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Developments with 3D Bioprinting for Novel Drug Discovery

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Abstract

Introduction: Although there have been significant contributions from the pharmaceutical industry to clinical practice, several diseases remain unconquered, with the discovery of new drugs remaining a paramount objective. The actual process of drug discovery involves many steps including pre-clinical and clinical testing, which are highly time- and resource-consuming, driving researchers to look for technological interventions that can improve the process efficiency. The shift of modelling technology from two-dimensions (2D) to three-dimensions (3D) for *in vitro* drug screening is one of such advancements. Three-dimensional models allow for close mimicry of cellular interactions and tissue microenvironments thereby improving the accuracy of results. The advent of this bioprinting technology for fabrication of tissue constructs has shown potential to improve 3D culture models.

Areas covered: The present review provides a comprehensive update on a wide range of bioprinted tissue models and appraise them for their potential use in drug discovery research.

Expert opinion: Efficiency, reproducibility, and standardization are some impediments of the bioprinted 3D models. In addition, vascularization of the constructs has to be addressed for the bioprinting domain, in the near future. While much progress has already been made with several seminal works, the next milestone will be the commercialization of these models after due regulatory approval.

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Declaration of Interest

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Keywords

3D bioprinting; drug discovery; tissue models; drug testing

1. Introduction

Drug discovery is an essential exercise of pharmaceutical research. However, existing drug discovery process is highly time consuming and expensive, with recent studies estimating 2.6 billion US dollars as the development cost of a new drug [1].The process of drug discovery commences from in silico design of several molecules followed by the screening of a library of these compounds to bind to a drug target. The molecules with strongest binding affinities are then tested in vitro and in vivo in pre-clinical phase. Several animal studies become necessary in pre-clinical testing. The clinical testing of drugs is also carried out in a phase-wise manner before a drug is approved for marketing. In the drug discovery process, *in vitro* efficacy and in vitro toxicity are the two common assays used to select leads from hits, and exclude compounds with unacceptable toxicological profile, often leading to optimization of the lead compounds. Thus, the number of compounds otherwise needed to be tested *in vivo* are reduced. However, false results may significantly defeat the drug discovery objectives. Therefore, regulatory agencies exercise cross-industry validation of any *in vitro* model before recommending its applications. Therefore, the accuracy of the model assumes prime importance. Moreover, several models are now being designed to offer phenotypic screening, which should allow for visualization of cell morphological parameters as end points of evaluating a response of a drug in the discovery phase. Lack of safety and efficacy are the principal causes of failures of the drugs which become evident only in Phases II and III of clinical trials. For certain years, regulators and industry have come to believe in the doctrine of “fail-early” to mitigate the risks associated with drug development. Most drug regulatory agencies have reiterated the need for continuous appraisal of technological advancements in order to reduce early-stage developmental costs of new drugs [2]. Since, drug discovery and development starts with screening of thousands of potential chemical entities to identify lead compounds with most efficient binding to a molecular target and validation of biochemical effect of this interaction and a majority of molecular targets are cellular substructures, cell-based assays have become a pivotal tool in early-stage drug screening process [3]. Moreover, drug discovery also entails study of drug solubility, permeability through intestinal cells, metabolism, and other pharmacokinetic parameters to ensure the “drug-ability” of the lead compounds. In vitro cellular assays also assume significance for predicting these properties and safety/toxicity of the screened molecular entities [4]. However, given the high rate of attrition in drug discovery cycle technological advancements can provide more precise, reproducible pre-clinical models that closely mimic the tissue biology, not only accommodating reductionist approaches but also knowledge of systems biology [5] along with recapitulating the native micro-environmental factors. Such advanced fabrication techniques thus become an important area of interest for drug discovery scientists [6].

Conventional cellular assays were performed in monolayer cell culture, wherein cells proliferated to spread over a rigid tissue culture polystyrene substrate in 2D. 2D cultures are

preferred for their ability to enable rapid manipulation, environmental control and visualization of cell growth compared to 3D cultures and is more relevant for modelling physiological barriers such as epithelial and endothelial barriers (i.e., lung, gut or vascular tissue). Over the years, the deficiencies of 2D culture in recapitulating cell bioactivities, tissue-specific architectures, cell-cell and cell-matrix interactions, and deviations from *in vivo* systems were recognized [7, 8, 9]. Meanwhile, the effect of extracellular matrix (ECM) as a key mediator on cellular behavior became evident, which led to increased efforts in fabrication of 3D culture models. Along with ECM/collagen, non-collagenous proteins, glycoproteins, immune and feeder cells, soluble factors as well as physical factors such as ECM stiffness, oxygen tension, and temporo-spatial growth factor gradients define a cell microenvironment. Such complexity of ECM cannot be mimicked in a 2D culture model. On the other hand, studies have demonstrated that 3D cultures can influence several cellular activities like cell survival, proliferation, differentiation, and mechano-responses [10, 11, 12]. The importance of 3D culture models is also bolstered by new insights, which show that ECM plays a more instructive role than mere structural support for cell growth [13]. In 3D cultures, aggregated cells, which are also referred to as spheroids or organoids, usually have volumetric depths more than 50 μm. Consequently, advantages of the 3D cultures have been seen over 2D counterparts. 3D cultures have shown more physiologically- relevant models due to physical constrain put by the microenvironment, cell-cell interactions, and mimicking of flow conditions as found in vivo. For example, certain tumor cells (e.g., human colon cancer cell line HCT116, MCF7 cell line and JIMT1 breast cancer cell line [14]) have been found to be more resistant to anticancer drugs when cultured in 3D models compared to 2D ones [15].

From a physiological viewpoint, enhanced glycolysis, hypoxia and acidic pH in the core of spheroids are often observed compared to 2D cultures. Hypoxia at the core of spheroids is associated with absence of angiogenic and anti-apoptotic factors, closely representing tumor core. On genetic and protein levels, differential expressions are also observed such as higher stromal cell-derived factor (SDF)-1 associated with circulating CXC receptor (CXCR) 4-positive endothelial progenitor cells [16]. Similarly, spheroids of HepG2 cells in 3D show higher cytochrome P450 activity and albumin production compared to 2D cultures, which might be useful for drug metabolism studies during drug discovery. Similar performances have also been observed in C3A hepatocytes for cytochrome P450 3A4 (CYP3A4) enzyme activities [17]. Another key difference between 2D and 3D cultures with profound implication in drug screening is the differences in localization of surface receptors of cells. Changing the dimensionality of culture system can also change signaling pathways activated in response to a particular chemical stimulant. For instance, in human epidermal growth receptor (HER2) cells, phosphoinositide 3-kinase (PI3K) pathway switched to mitogen activate protein kinase (MAPK) pathway when the culture models were changed from 2D to 3D [18]. Additionally, in 2D systems, most cells exist in the same cell cycle, whereas in 3D, core cells remain static compared to highly proliferating cells on the periphery [19]. Another important advantage of 3D models is the ability to co-culture heterotypic cells. Co-culture techniques can closely mimic the role of stromal cells and cancer stem cells in chemotherapy of cancer, as well as elucidate role of endothelial and perivascular cells in modeling angiogenesis [20]. Likewise, expression of various biomarkers is highly varied

when one cell type is cultured in the presence of other cell types. Amongst 3D systems, cells proliferate differently at locations with different stiffness. The aforementioned advantages of 3D culture system have motivated the fabrication of advanced 3D models.

Fabrication methods of 3D tissue models can be classified into scaffold based (anchorage dependent), scaffold free (anchorage independent) including more sophisticated microfluidics or micro-patterning platforms [21]. The *in vitro* models of organs with real-time responses at the tissue level can be monitored with microfluidic devices such as organ-on-chips [22], whereas micro-patterning mimics the substrate for the drug targeting, making it a reliable platform for personalized medicine (e.g., protein micro-patterning for drug discovery [23]). Scaffold-free techniques entail self-aggregation of cells, which can be created by several methods such as hanging drop, low attachment or liquid overlay, rotating flask, spinner flasks, magnetic levitation and microfluidic-based methods [24]. Hanging drop methods leverage gravity-driven aggregation of cells to form spheroids and have been useful models with appreciable tissue-size controls for studying tumor migration. However, these processes are time consuming and labor intensive, leading to being not particularly useful for high-throughput drug discovery [25]. Low attachment or liquid overlay method of 3D fabrication relies on a coating of agarose or poly (2-hydroxethyl methacrylate) (pHEMA) to reduce cell-matrix adhesion and improve cell-cell aggregation. This approach results in poor control over the desired construct size and requires 1–3 days for model formation [26]. The rotating flask and spinner flask methods are a set of bioreactor culture systems made of vessels in which cell suspensions are either rotated or agitated to prevent cell adhesion to matrix. Rotating flask method generally yields heterogeneous spheroids whereas spinner flask method is often associated with cell death [27]. Some other techniques have used magnetic levitation as an external force to induce cell aggregation, which however, suffer from low throughput [28]. More sophisticated microfluidics-based methods and chips have also been employed with the advantages of having provision for long-term perfusion as well as high throughput [29]. However, the flow conditions should be strictly controlled, and at the same time, the spheroids produced are sometimes difficult to be retrieved for analysis. To obtain high-throughput spheroids which are capable for real-time imaging, cells have also been cultured in computer-designed micro-wells coated with non-adhesive polymers. However, cell-seeding in each well is not uniform in the micro-molding technique [24, 30].

Apart from the scaffold-free culture methods, 3D models can also be fabricated using various natural or synthetic hydrogel materials (such as Matrigel™, collagen, fibrin, cellulose, gelatin, alginate, decellularized ECM) and poly(ethylene glycol) (PEG) and its derivatives respectively, which performed the role of ECM to house cells [31]. However, different hydrogel materials possess distinct characteristics in terms of degradation time, cell compatibility, stiffness of matrix, diffusion of nutrients, etc.

Though, it is now accepted that 3D cultures have vital advantages for drug discovery compared to 2D cultures, researchers are yet to obtain standardized fabrication methods, which can produce reproducible constructs. Ideally, the fabrication technique should allow rapid and cost-effective fabrication of 3D cultures. The 3D constructs for drug discovery should be highly reproducible, which is possible with 3D bioprinting due to its automation capabilities. Moreover, by eliminating the need of separate processing of any mold/substrate

material with cell components, fabrication times are largely reduced in 3D bioprinting compared to other biofabrication approaches. Additional requirements of *in vitro* models include the ability to form vascularized channels in constructs and amenability to allow co-culture to form heterotypic constructs mimicking tissue microenvironment. These constraints are not effectively addressed by other 3D fabrication methods and propel interest in 3D bioprinting to meet the requirements of *in vitro* models for drug discovery [21, 32, 33].

2. Advantages of bioprinting for drug discovery

Some of the conventional biofabrication strategies are hanging drop, liquid overlay, and magnetic levitation and rotating flask methods. Most of these methods requires some or the other from of manual cell seeding or required fabrication of a mold to achieve model fabrication [24]. Bioprinting is a high-throughput fabrication method amenable to high level of automation, which offers advantage of higher accuracy, resolution and precision in comparison to the conventional methods for fabricating 3D *in vitro* models [34]. Bioprinting enables facile fabrication of spatially-patterned co-culture models as well as reduces the probability of cross-contamination caused by handling of different cell types owing to constraining of the physical space. Precise control over delivery of genes and growth factors is enabled by bioprinting. Through bioprinting, fabrication of constructs with desired pore sizes conforming to architectural heterogeneity of a given tissue-type is also facilitated. Bioprinting also helps in fabricating constructs with controlled architecture in a high-throughput manner. Though vascularized constructs still remained to be achieved using bioprinting, it offers the best option amongst other methods to yield vascularized models [21]. Another important advantage is that bioprinting can be accomplished under physiologically-amenable conditions (e.g. temperature, humidity and pH). Cells can be bioprinted in tandem with other sensitive biological components including genes and proteins to modulate cellular behavior. It is thus observed that several bioprinted models have thus advanced into commercial landscape [24, 33]. On the other hand, fabrication of 3D models using bioprinting has to still improve high-throughput capability and the ability to fabricate miniaturized models below 50 μm dimensions reproducibly [35]. Presently, resolutions of bioprinted models are dictated by resolution of arm-stage movement of bioprinters [36]. However, recent advances, which leverage magnetic levitation for bioprinting of tissue spheroids offer increased scope of improving the throughput and resolution of bioprinted constructs [37]. Though several reports have highlighted the application of bioprinted constructs for pharmaceutical manufacturing [38], drug development [39], complex tissue constructs [40] or disease modelling [41], application of bioprinting in drug discovery through a wide range of tissues relevant for drug studies has not been comprehensively discussed or analyzed in the literature so far.

3. Components of bioprinting

The major components of bioprinting are (i) bioink, (ii) bioprinter, and (iii) the concomitant bioprinting process, starting from the designing of the tissue to the final accretion of the bioink for construction of target tissue. The bioink materials used in bioprinting processes are decellularised matrix components, microcarriers, hydrogels and cell aggregates which

are further classisfied into tissue strands, tissue spheroids and cell pellet [42]. The choice of scaffold-based or scaffold-free bioprinting is one of the first considerations, which provides different properties for specific applications. Scaffold-based bioprinting has shown greater commercial capability, affordability and ease of implementation. In scaffold-based approaches, cells are present in an immobilized state with restricted migration and distribution within the entire bioinks comprised of cells or/and hydrogels. Certain additives like low-acyl gellan gum, poloxamer or fluorosurfactants [43] may be required to suspend the cellular phase for prolonged bioprinting times. In addition, use of hydrogels imposes additional considerations on the characteristics of *in vitro* models. Cells cultured in hydrogels for a long term have shown reduced survival, functionality and phenotypic conformability. In contrast, the scaffold-free bioprinting approach allows the *in vitro* models to attain high cell densities. Cells at higher densities deposit their own ECM as they proliferate and self-assemble into micro-tissues. The exclusion of hydrogel support also ensures superior cell-cell interaction, which is vital for cell signaling. Scaffold-free bioprinting, though industrially less standardized, is advantageous in terms of cell functionality and phenotype stability for long-term cultures. Scaffold-free bioprinting can be performed using tissue strands, tissue spheroids or cell pellets [44, 45].

The selection of scaffold-based or scaffold-free approaches is determined by the application. Hydrogels are recommended in bioprinting applications for drug delivery since the degradation rate can be a parameter for controlled drug release. Similarly, for stem cell research, scaffold-based bioprinting is considered more suitable as crosslinking conditions of different hydrogels can be exploited to obtain biologically-relevant matrix compositions of stem cells [46]. However, in case of drug discovery research, scaffold-free methods are more useful to determine chemotherapeutic drug efficacy. One of the reasons is that cells in the core experience hypoxia, which resembles a tumor microenvironment. Likewise, scaffold-free bioprinting is also preferred for developing 3D co-culture models as hydrogel matrices often impede cell-cell cross-talks. From an industrial viewpoint, higher viscosities of hydrogels in the scaffold-based bioprinting approach usually hinder high-throughput fabrication, since higher viscosities are often associated with nozzle clogging. Shear-thinning rheology of bioinks is also required to ensure that bioink flows smoothly during bioprinting and at the same time, regains the structure after bioprinting. Shear-thinning behavior of bioinks is also important to assure the accuracy of bioprinted constructs, leading to 3D models with anatomically-complex shapes and architectures. To attain batch-to-batch repeatability, bioinks should have optimum yield stress [47], and shortened bioprinting durations in order to minimize sedimentation of cells in the bioink suspension.

For applications where scaffold-based bioprinting is required, the selection of hydrogel becomes the next important step. An appropriate hydrogel is selected by considering the physico-chemical properties including viscosity, cell-hydrogel adhesion property, toxicity of the hydrogel and its degradation products as well as the elastic modulus and mechanical strength. Another important consideration is the crosslinking mechanism. For bioprinting, optimization among rheology, biocompatibility and mechanical properties of bioinks are often necessary. The natural hydrogels are generally more cytocompatible. Tissue-specific hydrogels have also been preferable for bioprinting such as collagen type I for bone, collagen type II for cartilage, fibrin for angiogenesis, and Matrigel™ for cancer and cardiac

tissue development. On several occasions, functionalization with cell-recognizing motifs like arginyl-glycyl-aspartic acid (RGD) has shown improved outcomes in bioprinting with hydrogels.

The next important consideration of bioink is the crosslinking kinetics. Chemical, physical and enzymatic are the three principal mechanisms of crosslinking. Chemical crosslinking generally results in formation of constructs with the highest strengths due to strong intermolecular bonds. However, the intermolecular bonds also reduce the molecular pores for diffusion of nutrients and gases, compromising cell viability. In chemical crosslinking, higher concentrations of the crosslinker aid the bioprinting process but reduce the cellular migration [48]. Ideally, bioinks are expected to have very short crosslinking duration such that cells can be immobilized to a stable state at the earliest. Physical crosslinking relevant for bioprinting has been ionic gelation of alginate while photo-crosslinking is also commonly used with many bioinks, which results in rapid crosslinking without the use of any crosslinking bath. Owing to the quick crosslinking, photo-crosslinking can be conducted under high-throughput conditions, since increased gelation time reduces the throughput and impedes fabrication of multi-layered models. Most physical gelation is achieved at shorter times with minimal fluctuations in viscosity during bioprinting compared to chemical crosslinking. However, physically-crosslinked constructs are weaker and reversible compared to chemically crosslinked constructs. On the other hand, enzymatic crosslinking results in the same biocompatibility as the polymer bioink and can be used to crosslinked several hydrogels like fibrin, gelatin, hyaluronic acid and collagen using enzymes such as transglutaminase, horseradish peroxidase (HRP) and thrombin, This method has the minimum concerns with the toxicity of the crosslinking mechanism, it is not cost effective and poses difficulties in implementation [49].

4. Bioprinting modalities

Bioprinting modalities are classified into three types based on the principle of bioink deposition, which are droplet-, extrusion-, and laser-based bioprinting [50]. The principle of operation for the bioprinting modalities are schematically illustrated in Figure 1. Droplet-based bioprinting (DBB) refers to techniques that perform non-contact deposition of cell-laden bioinks in the form of precisely controlled droplets.

DBB was first introduced through modifications of inkjet printing technology in the late 80s and laid the foundation for bioprinting [51]. Along with common inkjet drop-on-demand deposition techniques, acoustic-, thermal-, piezo-devices for droplet ejection, electrohydrodynamic jetting and micro-valve-assisted droplet generation are also part of DBB [52]. DBB offers cell viability in the range of 70–90%. A wide range of deposition rates (1–10,000 Hz) are achievable. Besides, DBB holds other advantages such as low bioprinter costs, anatomical resolution and high-throughput ability. On the other hand, nozzle-clogging caused by highly viscous bioinks or high cell density constrains the application of DBB. In addition, poor uniformity of droplet volume, which may range from <1 pL to >300 pL, reduces the bioprinting resolution [53].

Extrusion-based bioprinting (EBB) employs a computer-controlled robotic system for continuous dispensation of bioink filaments [54]. Extrusion is performed by mechanical or pneumatic dispensing. The shear stress experienced by cells due to extrusion forces has been associated with diminished cell survival in EBB. Other limitations include limited resolution of bioprinted constructs, and alteration from bench to bedside, and the problem of sustained delivery of cells in a biomimetically-controlled environment to the transformation of the technology into a commercialized product. Despite all this, it has the advantage of bioprinting structurally-integrated and mechanically-sound constructs and is also the most economically-efficient modality, which is readily affordable [54].

Laser-based bioprinting (LBB) is identified as techniques, which employ laser energy to precisely pattern bioinks [55]. LBB can be further divided into two methods, one of which involves photopolymerization techniques that include two-photon polymerization or stereolithography, and the other one is based on cell transfer (e.g., laser induced forward transfer (LIFT) or laser guided direct writing). In comparison to DBB or EBB, LBB is devoid of clogging or cell-damage concerns apart from providing high resolution of *in vitro* models. However, bioprinter costs in this modality are higher.

Thus, it can be concluded that each technique has advantages and disadvantages with respect to cell survival, range of printable bioinks, ease of operation, resolution of bioprinted constructs, cost of equipment, practicality and throughput of the process [56]. In recent years, integration of microfluidics and development of multi-material, embedded bioprinting systems are enabling fabrication of constructs with higher resolution and high throughput [57, 58, 59]. The comparison of different printing modalities and their applications in drug screening has been summarized in Figure 1.

5. Bioprinted *in vitro* tissue models for potential application in drug discovery

Compared to any other biofabrication methods (such as methods such as hanging drop, microwell, micro-patterned matrices, microfluidics, acoustic force, and magnetic force-based techniques), 3D bioprinting offers the ability to precisely position multiple cell types as per tissue design. Depositing multiple cell types offer the opportunity to fabricate a 3D construct considering co-culture and vascularization possibilities. The complex spatial positioning is also complimented with versatility to process several materials. It also provides the added benefit of increased cell-matrix interactions ensuring the viability of cells for a longer period of time. Bioprinting is also a high-throughput, scalable, highly reproducible, and automated technique compared to other biofabrication techniques. The high-throughput fabrication advantage of bioprinting for generating spheroids has been demonstrated in recent studies [60, 61]. Bioprinted constructs can be easily perfused, which is a critical requirement for conducting drug screening. Additionally, increasing emphasis is directed towards generation of personalized diseased models for patient-specific drug discovery and therapeutic planning [62]. In this direction, bioprinting has shown potential especially with the use of induced pluripotent stem cells (iPSCs) as cell component, though demonstration of completely functional tissue made of bioprinted iPSCs still remains to be

performed [63]. Considering these advantages, several in vitro models for drug testing have been fabricated as summarized in Table 1 and detailed below.

5.1 Tumor Models

It is well known that the tumor microenvironment plays a significant role in governing the action of various drugs on the tumor. Bioprinting has shown promise in fabrication of tumor models with increased biological resemblance over their 2D counterparts. This has been evidenced in a glioma model (Figure 2A) fabricated using glioma stem cell-laden alginate/gelatin/fibrinogen bioink. The model showed increased resistance to the drug temozolomide as compared to 2D culture model [64]. 3D bioprinted constructs have also shown the presence of hypoxic core [65] and necrosis resembling *in vivo* conditions. The optical projection printing system has been shown to be useful to generate embryonic bodies of iPSC, which showed expression of several key pluripotency markers, **as depicted in** Figure 2B. In another study, MRC-5 fibroblasts and human ovarian cancer (OVCAR-5) cells have been bioprinted by DBB on Matrigel™ to form high-throughput, reproducible multicellular constructs with controlled spatial patterns [66]. The results showed that multicellular acini of ~100–500 μm^2 were formed at Day 1, but became more heterogeneous over a 15-day period as both cell proliferation and acini fusion occurred simultaneously in this period. The overall size of pattern was consistent with those obtained with manual pipetting and *in vivo* tissues. Moreover, acini size could be controlled by manipulating droplet size and cell concentration. Bioprinted tumor tissue models for cancer cell migration have also been obtained using a laser-based bioprinting with HeLa and 10T1/2 (non-cancerous fibroblast) cells in a poly(ethyleneglycol diacrylate) (PEGDA) bioink. Models containing channels with a width in the range of 25–120 μm were fabricated and it has been shown that HeLa cells migrated to different extents as a function of channel diameter, whereas no effect on fibroblast migration was observed [21].

In the domain of scaffold-free bioprinted *in vitro* models, Organovo Company has met certain commercial success. The Organovo 3D NovoGen Bioprinter system has enabled the fabrication of a model of breast cancer in which cancer cells were surrounded by a stromal milieu comprising of endothelial cells, fibroblasts, and adipocytes [67]. The *in vitro* model remained viable for around 14 days and there was a distinct compartmentalization inside these models. Such models have been applied to test the response of certain hormonal drugs such as prolactin, estradiol and human growth factor, as well as certain chemotherapeutic agents like paclitaxel, tamoxifen, methotrexate, tamosifen and cisplatin [67]. In another study, 3D neoplastic tissues for cervical anti-cancer drug screening have been developed (Figure 2C) using EBB with HeLa cells embedded in gelatin/alginate/fibrinogen to test their sensitivity to paclitaxel. The model showed enhanced chemosresistance compared to the 2D planar model [68]. In another work by Zhou and co-workers, *in vitro* model has been developed to study interactions of breast cancer cells and bone stromal cells using tabletop stereolithography with gelatin methacryloyl(GelMA)-nanocrystalline hydroxyapatite bioink. The results showed that breast cancer cells proliferated at the expense of other cell types while secreting enhanced vascular endothelial growth factor (VEGF) [69]. A pattern of growth observed in this model has been represented in Figure 2D. Though these studies have

established the utility of bioprinting in cancer drug discovery, effect of several parameters like layer height and pore size remain to be optimized.

5.2 Skin

The commercial demand for *in vitro* 3D skin models are established especially because of large inter-species variations. Bioprinting is the ideal fabrication method to create skin constructs for pharmaceutical and dermatological testing due to the inherent multilayered, multicellular composition of human skin. Several groups have informed varied success to obtain dermal equivalents. A direct cell printing method was utilized to create multi-layered models containing fibroblasts, keratinocytes, and a collagen-based hydrogel as the structural component to mimic skin layers [70]. The stratified skin was created by depositing nebulized sodium bicarbonate, a crosslinking agent, after the deposition of a layer of collagen and cells. Both cell types were shown to maintain viability and proliferation, and yielded a stratified structure. Bioprinting with collagen/fibroblasts bioink, followed by deposition of melanocytes and keratinocytes to get functional skin constructs, has also been reported [71]. Exposing bioprinted multilayered constructs to air-liquid interface has been shown to promote tissue maturation and stratification, which are confirmed by expression of skin specific biomarkers [72]. Bioprinted constructs using LBB modality and HaCaT keratinocytes/NIH3T3 fibroblasts embedded in collagen/Matrigel™ matrix has shown to express connexins, pan-cadherin and laminin [73]. Some key findings of the study have been depicted in Figures 3A1–6. Progress has also been made towards standardizing production of skin equivalents using primary human dermal fibroblasts and evaluated by collagen remodelling up to 42 days of culture [74]. Moreover, cell sedimentation during bioprinting can be a cause of inhomogeneities in the constructs. Chahal et al. has considered the problem and proposed a solution by utilizing a suspension of cells with Ficoll® PM400, which is a highly-branched hydrophilic polymer of sucrose with an average molecular weight of 400,000 to increase viscosity of the bioink. Though, the induced buoyancy was successful in reducing cell sedimentation, optimization was needed considering the fact that higher viscosity is associated with increased buoyancy resulting in higher shear stresses on the cells [75]. In a step to further automate the process for industrial applications, integration of EBB and DBB to produce a skin construct with a transwell system has been reported. After two weeks of *in vitro* culture, the model demonstrated a stratified epidermis layers along with a stable fibroblast-stretched dermal layer as shown in Figure 3B. The fabrication of the construct was found to be 50 times lower in cost using only a tenth of medium compared to conventional culture [76]. Using fibroblasts as cellular component for magnetic 3D biopriniting, toxicity assay of five different drug types- all-trans retinoic acid, dexamethasone, doxorubicin, 5′-fluorouracil, and forskolin has also been performed [77].

5.3 Cornea

Several anterior eye diseases are treated by drugs which are delivered by topical ocular administration. Cornea poses the major barrier for absorption of these drugs. In this drug discovery segment, most transcorneal drug permeation studies are performed in cornea obtained by excision from animals, indicating the strong need for fabrication of corneal *in vitro* models [78]. Cornea consists of a serial arrangement of lamellae of collagen that provides the required shape and strength. The kind of complexity in the structure such as

convoluted patterns, undercuts and concavity, which are represented in the form of points, lines and circles [79], can only be recapitulated by the appropriate use of bioprinting. Recently, a bioprinted corneal model using EBB with collagen/alginate/keratinocyte bioinks has been successfully generated [80]. The corneal equivalent was designed based on images from a Scheimpflug camera and modelled by a finite element method. Bioprinting was then performed leveraging the freeform reversible embedding of suspended hydrogels (FRESH) method for low viscosity bioinks. Alginate and different compositions of collagen were tested for bioprinting, and viability of keratinocytes was demonstrated after a week-long culture. However, a detailed analysis of cell phenotype is to be reported in order to translate the technology into drug discovery applications.

5.4 Intestines

The human intestine is a very important site for drug absorption. The role of early phase screening of new chemical entities in terms of their physicochemical properties for efficient absorption has been emphasized. Using the Organovo 3D NovoGen bioprinter system, an *in vitro* intestinal tissue model containing epithelial cells of human intestinal origin and myofibroblasts was fabricated. The model has been shown to possess polarized columnar epithelium with tight junctions and specialized cells with expression of cytochromes P450 (CYP450). The tissue model demonstrated the barrier function as found in native physiology as well as the presence of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) transporters. The toxicity assessment has been done with the help of indomethacin, an anti-inflammatory drug. The barrier function was assessed by transepithelial electrical resistance (TEER). Combining these results, it could be considered that such a model could be utilized for modelling diseases such as Crohn’s disease and inflammatory bowel disease, and can be a viable method for pre-clinical investigations concerning early biopharmaceutics/toxicity studies [81].

5.5 Vascular networks

Vascular networks are the principal components of organs governing drug distribution. In addition, any vascular injury observed during pre-clinical assessment also contributes to attrition. Therefore *in vitro* vascular models have a predominant role to play in drug discovery. However, fabrication of vascular networks is still a challenge for most techniques [82]. Using bioprinting, several vascular and vascularized tissues have been fabricated [82], which can be applied for drug testing. Bioprinting vascular constructs have involved two principal approaches which are direct fabrication of hollow constructs and indirect bioprinting with fugitive bioinks [83]. Indirect bioprinting is performed by depositing a cylindrical structure followed by bioprinting of bulk hydrogel. The sacrificial ink is then dissolved to leave behind hollow channels. Gelatin and Pluronic have been the most common fugitive inks, with Pluronic preferred for yielding greater spatial resolution, superior construct uniformity and less toxicity [84]. The effects of Rho kinase (ROCK)-inhibitors on angiogenesis and cellular migration have been elucidated in bioprinted constructs of mesenchymal stem cells (MSCs) [85, 86].

5.6 Muscle

The muscle tissues have a very complex structure comprising of various kinds of cell types and different mechanical properties exhibiting contractile force. Various bioprinting approaches have been thus established to closely mimic complex anatomy of muscle. Murine C2C12 cells were mixed with alginate/Pluronic bioink to create muscle constructs *in vitro* using EBB. These 3D constructs have been used in several studies of drug testing based on the measurement of contractile force and the cell viability of above 80% was observed, along with the substantial amount of myogenic differentiation indicated by the positive expression of myogenic markers such as MyoD, Myogenin and alpha-sarcomeric actin [87]. Use of skeletal-muscle derived bioink can provide enhanced potential for potential for fabricating biomimetic muscle constructs [88]. In vitro muscle models will have enhanced applicability for testing of drugs, such as like cardiotoxin, against muscle injuries [89], muscular dystrophy [90] or for evaluating absorption of drugs intended to be developed as intra-muscular injections for depot action [91].

5.7 Cardiac tissue

Bioprinting has shown promise for the development of contractile cardiac tissue with scalable and biologically-mimicked cellular organization. A fabrication strategy has been reported based on EBB by means of integrated tissue-organ printing (ITOP) system [92]. Fibrin bioink suspended with rat heart-origin primary cardiomyocytes has been bioprinted by pressurized air with sacrificial hydrogel and a supporting polymeric frame through a 300 μm nozzle. Consequently, the maturation and development of the cardiac tissue was seen with the help of immunostaining of connexin 43 and α-actinin, indicating tissue formation of required density and the presence of electromechanically-coupled cardiac cells (Figure 3C) [93]. The constructs exhibited contraction within the physiological frequency range (i.e., from around 10 to 2,000 Hz [94]) and patterns after treatment with known cardiac drugs such as epinephrine and carbachol. The safety and effectiveness of drug discovery could be improved if the cells incorporated in the constructs were taken from human. Further, cardiac tissue maturation gained momentum in response to inhibition of Notch signaling pathway [95]. All these results, some illustrated in Figure 3D, demonstrated the feasibility of bioprinting functional cardiac tissues for pharmaceutical discovery applications. In another work, constructs using the NovoGen MMX Bioprinter™, gelatin methacryloyl (GelMA), alginate and a photoinitiator Irgacure 2959 with human umbilical vein endothelial cells (HUVECs) as cell components were also bioprinted and integrated with a microfluidic bioreactor to form cardiac tissue constructs [96].

5.8 Liver

Bioprinting technology has also been used to fabricate liver tissues that stably maintained metabolisms of drugs, glucose and lipid. The bioprinted liver tissue exhibited expression of hepatic drug transporters and metabolic enzymes for several weeks [97]. Glucose production *via* cyclic AMP (cAMP)/protein kinase A signaling was demonstrated, which was suppressed by insulin administration. Bile acid secretion was also observed through a sinusoid-hepatocyte-bile duct route from the bioprinted liver tissue. Cardio- and hepato-toxicity remain the most primary reasons for late stage failures and post-market withdrawal

of various drugs, making the studying of drug toxicity in both organs very important. In another groundbreaking work on a bioprinted liver tissue model as a drug discovery platform, drug-induced liver injury (DILI) against dose response of trovofloxacin and levofloxacin has been investigated [98]. Hepatocytes and non-parenchymal cells were bioprinted and matured into liver tissues as evidenced by endothelial CD31+ expression, desmin-positive, smooth muscle actin-negative dormant stellates and presence of hepatocyte-hepatocyte junctions (Figures 4A1–2). The hepatotoxicity of trovofloxacin was not detected by standard pre-clinical models, while levofloxacin is a non-toxic structural analogue of trovofloxacin. Trovofloxacin induced significant dose-dependent toxicity at the clinically-relevant doses (≤ 4 μM) in the bioprinted liver. Additionally, the experiments contrasted previous studies as trovofloxacin toxicity was observed without any lipopolysaccharide stimulation or the presence of any resident macrophages. Hence, it was demonstrated conclusively that the bioprinted liver tissue could not only effectively model the compound that induced liver fibrosis, but also distinguish related compounds with respect to their distinctive toxicity profiles [99]. A two-compartment bioprinted human liver organoid with centimeter scale were reported by Norona et al. using human hepatocytes, hepatic stellate cells and endothelial cells, which were cultured in a 24-welled Transwell® system [100]. These organoids revealed high cell viability, albumin secretion and CYP3A4 activity for 28 days, and were also able to detect the toxicity of transforming growth factor (TGF)-β1, and certain fibrogenic compounds like methotrexate and thioacetamide. Jeon et al. have fabricated bioprinted liver tissues containing HepG2 cells in alginate and found better growth and expression of liver-specific genes in the bioprinted tissues relative to 2D monolayers [101]. To completely model liver *in vitro*, development of vascularized liver tissue is essential. Bioprinting has been applied to advance the research in this direction. HepG2/C3A cells encapsulated in a GelMA hydrogel were fabricated using sacrificial inks to create hollow microchannels inside liver tissue constructs. HUVECs were subsequently seeded onto the hollow microchannels. As a consequence, permeability of biomolecules into the liver model was down-regulated. Interestingly, it was also seen that the HUVECs seeded in the microchannels increased the viability of HepG2/C3A cells (Figures 4B1–4) [102].

A liver-on-a-chip platform built by interfacing a Novogen MMX bioprinter with a bioreactor has been reported for long term culture of human HepG2/C3A spheroids [103]. Hepatic spheroids were encapsulated within photocrosslinkable GelMA hydrogels and their viability was demonstrated throughout a 30-day culture. Secretion rates of ceruloplasmin, albumin, transferrin, and α-1 anti-trypsin were monitored. The functionality was also confirmed by immunostaining for the hepatocyte markers like tight-junction protein ZO-1, cytokeratin 18, and multidrug resistance protein (MRP)2 bile canalicular protein. Further, treatment with 15 mM acetaminophen was shown to induce a toxic response confirming the utility of this model for drug discovery application. A recent progress has also been made with digital light processing-based bioprinting towards development of bioprinted tissues, resembling the liver modules using human-induced pluripotent stem cells-derived hepatic progenitor cells with adipose- and endothelial-derived stem cells to create a hexagonal architecture [104]. Several specific liver functions such as albumin and urea secretion, and E-cadherin, P450 expressions were shown to be elevated in tri-cultures as compared to 2D or 3D monocultures over multiple weeks after bioprinting. Expression of such markers in the constructs can be

visualized in Figure 4C. In other studies, bioprinted liver micro-tissue using automated direct cell writing with HepG2-embedded in alginate were deposited over a Polydimethylsiloxane (PDMS) microfluidic tissue chamber. Metabolism of 7-ethoxy-4-trifluoromethyl coumarin (EFC) was used as performance indicator for the microfabricated tissue. Similarly, co-extruded construct of HepG2 and human epithelial cells has been investigated with perfusion integration to evaluate the metabolism of amifostine, an anti-radiation pro-drug [105, 106].

5.9 Alveolar model

The respiratory system is the vital point of entry for the different kinds of antigens in form of aerosols, smoke, or other types of harmful particulates in the air. There has been a rapid increase in the respiratory diseases, due to the advent of increasing air pollution and the induced more and more suspension of particulates in the air. Even though it is known that there are certain types of barriers in the epithelial barrier system, antigens can still cross barrier systems and enter into the lungs and cause deadly diseases such as asthma, chronic bronchitis, emphysema, and pneumonia. Hence, it is important to create 3D models to get a detailed view of the antigens passing through these barriers [107]. Horvath et al. utilized a valve-based bioprinting method with type II alveolar epithelial cells and endothelial cells separated by Matrigel™ layer [108]. Though both cell types maintained appreciable cell viability, better barrier function was observed in bioprinted constructs compared to manually seeded controls, and epithelial cells showed greater barrier control over endothelial cells. Intensive studies with such an *in vitro* model helps in understanding cellular responses and the pathway that eventually leads to such diseases. Moreover, several drugs for treatment of respiratory diseases are required to be delivered via intra-nasal or pulmonary route of administration, resulting in the importance of risk assessment with respiratory system. The success with engineering a 3D air-blood tissue barrier is expected to eventually aid the drug discovery process.

5.10 Kidney

In vitro kidney tissue models assume importance in drug discovery and screening as nephrotoxicity is a significant concern with many drugs. Drug development data show that nephrotoxicity is associated with failure rate of 2% of drugs in preclinical phase and as high as 20% of drug attrition in Phase III trials [109]. Proximal tubule of the kidney is associated with most of the drug-induced injuries, and has received attention for fabrication of 3D models. Preservation of apical brush border, polarity, and receptor-mediated transport processes are important aspects for standardization of *in vitro* models. Lewis and co-workers has shown application of EBB method for creating proximal tubules on an *in vitro* model [110], which could be maintained for around two months in a perfusion chip. The bioink was comprised of gelatin/fibrin as the ECM and Pluronic as the sacrificial ink to bioprint the lumen structure. The bioprinted 3D perfusable model showed epithelial-like morphology. Further, the model demonstrated dose-dependent disruption in epithelial barrier on treatment with cyclosporine A, a known nephrotoxic drug. The Organovo company has also provided an ExVive™ kidney tissue, which was comprised of an apical layer of polarized primary renal proximal tubule epithelial cells interfaced with an interstitium of endothelial cells and

primary renal fibroblasts [111]. The model has shown utility for dose-dependent toxicity analysis of several drugs like cisplatin and resazurin.

6. Conclusions

Undoubtedly, major breakthroughs have been achieved in fabrication of 3D models by bioprinting with superior functionalities compared to conventional approaches. Bioprinting techniques have shown that it is much more feasible for high-throughput industrial applications compared to other 3D fabrication methods. Commercial launch of bioprinted liver and kidney tissues are encouraging; however, vascularization and miniaturization of constructs still remains a crucial challenge. On the research frontier, integration of bioreactors along with on-chip perfusion capabilities through microfluidics should be taken up as the next important objective of biofabricating *in vitro* models. In the commercial prospective, bioprinters are expected to become more user-friendly and low-cost with increased user base. In parallel, standardization of the models with respect to identical markers for specific tissue formation should be demonstrated for each future studies. With the rapid advancement in bioprinting technologies, bioprinted models for drug discovery are expected to be soon adopted by the pharmaceutical community.

7. Expert Opinion

Three-dimensional models for *in vitro* drug discovery have achieved significant progress with the rapid evolution of bioprinting technologies [21]. Cell-based assays remain a key bottleneck for drug discovery due to ethics arising with animal rights activities, concerns of inter-species variation and the functional limitations of conventional 2D cultures. Cell-based assays are involved in all major steps of drug discovery such as toxicity assays, lead optimization, and metabolic screening. Along with mammalian cell-laden bioink, bioprinting has shown potential for high-throughput screening with microarrays and bacteria bioprinting for anti-microbial drug discovery [112, 113]. However, a major debate still remains to be settled on how much physiological mimicry is expected from an *in vitro* model for the particular stage of drug discovery. It has been appreciated that, compared to tissue engineering, long-term culture may not be always required from an *in vitro* culture if the objective is to identify drug permeation through biological barriers. Therefore, product specifications and performance should be framed for each established cell-based assay. This exercise will call for a large participation from the pharmaceutical community including biomedical engineering, molecular biology and biomanufacturing scientists. Secondly, a strong correlation is also required to be established between *in vitro* models with *in vivo* counterparts. Correlation would be expected to be established with respect to two major approaches. One aspect concerns the growth and functionality of cells in an *in vitro* model. automated image analysis [114, 115] and non-invasive sensors can be employed for these purposes. For example, an integrated construct printed by microelectromechanical device with functional sensing properties to record drug analysis has been demonstrated, in which a modified Hewlett-Packard thermal inkjet printer was applied to deposit C2C12 cells over cantilevers [116]. Confluent myotubes on cantilevers were observed after seven days, which showed contraction upon excitation with electrical pulses and contraction blockage on treatment with veratridine, an alkaloid drug [117]. On the other hand, the correlation

between *in vitro* and *in vivo* drug performances would also required to be studied. For example, the TEER should be applied to quantitatively define integrity of tight junctions in real-time for several epithelial barrier models like intestinal and respiratory tract, and blood-brain barrier (BBB) before applying them for drug studies [118, 119]. In this regard, artificial intelligence and big data analytics may be promising tools to be employed. Though challenging, an attempt can be made by the food and drug administration (FDA) to allow comparison of experimental results of new 3D bioprinted models retrospectively with all the pre-clinical data or open databases that were administrated by the regulatory agency for accelerating the progress [120, 121, 122, 123]. Indeed, each *in vitro* model should also be approved by FDA; however, being advantageous due to automation and repeatability, bioprinted constructs are expected to obtain FDA approvals.

In the bioprinting domain, vascularization of the constructs has to be addressed in the near future. The blood vessel is the connective tissue that is responsible for distribution of drugs in organs, and hence tissue construct without vascularization is considered lack of biomimicry. Currently, most researchers usually create macro-size channels using fugitive inks (i.e., gelatin, Pluronic, agarose) as sacrificial templates in bulk hydrogels [54] followed by seeding endothelial cells (such as human umbilical vein endothelial cells (HUVECs)) onto the created channel network, and lining these channels with endothelial cells through shear stress induction via perfusion. Bioprinted constructs with such an architecture show excellent connectivity of macro-channels with slow degradation rate enabling endothelial cells to grow and allow vascularization [124]; however, limited work has been done to incorporate micro-size capillaries branching from such macro-channels. The incorporation of micro-capillarization is important to diffuse drug compounds into the surrounding functional tissue (in the form of organoids or cells laden in hydrogels) during drug screening. Thus, bioprinted macro-vessels and biologically-derived micro-size capillaries should be interconnected in order to mimic the native tissue anatomically, mechanically and biologically.

Fortunately, much progress has already been made with several seminal works, and the next milestone will be the commercialization. Bioprinting is expected to reduce the cost of drug discovery while improving precision of the process. Although bioprinted models may not be necessitated for all step of drug discovery, the efforts to demonstrate their capability to complement existing techniques have brought forth fruitful outcomes.

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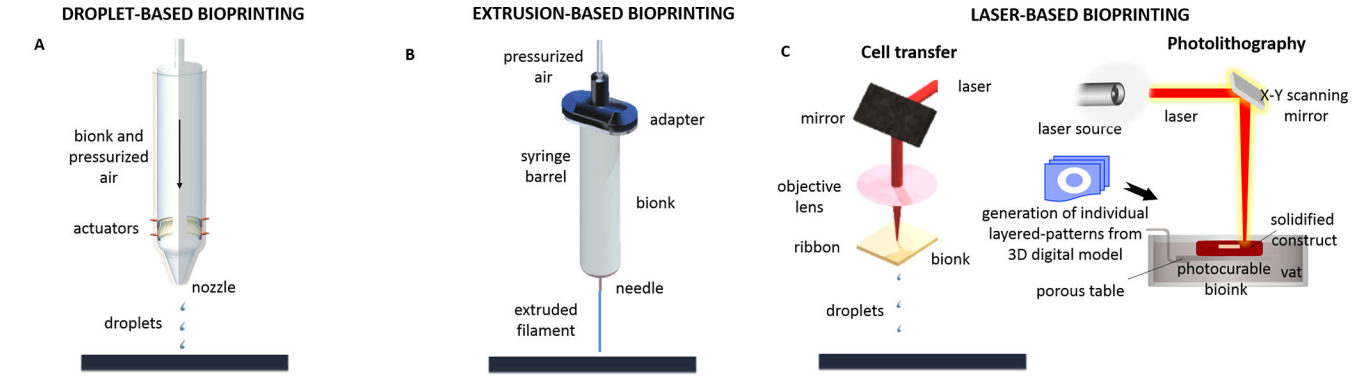
Article highlights:

- Bioprinting offers advantage of higher accuracy, resolution and precision in comparison to the conventional methods for fabricating 3D *in vitro* models.
- 3D models of various tissues have been bioprinted, and especially, the commercial launch of bioprinted liver and kidney tissues are promising.
- Vascularization of constructs still remains a crucial challenge.
- Standardization of the models with respect to identical markers for specific tissue formation should be demonstrated for future studies.
- Bioprinted models for drug discovery are expected to be soon adopted by the pharmaceutical community with the rapid advancement in bioprinting technologies.



Comparison of strengths and limitations	Strengths	Limitations
	Compatibility with lower viscosities in range of 3.5-12 mPa/s	Nozzle clogging in high cell densities
	High resolution (1-300 picoliter/droplet)	No uniformity in droplet size
	Compatibility with various biological factors such as living cells, DNA, biochemical etc.	Cross-contamination when bioprinting of multiple bioink solutions takes place simultaneously
	High-throughput	
	Commercially available	
Bioprinted tissue models	Tumor, skin and vascular networks models	

Comparison of strengths and limitations	Strengths	Limitations
	Compatibility with viscosities in range of 30- 6x107 mPa/s	High cell damage owing to high shear stress
	Enables scaffold-free bioprinting including tissue spheroids and tissue strands	Low-resolution
	Suitable to prepare organ-on-a-chip models for drug testing	Not practical for high-throughout bioprinting of tissue models
	Facilitates vascularization using fugitive ink	Limited to control cell-cell and cell-matrix interactions
	Commercially available	
Bioprinted tissue models	Tumor, skin, cornea, vascular networks, muscle, cardiac, liver, alveolar and kidney models	

Comparison of strengths and limitations	Strengths	Limitations
	Compatibility with viscosities 1-300 mPa/s	Labor intensive and time-consuming process
	Nozzle-free	Hard to bioprint heterocellular models
	High resolution	High cost
	Less cell damage	
Bioprinted tissue models	Tumor, skin, vascular networks and liver models	

Figure 1.

Schematic illustration of **(A)** droplet-, **(B)** extrusion-, and **(C)** laser-based bioprinting modalities.

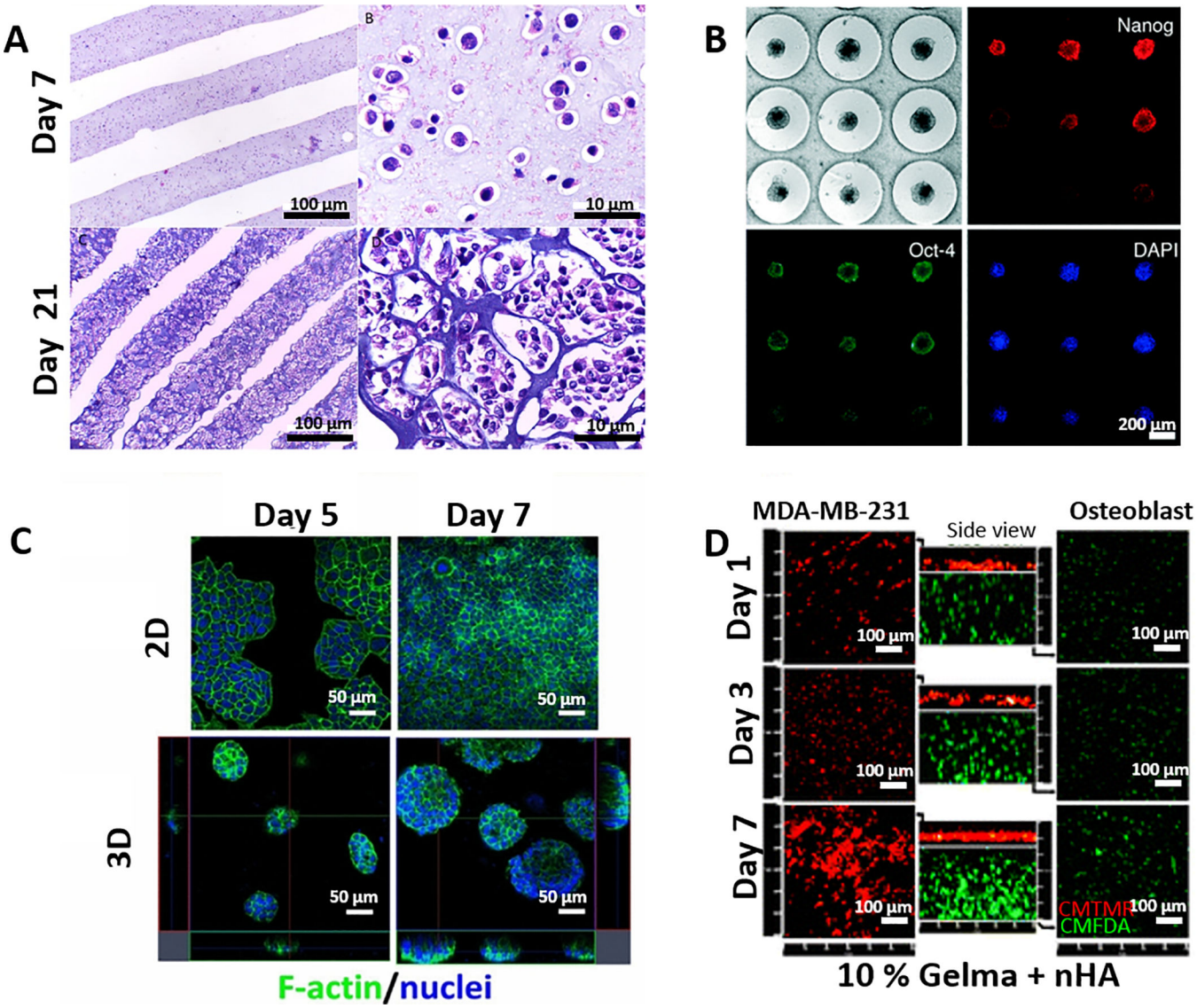


Figure 2.

Bioprinted tumor models **(A)** Hematoxylin-eosin (H&E) staining of 3D bioprinted glioma cells cultured *in vitro* at Day 7 and Day 21 (reproduced from Ref. [64] with permission of IOP publishing); **(B)** an optical image and Nanog, Oct-4 and DAPI immunofluorescent images showing pluripotency, non-differentiated and nucleous of cells at Day 3 (reproduced from. [65] with permission of the Royal Society of Chemistry); **(C)** 2D and 3D cultures of Hela cells in gelatin/alginate/fibrinogen blend at Day 5 and Day 7 (reproduced from Ref. [69] with permission of the American Chemical Society); **(D)** confocal microscopy images of co-culture of MDA-MB-231 and human osteoblast cells in 10 % Gelma and nanocrystalline hydroxyapatite (nHA) (reproduced from Ref. [68] with permission of IOP publishing).

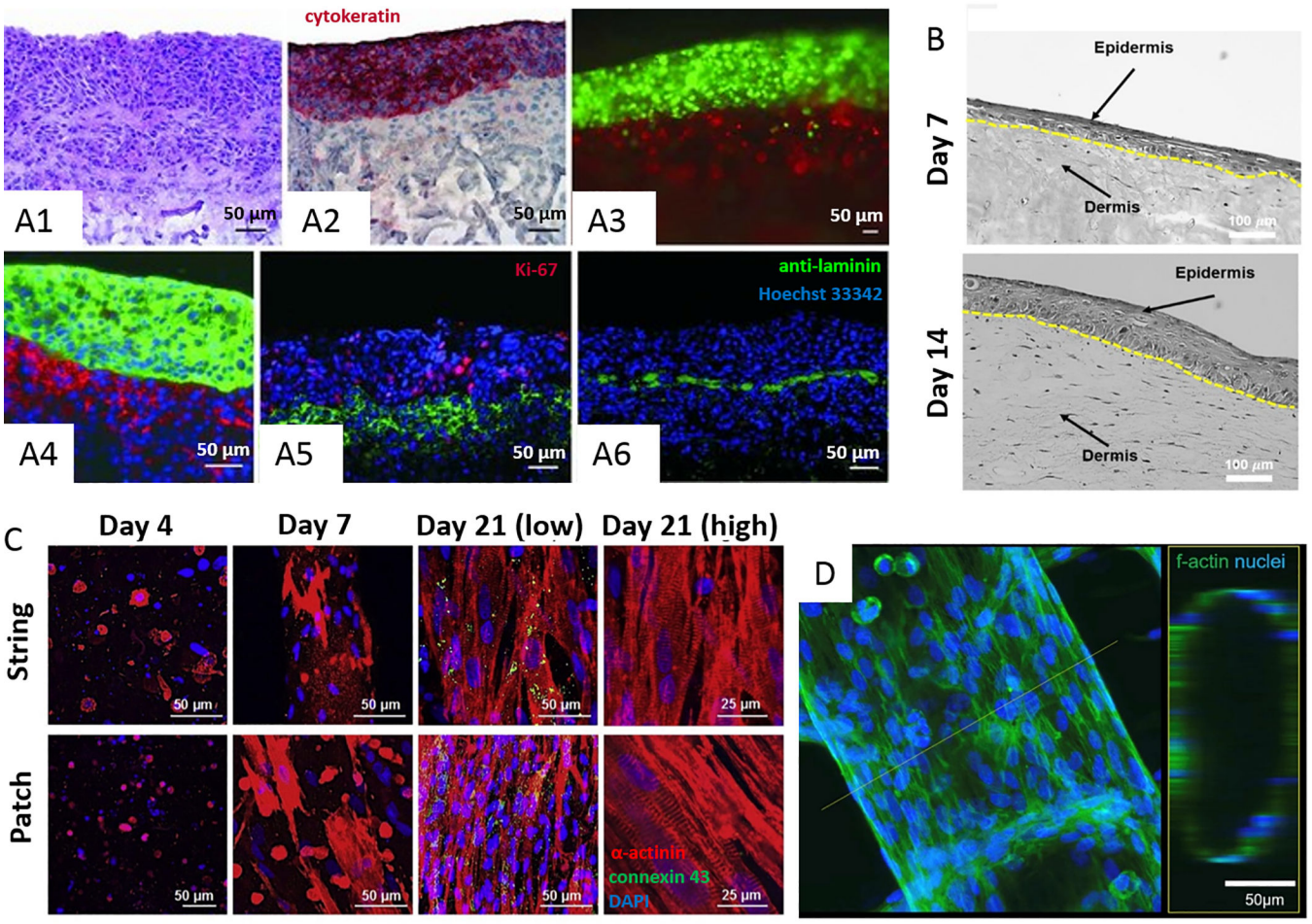


Figure 3.

Bioprinted skin and heart models **(A1)** a H&E staining image of bioprinted murine fibroblast (NIH 3T3) and human keratinocyte (HaCaT) construct on Matriderm™, **(A2)** immunoperoxidase staining of cytokeratin demonstrates keratinocytes where all nuclei stained with Hoechst 33342 (blue), **(A3-A4)** fluorescence images showing distribution of fibroblasts (red) and keratinocytes (green) where cell nuclei are stained in Hoechst 3342 (blue), **(A5-A6)** while Ki-67 (red) staining represents the proliferating cell nuclei, anti-laminin (green) staining demonstrates the basement membrane (reproduced from [73] with permission of John Wiley and Sons); **(B)** H&E stained samples of skin model at Day 7 and 14 (yellow dash line represents the interface between epidermis and dermis) (reproduced from Ref. [76] with permission of IOP publishing); **(C)** immunofluorescent images of a string and patch forms of the bioprinted cardiac tissues express actinin (red), connexin (green) and cell nuclei (blue) at Day 4, Day 7, Day 21 (with low and high magnification) (reproduced from [93] with permission of Elsevier); **(D)** projection and 3D rendering confocal images showing the distribution of encapsulated HUVECs inside the bioprinted microfibers in the endothelium at Day 14 (reproduced from [96] with permission of Elsevier).

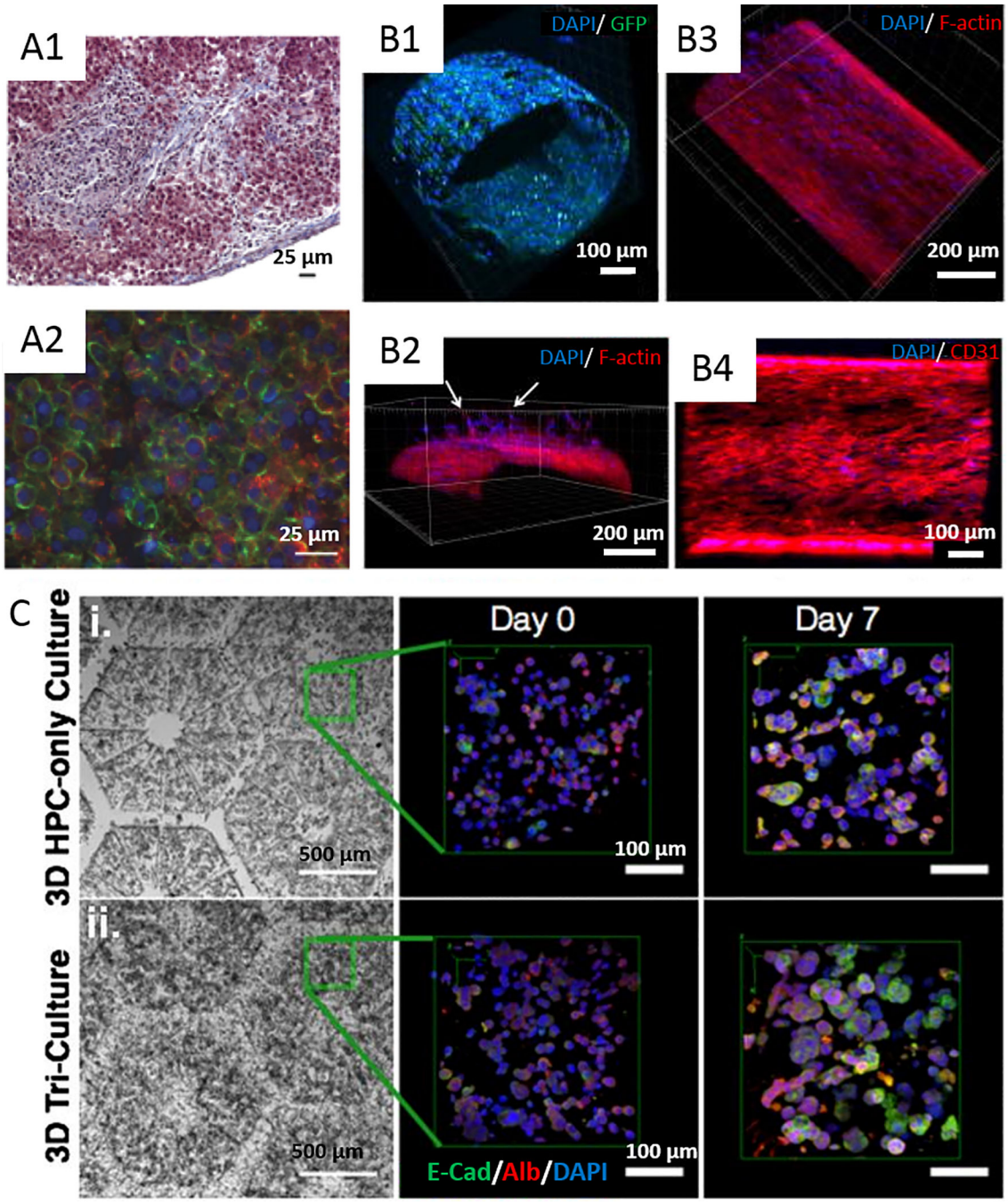


Figure 4.

Bioprinted liver models **(A1)** Masson`s trichrome staining of 3D liver tissue showing ECM deposition, **(A2)** IHC staining represents E-cadherin (green) and albumin (red) expressions (reproduced from [99] with permission of PLOS One); **(B1)** DAPI and GFP-HUVEC layers of 3D vascularized liver tissue model **(B2-B3)** top and cross-sectional views of in GelMa hydrogel after 2 days, **(B4)** DAPI and CD31 staining of bioconstruct from top view (reproduced from [102] with permission of AIP Publishing); **(C)** optical and confocal images showing E-cadherin, albumin, and DAPI staining at Day 0 and Day 7 (reproduced

from [104] with permission of National Academy of Sciences of the United States of America (NAS)).

Table 1.

Bioprinted organ/tissue models for drug discovery purpose

Organ/Tissue Model	Bioprinting modality	Bioink	Cell Types	Drugs Tested	References
Glioma	EBB	Alginate/gelatin/ fibrinogen	Breast cancer cells	Temozolomide	[64]
	DBB	Matrigel	OVCAR-5 cells and MRC-5 fibroblasts	Prolactin, estrodine	[66]
3D neoplastic tissues	EBB	Alginate/gelatin/ fibrinogen	HeLa cells	Paclitaxel	[68]
Skin	LBB	Collagen/ Matrigel™	HaCaT keratinocytes/NIH3T3 fibroblasts	all-trans retinoic acid, dexamethasone, doxorubicin, 5′-fluorouracil, and forskolin	[73, 77]
Cornea	EBB	Collagen/ alginate	keratinocytes	-	[80]
Intestine	EBB	Scaffold-free	Epithelial cells of human intestinal origin and myofibroblasts	Indomethacin	[81]
Vascular network	Indirect bioprinting	Gelatin/ Pluronic	MSCs	Rho-kinase inhibitors	[84, 85,86]
Muscle	EBB	Alginate/ Pluronic	C2C12 cells	Cardiotoxin	[87, 89]
Heart	EBB	Fibrin	Rat heart origin primary cardiomyocytes	Epinephrine and carbachol	[93]
Liver	Sacrificial bioprinting	GelMA	-HepG2/C3A cells, HUVECs	-Acetaminophen, Trovofloxacin,	[99, 102]
	DBB	Alginate	-HepG2 and human epithelial cells	-levofloxacin -Amifostine	[105, 106]
Alveolar model	Valve-based printing	Matrigel™	Type-II alveolar epithelial cells and endothelial cells		[108]
Kidney	EBB	Gelatin/fibrin as ECM and Pluronic as sacrificial ink	Proximal tubule epithelial cells	Cyclosporine A, cisplatin, resazurin	[110]