating a wide range of cell types, including primary cells, stem cells, and cancer cells. This versatility allows for the development of various disease models and the study of different biological processes, such as tumor growth, drug resistance, and tissue regeneration. Another significant advantage is the ability to rapidly generate spheroids, which can be used to assess drug efficacy and toxicity in a timely manner. This speed is crucial for accelerating the drug discovery process and evaluating potential therapeutics more efficiently. Additionally, scaffold-free models support the formation of more homogenous cell aggregates, which is important for consistency in experimental outcomes [53]. They also

facilitate long-term culture, enabling chronic exposure studies and providing insights into long-term drug effects and cellular adaptation mechanisms.

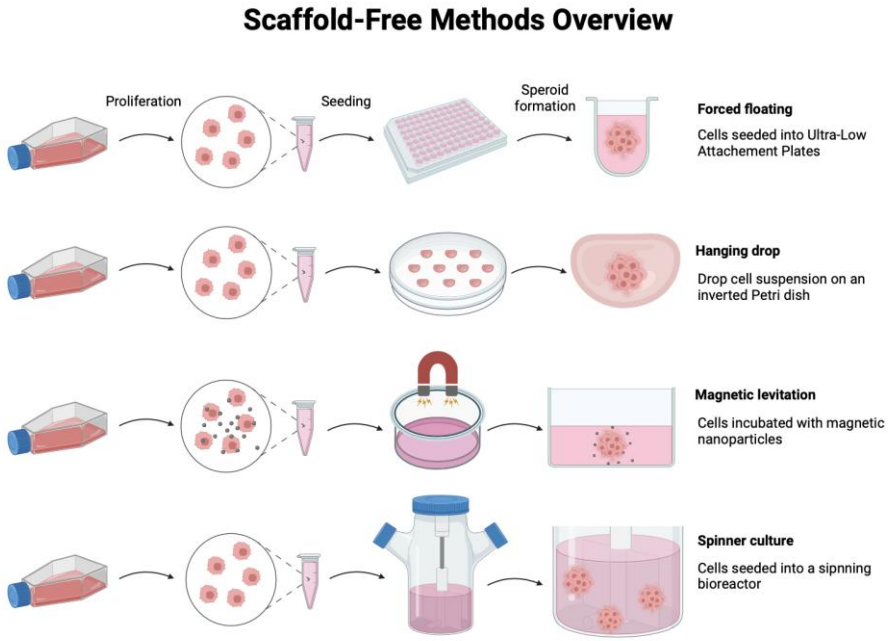


**Fig 4** Scaffold-Free Methods Overview. All spheroid cultures are formed from primary sources or cell lines and proliferation. After they are being seeded in various techniques, with the usage of different types of forces, cells start to aggerate and form spheroids.

Scaffold-free 3D models, while advantageous, have several limitations that need to be addressed. One major challenge is the difficulty in controlling the size and shape of spheroids, which can lead to variability in experimental results. Ensuring uniformity and consistency across different samples remains a significant hurdle. Another limitation is the lack of structural support, which can result in less stable and less robust tissue models. Without a scaffold, it can be challenging to maintain the integrity of larger or more complex tissue constructs over time, limiting their use in certain applications. Scaffold-free models also struggle with nutrient and oxygen diffusion, particularly in larger spheroids. The absence of a vascular network can lead to the development of necrotic cores, reducing the viability and functionality of the tissue model. This limitation affects the ability to accurately model physiological conditions and drug responses. Furthermore, the scalability of scaffold-free models can be problematic. While they are suitable for high-throughput screening, the production of large-scale or more complex tissues for therapeutic applications remains challenging. Developing methods to scale up these models without compromising their integrity and functionality is an ongoing area of research.

**Forced Floating**

The Forced Floating technique involves culturing cells in ultra-low attachment plates to promote spheroid formation. By preventing cell adhesion to surfaces with surface coating, cells are held in a suspended culture causing cells to form cell-cell attachment, which leads to aggregation and spheroid formation [54].

Bell et al. investigated whether primary human hepatocyte (PHH) spheroids could serve as a viable model for studying drug-induced liver injury (DILI) [55]. Their research primarily focused on assessing the functional and metabolic activity of PHH spheroids, which is crucial for DILI drug screening. The study involved monitoring major cytochrome P450 enzymes (CYPs), particularly five major CYPs, and the formation of drug metabolites. CYPs are responsible for the oxidative transformation of a wide variety of drugs and endogenous compounds [56]. Notably, the research demonstrated stable hepatic function in PHH spheroids by sustained ATP levels, albumin secretion and preserved CYP enzyme activity. These findings suggest the potential suitability of PHH spheroids for chronic drug toxicity assays.

The forced floating technique for 3D cell culture offers advantages such as promoting natural cell-cell interactions, simplicity, cost-effectiveness, versatility, and scalability. However, it also faces limitations including difficulty in controlling spheroid size and shape, lack of structural support, issues with nutrient and oxygen diffusion, scalability

challenges for complex tissues, and reproducibility concerns. Addressing these limitations will enhance the utility of the forced floating technique in drug discovery.

**Hanging Drop**

The Hanging Drop technique involves cellular cultivation within droplets suspended on the interior of culture dish lids. Droplets hang on the surface with surface tension utilizing gravitational forces to instigate spontaneous cellular aggregation within suspended droplets of culture medium. Among scaffold-free techniques, Hanging Drop is distinct in its ability to control the size of the resulting spheroids.

Michael et al. developed innovative approach to enhance the hanging drop method as the Paper Hanging Drop Chip (PHDC), facilitating the stable culture of 3D spheroids for over ten days [57]. A key feature of PHDC is its ability to allow uninterrupted and periodic medium exchange, enabling efficient nutrient and oxygen transport through its porous substrate. This was demonstrated to yield results consistent with conventional hanging drop cultures in drug screening assays. Further advancement led to the creation of Networked PHDCs (N-PHDCs), combining multiple units. This advancement is significant in mimicking complex *in vivo* microphysiology, thereby offering a viable tool for multispheroid-based studies that closely emulate human *in vivo* systems.

Limitations associated with media exchange in the hanging drop technique have prompted the development of alternative approaches for 3D spheroid formation. Liu et al. introduced the SimpleDrop Chip, a novel microfluidic device constructed from polydimethylsiloxane (PDMS) with integrated micro-chambers [32]. These chambers facilitate the introduction of cell suspensions, culture medium, and even drugs for anti-cancer drug assays. The SimpleDrop Chip offers potential advantages for drug testing, particularly in fluorescent dye-based (immunofluorescence) analyses, due to its simplified handling compared to the hanging drop technique.

The hanging drop method for 3D cell culture offers advantages such as high reproducibility, minimal equipment requirements, natural cell-cell interactions, versatility, simplicity and ease of observation. However, it also faces limitations including difficulties with media exchange, scalability issues, size limitations of spheroids, uncontrolled spheroid size and formations, handling and stability challenges, and the need for specialized plates for optimization [32]. Addressing these limitations will enhance the utility of the hanging drop method in drug discovery.

**Magnetic Levitation**

The Magnetic Levitation method, utilizing magnetic nanoparticles (MNP), is a pivotal technique for regulating nutrient distribution within cell cultures, subsequently influencing cell aggregation patterns. This process magnetizes cells through electrostatic forces and non-specific binding to the cell membrane [58]. The controlled manipulation of MNPs via magnets facilitates cell levitation, enhancing cell-to-cell interactions and promoting spheroid formation. There are two distinct approaches to this method: Positive magnetophoresis / Paramagnetic manipulation and Negative magnetophoresis. Positive magnetophoresis involves integrating MNPs into cells, while Negative magnetophoresis levitates cells without MNP labeling [59], [60]. Souza et al. were pioneers in applying this method, aiming to develop a 3D cell culture system that employs magnetic levitation in conjunction with phage-based hydrogels containing nanoparticles [61] This system is designed to closely mimic *in vivo* protein expression and is conducive to long-term multicellular studies.

Roth et al. developed the Spatially Patterned Organoid Transfer (SPOT) platform, a novel approach leveraging magnetic levitation for optimized organoid cytoarchitecture conservation [62]. This platform utilizes iron-oxide nanoparticle-laden hydrogel and magnetized 3D printing, enabling controlled assembly of organoids. Consequently, SPOT facilitates the creation of well-organized assembloids that replicate key aspects of developmental processes and disease etiologies.

The magnetic levitation technique for 3D cell culture offers advantages such as rapid formation of spheroids, natural cell-cell interactions, minimal physical handling, versatility, cost-effectiveness, and enhanced diffusion. However, it also faces limitations including challenges with uniformity and reproducibility, potential interference from magnetic particles, limited size of constructs, scalability issues, handling and optimization complexities, and the need for specialized equipment. Addressing these limitations will enhance the utility of the magnetic levitation technique in drug discovery.

**Spinner Culture**

The spinner culture technique involves culturing cells in bioreactors with constant stirring, promoting cells to form cell-cell adhesion and aggerate. These spinner cultures are mostly used in mass productions of spheroids. Magnetic spinners facilitate stirring, ensuring even distribution of oxygen and nutrients. Critical parameters in this technique

include the type and size of the paddle and impeller, as well as the stirring rate, which are essential for maintaining cell-cell attachment and minimizing stress on the cells [63], [64].

As a result, it is more advantageous to study 3D cell culture forms in order to increase the reliability of the unreliable and unrealistic results produced by 2D *in vitro* cell forms in pharmacokinetic studies. 3D culture forms can be used to evaluate both the impact of the tumor microenvironment and the possible effects of pharmacokinetics on tumor forms.

The spinner culture technique for 3D cell culture offers advantages such as enhanced nutrient and oxygen supply, scalability, cost-effectiveness, rapid formation of spheroids, versatility, and suitability for long-term culture. However, it also faces limitations including shear stress, control over spheroid size, complexity of optimization, handling and sampling challenges, limited structural complexity, and scalability issues for complex tissues. Addressing these limitations will enhance the utility of the spinner culture technique in drug discovery.

## Discussion

While 3D cell cultures offer numerous advantages over their 2D counterparts, certain drawbacks currently limit their widespread adoption in drug discovery. Developing 3D models can be more expensive, time-consuming, and intricate, with variability observed across cell lines that can hinder reproducibility [27]. Despite these challenges, 3D models are demonstrating promising results in the drug development pipeline, leading to their emergence as a preferred method. Furthermore, the application of 3D models is expanding into the fields of organ transplantation (regenerative medicine) and personalized medicine, where patient-derived tumor models hold immense potential [65]. Future advancements aim to refine 3D models, making them more user-friendly, cost-effective, and ultimately more clinically relevant.

Scaffold-based models are gaining traction in drug discovery due to their advantages, including high reproducibility, the ability to incorporate co-cultures, and the potential to mimic *in vivo* cellular organization. However, achieving cell-specificity and reproducibility with these models necessitates extensive research and development [50]. Different cell types require unique combinations of growth factors and ECM components, making scaffold development a complex process that demands significant time, resources, and advanced technologies. 3D bioprinting is a cutting-edge technology with immense potential for creating complex tissue models. It utilizes a layer-by-layer deposition of cell-laden or non-laden hydrogels to create complex 3D structures that replicate the architecture and function of natural tissues and organs. The tumor microenvironment (TME) plays a critical role in drug efficacy, necessitating the development of *in vitro* models that faithfully recapitulate its characteristics. Innovative 3D bioprinting technology offers a promising avenue for precisely controlling and mimicking the TME, encompassing all its components, including both mechanical and fluidic forces [10]. However, a major challenge lies in constructing and utilizing intricate, composite tissue structures that resemble solid organs. This challenge arises from the interplay of numerous factors, including the selection of appropriate cells, bioink composition, the type of bioprinter and the bioprinting technique employed [66].

Gelatin methacryloyl (GelMA) is a widely used biomaterial in 3D bioprinting due to its tunable properties. The concentration of GelMA plays a critical role in mimicking *in vivo* tissue structure and cellular behavior. Arya et al. demonstrated that 10% GelMA hydrogels exhibited morphological features similar to decellularized human breast tumor tissue, as confirmed by compression testing and microscopic evaluations [67]. Interestingly, a decrease in GelMA concentration resulted in enhanced cell proliferation, with the highest rates observed in 5% GelMA hydrogels. However, the superior mechanical properties of 10% GelMA hydrogels make them more suitable for long-term cell culture, making them a favorable matrix for mimicking the *in vitro* breast microenvironment. This study also highlights the importance of 3D models in drug discovery. When compared to traditional 2D models, 3D spheroid models derived from breast cancer cells exhibited decreased sensitivity (higher IC50) to paclitaxel, a first-line chemotherapeutic drug for advanced breast cancer. This finding suggests the development of drug resistance within the 3D microenvironment, potentially due to the role of cell-cell and cell-ECM interactions in spheroid formation. Cell adhesion-mediated drug resistance (CAM-DR) is currently being investigated through the lens of integrin-mediated adhesion [33]. Integrins are cell adhesion molecules that can influence cell survival and potentially inhibit apoptosis. While studies suggest a link between integrin signaling and drug resistance, further investigation is necessary to fully elucidate the underlying mechanisms.

3D organoid cultures offer significant advantages over traditional 2D models in drug development. They effectively mimic *ex vivo* organ development and function, making them valuable tools for disease modeling. Additionally, organoids exhibit well-differentiated behaviors, enabling predictions of therapeutic response and

pharmacokinetic (PK) studies. These features facilitate rapid and multiplexed assays, streamlining the drug development process [46]. However, despite these advantages, recent research has highlighted a critical factor: the composition of the culture medium. Studies have shown that growth factors and molecular inhibitors within the medium can significantly influence organoid drug response [68], [69]. This finding underscores the importance of optimizing culture media to achieve reliable and informative results in drug development using organoid models.

Microfluidic devices offer a valuable approach in drug discovery while holding current limitations. The fabrication process typically involves specialized equipment and materials, requiring significant technical expertise. This complexity translates to higher costs compared to traditional 2D cultures. Maintaining consistent results in 3D cell cultures presents a significant challenge due to the inherent complexity of these systems [29]. The sensitivity of 3D cultures to even minor perturbations extends from the biological variability of primary cells to subtle variations in culture conditions. The intricate nature and specialized equipment requirements inherent to scaffold-based 3D cultures present significant barriers to their large-scale adoption in industrial and clinical applications.

Microfluidic devices offer a distinct advantage for 3D cell culture by employing oxygen and growth factor-permeable materials, a feature that addresses hypoxia, a major challenge encountered in scaffold-free models [41], [48]. OoCs and HoCs represent a significant advancement in mimicking human physiology, aiming to replicate organ size, interactions, and functions. However, achieving accurate relative size scaling across individual OoCs within these multi-organ systems remains a challenge, as highlighted by Wikswo et al. [70]. Allometric and functional scaling approaches are crucial for overcoming this limitation and enabling OoCs/HoCs to effectively replicate human drug response and pharmacokinetics.

Another critical limitation of OOC chips lies in the control of essential factors for cell function. Due to the inherent complexity and heterogeneity within a chip, meticulously regulating parameters like pH, temperature, organ-specific growth factors, oxygen levels, and fluid flow is crucial for optimal cell proliferation and 3D structure formation [71]. To address this challenge, researchers are exploring the integration of sensors within OOC models. These sensors would enable real-time monitoring and precise control of these critical parameters without disrupting the cultured tissues, cells, or organoids. Integration of sensors within OoC models is another key advantage, allowing for continuous monitoring and control of crucial parameters without disrupting the cultured tissues/cells/organoids [49]. Notably, nutrient/fluid flow rate is a critical parameter. Excessive flow rates can lead to cell disintegration, hindering organoid/tissue formation and potentially generating misleading results in pharmacokinetic studies. OoCs offer several advantages over 2D models. Their more accurate flow control translates to efficient nutrient utilization and enables rapid, multiplexed analyses.

Scaffold-free spheroid models hold promise for 3D cell culture, but a critical limitation remains: the risk of spheroid disintegration [58]. Magnetic levitation emerges as a solution, minimizing this disintegration risk while effectively producing homogeneous and compact cell aggregates. Compared to other 3D culture techniques, magnetic levitation offers significant advantages. It requires minimal specialized equipment, facilitating its implementation across diverse cell lines and enabling easier large-scale production. Furthermore, this versatile technique demonstrates efficacy in various applications within biotechnology, pharmaceutical development, stem cell research, and personalized medicine. However, a limitation associated with paramagnetic manipulation, a method used in magnetic levitation, is the potentially time-consuming and labor-intensive process of magnetically labeling cells [60].

Spinner cultures offer a valuable tool for large-scale production of spheroids due to their automation capabilities, facilitating rapid and efficient aggregation. This automation is particularly advantageous for companies managing numerous drug development projects. However, this rapid process can potentially lead to variability in spheroid size and shape. Additionally, prolonged stirring within the culture can have detrimental effects on cell physiology, potentially causing spheroid disintegration. This size variation can pose significant challenges during drug screening assays [41], [63]. While spinner cultures offer automated control of pH and dissolved oxygen levels compared to static plate systems such as forced floating, the automated stirring itself can have both advantageous and limitations. One of the limitations is that high stirring rates can disrupt spheroid integrity, while low rates can lead to cell sedimentation, impeding spheroid formation [64]. Another limitation of spinner cultures is the requirement for a larger volume of culture medium compared to other 3D culture techniques. This increased media volume translates to higher operational costs [41].

The hanging drop method has traditionally been employed for spheroid formation due to its high reproducibility and ability to generate single spheroids. However, limitations such as difficulties in media exchange using standard culture dishes, the high cost and inflexibility of specialized hanging drop plates, and the extensive time required

for optimization have hindered its widespread use [57]. Furthermore, the hanging drop method is limited by the restricted size of the spheroids that can be formed due to the nature of the technique. Additionally, media exchange can be challenging due to the setup, making it less suitable for drug assays where frequent addition or removal of compounds is necessary [32].

The Paper Hanging Drop Chip (PHDC) presents another option, offering advantages in simplicity and cost-effectiveness [57]. Additionally, PHDC enables spheroid culture for extended periods, up to 10 days. However, a limitation of PHDC is its white paper color, which hinders the optical imaging of non-fluorescent cells.

The SimpleDrop Chip is an innovative approach to 3D cell culture. It eliminates shear forces typically encountered in other techniques, making it a potentially gentler and more biocompatible approach for cell cultures [32]. Additionally, the polydimethylsiloxane (PDMS) material contributes to the environmental friendliness of this method compared to some traditional methods.

Ultra-Low Attachment (ULA) plates, also known as forced floating, have emerged as a more favorable alternative due to their ability to address these drawbacks. ULA plates offer a simpler and more cost-effective approach, facilitating media exchange and streamlining the process [72].

While the hanging drop method remains favored for certain applications due to its high reproducibility, ULA plates represent a more practical and adaptable choice for many researchers.

## Conclusion

Three-dimensional (3D) models offer significant advantages over traditional 2D and animal models, making them highly favorable in drug development. By successfully replicating *in vivo*-like structures with accurate cell-cell and cell-ECM interactions, 3D models have shown promising results in pharmacokinetic and toxicology assessments [9]. The advantages of 3D models are evident in their ability to replicate key aspects of human tissue dynamics and drug behavior, reducing the ethical and practical limitations associated with animal testing and thereby addressing the high attrition rates observed in clinical trials.

3D models can be produced in three main forms: scaffold-free models, scaffold-based models, and organoid models. Scaffold-free models were the first to be developed and offer advantages such as lower complexity, cost-effectiveness, reproducibility, and accessibility. Despite these benefits, they face challenges like lack of size controllability, which hinders uniformity and consistency across samples, and structural fragility, increasing the risk of spheroid disintegration. Scaffold-based models address some of these limitations by using scaffolds to control size and provide structural support, making them less fragile [12]. However, these models are more complex, requiring substantial resources and specialized knowledge [27]. The high cost of materials and the need for interdisciplinary collaboration among engineers, biologists, and chemists add to the challenges associated with scaffold-based models, hindering their large-scale adoption in industrial and clinical applications. Organoid models, particularly OoC and HoC systems, have shown promising results in drug assessments. While these models offer significant potential for mimicking human physiology and studying drug responses, achieving relative size scaling across individual OoCs remains a major challenge [42]. The use of allometric and functional scaling approaches is necessary for accurate results in drug assays, necessitating further research to optimize their use and address existing limitations.

In conclusion, while 3D models provide a promising alternative to traditional methods, continued advancements and interdisciplinary efforts are essential to overcome current challenges and enhance their application in drug development.

**Future studies**

Future research should focus on overcoming the current limitations of 3D cell culture models to maximize their impact on drug development. The biggest problems 3D models currently face include lack of reproducibility, scalability, biological and disease relevance, high costs, and the interdisciplinary knowledge required for further development. Key areas for future studies include technological advancements, reproducibility and scalability, enhanced predictive models, interdisciplinary collaboration, and regulatory and ethical considerations.

Technological advancements are essential for improving the mechanical and biological properties of scaffolds and bioinks used in 3D bioprinting [27]. Previous studies have shown that the extracellular matrix (ECM) significantly affects drug responses [7]. Therefore, further research into the ECM and its compounds is needed to create more accurate models [23], [24]. Additionally, research should prioritize developing more sophisticated microfluidic devices that better mimic the dynamic interactions within human tissues. One of the main issues with current microfluidic devices is the lack of flow control, which leads to inaccurate drug responses. Integrating biosensors

offers a promising future for system optimization, which is essential for drug discovery [49]. These innovations will contribute to creating more accurate and reliable 3D models.

Reproducibility and scalability are crucial for the widespread adoption of 3D culture techniques. Reproducibility is a concern for their use in high-throughput screening (HTS) and high-content screening (HCS), which are important steps in drug discovery [28]. To address this, there is a need for the development of a versatile 3D culture system capable of seamless integration into mainstream drug discovery pipelines while also permitting precise modulation to replicate the tissue-specific attributes of an *in vivo*-like microenvironment. Additionally, making these technologies scalable and more accessible is vital for their extensive use in research and industry [70]. Improving scalability requires the development of universally adapted scales based on allometric and functional scaling.

Enhancing the predictive accuracy of these models for human drug responses, particularly in personalized medicine, is another important area for future research. Using patient-derived cells in organoid and other 3D models can tailor drug development and testing more closely to individual patient profiles, improving the relevance and effectiveness of therapeutic strategies [65].

Interdisciplinary collaboration is essential to address the multifaceted challenges associated with 3D cell culture models. Collaboration between biologists, engineers, material scientists, and clinicians can further our understanding of the complex interactions within these models and their applications in various fields of biomedical research [29]. This collaborative approach will drive innovation and overcome technical and methodological barriers.

Addressing regulatory and ethical aspects is also important for the adoption of 3D cell culture models in drug development. These models have the potential to meet regulatory standards and reduce ethical concerns associated with animal testing, paving the way for their acceptance and implementation in the pharmaceutical industry. By addressing these areas, future advancements can make 3D cell culture models more user-friendly, reproducible, versatile, cost-effective, and clinically relevant, ultimately enhancing their role in the drug development pipeline and beyond.

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