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Review article

3D bioprinting for drug discovery and development in pharmaceutics

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ABSTRACT

Successful launch of a commercial drug requires significant investment of time and financial resources wherein late-stage failures become a reason for catastrophic failures in drug discovery. This calls for infusing constant innovations in technologies, which can give reliable prediction of efficacy, and more importantly, toxicology of the compound early in the drug discovery process before clinical trials. Though computational advances have resulted in more rationale *in silico* designing, *in vitro* experimental studies still require gaining industry confidence and improving *in vitro-in vivo* correlations. In this quest, due to their ability to mimic the spatial and chemical attributes of native tissues, three-dimensional (3D) tissue models have now proven to provide better results for drug screening compared to traditional two-dimensional (2D) models. However, *in vitro* fabrication of living tissues has remained a bottleneck in realizing the full potential of 3D models. Recent advances in bioprinting provide a valuable tool to fabricate biomimetic constructs, which can be applied in different stages of drug discovery research. This paper presents the first comprehensive review of bioprinting techniques applied for fabrication of 3D tissue models for pharmaceutical studies. A comparative evaluation of different bioprinting modalities is performed to assess the performance and ability of fabricating 3D tissue models for pharmaceutical use as the critical selection of bioprinting modalities indeed plays a crucial role in efficacy and toxicology testing of drugs and accelerates the drug development cycle. In addition, limitations with current tissue models are discussed thoroughly and future prospects of the role of bioprinting in pharmaceutics are provided to the reader.

Statement of Significance

Present advances in tissue biofabrication have crucial role to play in aiding the pharmaceutical development process achieve its objectives. Advent of three-dimensional (3D) models, in particular, is viewed with immense interest by the community due to their ability to mimic *in vivo* hierarchical tissue architecture and heterogeneous composition. Successful realization of 3D models will not only provide greater *in vitro-in vivo* correlation compared to the two-dimensional (2D) models, but also eventually replace pre-clinical animal testing, which has their own shortcomings. Amongst all fabrication techniques, bioprinting- comprising all the different modalities (extrusion-, droplet- and laser-based bioprinting), is emerging as the most viable fabrication technique to create the biomimetic tissue constructs. Notwithstanding the interest in bioprinting by the pharmaceutical development researchers, it can be seen that there is a limited availability of comparative literature which can guide the proper selection of bioprinting processes and associated considerations, such as the bioink selection for a particular pharmaceutical study. Thus, this work emphasizes these aspects of bioprinting and presents them in perspective of differential requirements of different pharmaceutical studies like *in vitro* predictive toxicology, high-throughput screening, drug delivery and tissue-specific efficacies. Moreover, since bioprinting

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techniques are mostly applied in regenerative medicine and tissue engineering, a comparative analysis of similarities and differences are also expounded to help researchers make informed decisions based on contemporary literature.

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1. Background: current challenges in drug discovery

The process of launching a new drug is generally comprised of two major stages: drug discovery in the preclinical phase and drug development in the clinical phase [1]. The former involves identification of suitable molecular candidates from a large number of possible compounds that will react with a biochemical target. Multiple steps and multiple cycles of testing are required to study the interaction of the compound with the target. Further development addresses the validation of the safety and efficacy of the candidates through phase I–III and phase IV trials, before and aftermarket approval, respectively. Integrating drug delivery strategies with drug discovery and development processes is considered imperative early in the pipeline by using models that will simulate their future [2]. Although there have been many advancements in the pharmaceutical industry, a high attrition rate remains as the main reason for the tremendous time and cost incurred in pharmaceutical research [3].

During the drug discovery period, one to three candidates are selected from thousands of compounds while only one new molecular entity (NME) ultimately gets launched from almost twenty-four candidates in development [4]. Since clinical development itself contributes to almost 60% of the total cost as well as the majority of the discovery cycle time, reducing the attrition rate of drug candidates in clinical development (mainly Phase II and Phase III) presents the greatest challenge and opportunity for pharmaceutical research and development (R&D) [4]. To improve R&D productivity, a paradigm called "quick-win, fast-fail" is gaining wider implementation to reduce the costs and time of development cycle, as depicted in Fig. 1, by minimizing technical uncertainty in early development. Additionally, an analysis of combined 2000–2010 data obtained from four principal pharmaceutical companies revealed that of the 605 terminated compounds from 808 proposed compounds, non-clinical toxicology was the highest cause of attrition, accounting for 240 (40%) of the failures [3]. The continuing high rate of non-clinical toxicology failures may be attributed to mechanisms that are harder to extrapolate from *in vitro* data and call for more predictive toxicity assays. Development of novel approaches to increase the value of *in vitro* studies can decrease the pre-human trial costs and enhance the early identification of toxicity of a compound significantly.

In drug discovery, *in vitro* efficacy assays are implemented to screen leads from hits, while *in vitro* toxicity assays are implemented to exclude compounds with unacceptable toxicities and

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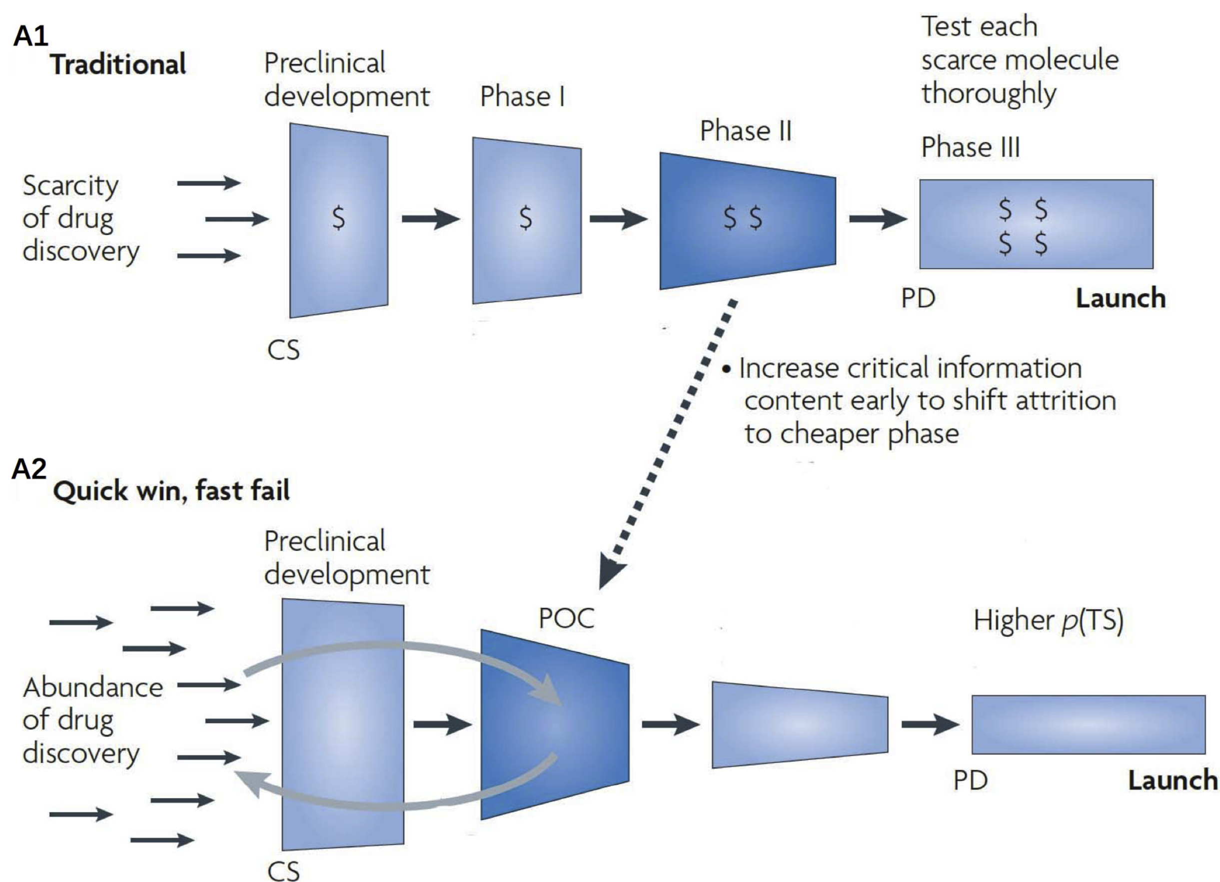

Fig. 1. (A1) The traditional paradigm of drug development and an alternative development paradigm referred to as quick win, fast fail (A2). In this alternative, technical uncertainty is intentionally decreased before the expensive later development stages (Phase II and Phase III) through the establishment of a proof-of-concept (POC). This results in a reduced number of new molecular entities (NMEs) advancing into Phase II and III, but those that do advance have a higher probability of technical success (*p*(TS)) and launch. CS: candidate selection; PD: product decision (reproduced/adapted with permission from [4].

help in optimization of less toxic lead. These *in vitro* assays reduce the range of compounds to be tested for the subsequent process. Although these assays are simplified and rapid, a non-mimetic *in vitro* assay may fail to identify valuable compounds and provide false leads for succeeding stage, which undoubtedly increases attrition and cost. By far, most of the conventional *in vitro* drug discovery assays are performed in two-dimensional (2D) monolayer cell culture systems, which do not simulate the exact *in vivo* conditions for accurate evaluation of cellular responses to drugs [5], as detailed in the next section. In 2D cell culture systems, drug effects are often altered and some non-predicted or misleading results are obtained. Therefore, it is imperative to develop *in vitro* cell-based systems, which would be able to accurately predict efficacy and safety *in vivo*. Developing technologies to fabricate three-dimensional (3D) constructs may provide potential solutions to address the problems of drug discovery.

2. 3D models in pharmaceutics

The majority of existing cell-based drug assays utilizes the 2D monolayer culture method in which cells attach on flat or rigid substrate comprised of glass or polystyrene. Cells proliferate at a rapid rate to a sheet-like confluency. This time-tested 2D monolayer culture method proved to be a valuable tool for various cell-based studies; however, *in vivo* cells reside in a 3D environment surrounded by other cells and the extracellular matrix (ECM), thus 2D culture systems fail to faithfully recreate an *in vivo* cell environment [5]. Therefore, in order to overcome these limitations and improve the results of cell assays, two major 3D culture systems have been developed in the past decade including 3D scaffold-based [6,7] and scaffold-free [8] systems. Scaffold-based models are generated by stacking cell sheets, seeding individual or aggregated cells on a prefabricated scaffold or by embedding them in ECM-like matrixes before polymerization or solidification [9]. Scaffolds function to support cell adherence, growth, differentiation and migration. Commonly used scaffold materials include decellularized extracellular matrix (dECM) and a myriad of natural or synthetic polymers, exhibiting a wide array of mechanical, biocompatibility and toxicity properties [9,10]. In scaffold-free systems, cells are allowed to proliferate without use of any exogenous structures. In this modality, cellular self-assembly through cadherin-mediated adhesion leads to formation of the 3D constructs [11,12].

A number of methods have been developed to fabricate *in vitro* 3D tissue models, including hydrogel culture [13,14], and bioprinting [15], hanging drop method [16], microwell-based method [17], micro-patterned matrices [18], microfluidics-based method [19], acoustic-based method [20], magnetic force [21]. Among the 3D tissue models, spheroids are the basic and most commonly utilized model. Spheroids can be formed using hanging drop method [16], rotating wall vessels [22], spontaneous formation [23], or surface modification [24] method. Additionally, 3D models can also be micro-fabricated by high-throughput microarrays using various methods including micro-well, surface patterning, microfluidics and cell printing. So far, a large number of 3D constructs or microarrays of physiological organs such as skin [25,26], heart

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[27,28], kidney [29], liver [30,31], lung [32] and disease models such as pulmonary edema [33], and tumors [34] have been engineered for acute or chronic drug assays and high-throughput screening (HTS) of lead compounds for pharmaceutical and cosmetics development [35].

Considering the numerous advantages, a large number of 3D culture systems, including microarrays, with various ECMs compositions have been fabricated for different pharmaceutical applications such as for targeted drug delivery [36], drug efficacy or toxicity studies [31,37], and HTS [38]. However, given the complexity and specificity of 3D cellular niches, creation of biomimetic constructs with appropriate topological and mechanical simulation is still a significant fabrication challenge. Also, due to the complexity of the specific setup required for fabrication of 3D models, the 3D tissue culture models may not all be ideally suited for routine drug testing by the pharmaceutical industry. At present, only a few human 3D co-culture models are available for use in industrial drug testing [39–42]. There is still an imperative need to fully exploit the value of culturing cells in 3D models.

Despite their benefits, 3D models still suffer from several limitations [43,44]. One of the major limitations arises with the incorporation of multiple cell types resulting in more heterogeneity and data variability compared to 2D models. Moreover, 3D models lack standardization in size and volume [45]. Second, some naturally-derived ECM matrices exhibit significant batch-to-batch variations in biological properties yielding inconsistent experimental results [43]. Although synthetic matrices show more consistent performances, low bio-compatibility limits their utilization. Some studies using standardized microfluidics-based systems or microarray-based high-throughput systems have reported irregularities of specific ECM components and natural scaffolds [46]. Third, 3D culture is more expensive and laborious for large-scale studies or high-throughput assays than traditional 2D culture. However, the foremost limitation of 3D culture is the lack of vascularization, which plays a prominent role in determining cell behavior due to limited transportation of oxygen, nutrients, drugs and intercellular factors throughout the 3D structure [47]. Finally, functional 3D tissue models lack the ability to form hierarchical, ordered architectures and structures that recapitulate the organization of native tissues from *in vitro* cultured cells.

Recent studies indicate that cell–cell and cell-ECM communications are key factors indistinguishing between 2D and 3D systems as well as between different 3D systems [48]. To overcome the shortcomings of 3D models, these cellular communications should be established in a biomimetic manner, providing human cells with the specific niche, supplying appropriate topological cues and stimulating mechanical stresses as *in vivo*. Among various methods developed to engineer 3D culture systems, bioprinting offers great potential to mimic the *in vivo* cell–cell and cell-matrix communications.

3. Bioprinting in pharmaceutics

Bioprinting has gained tremendous interest worldwide in the past few years, making a revolutionary impact on biomedical sciences [49–51]. Bioprinting is defined as the synchronous positioning of biomaterials and living cells in a prescribed layer-by-layer stacking organization to fabricate 3D constructs [52]. It offers great precision in the spatial and temporal placement of living cells, proteins, DNA, drugs, growth factors and other bioactive substances in order to guide tissue generation and formation. The biomaterial solutions used in bioprinting are referred to as "bioink." The four main types of bioink materials employed in bioprinting technology include cell aggregates (tissue spheroids, cell pellet and tissue strands), hydrogels, micro-carriers, and decellularized matrix components [53]. Like other biofabrication techniques, bioprinting can also be performed in two ways, namely (i) scaffold-based and (ii) scaffold-free bioprinting [54]. In the former, cells are bioprinted within exogenous biomaterial matrix such as dECM or hydrogels. In the latter, cell pellets or pre-aggregated cells are spatially confined in printed or in mold structures to allow their self-assembly. Typical steps for bioprinting process include medical imaging and processing for computer-aided design models, the selection of bioink, bioprinting and *in vitro* or *in vivo* use of bioprinted constructs [55].

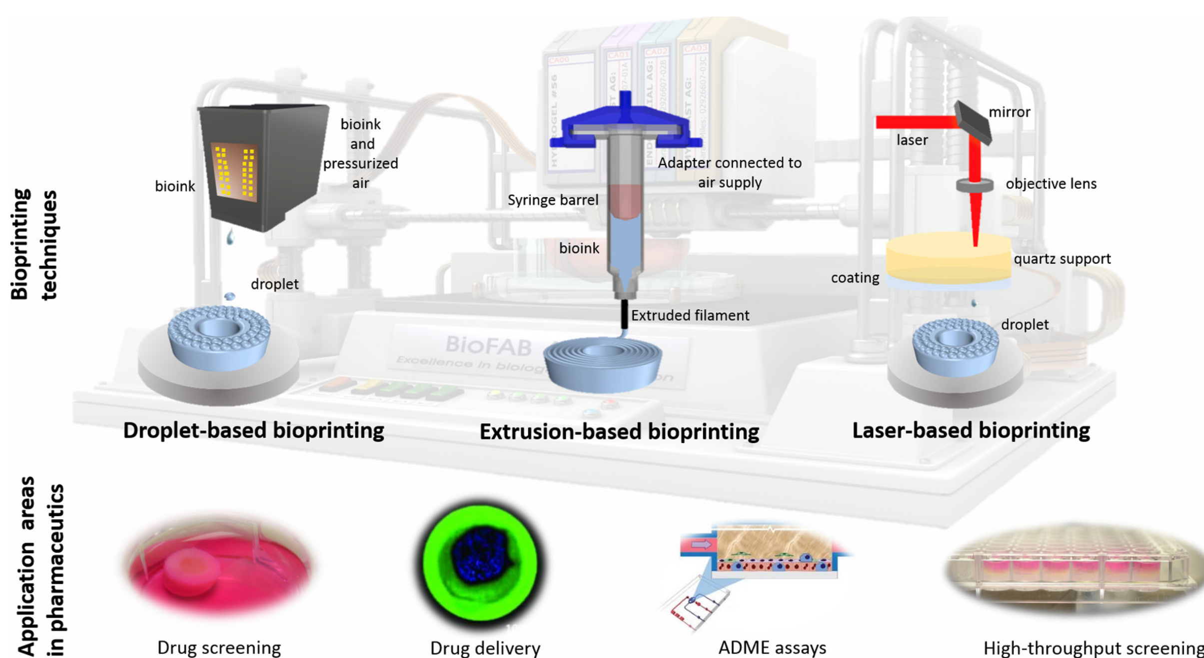

Fig. 2. Modalities of bioprinting processes and their utilization in tissue fabrication for drug discovery and development for pharmaceutics (drug screening image reproduced/adapted with permission from [161], and ADME assay image reproduced/adapted from [162]).

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3.1. Modalities of bioprinting

Based on the method of deposition and patterning of biological materials, there are three main types of bioprinting modalities namely droplet-, extrusion-, and laser-based bioprinting. Fig. 2 shows the bioprinting modalities and their utilization in fabrication of tissues for drug discovery and development.

3.1.1. Droplet-based bioprinting (DBB)

Droplet-based bioprinting was introduced in the late 1980 s providing a foundation for the development of future bioprinting technologies [56]. Inkjet printers (also known as drop-on-demand printers) are one of the most commonly used type of droplet-based bioprinters [57]. Compared to the commercially available ink-based printers, the bioinks in the cartridges consist of biomaterials such as living cells, DNA, RNA, bio-chemicals; instead of paper, the deposition surfaces are electronically controlled 3D stages or even *in situ* surfaces for *in vivo* bioprinting (see Fig. 2A). Inkjet-based bioprinters use thermal, piezo, or acoustic forces to eject the droplets onto the supporting substrate. The deposited droplets are solidified to form 3D structures by using different chemical or physical crosslinking mechanisms such as crosslinking agents, pH and ultraviolent (UV) radiation. The minimum cell viability in DBB is generally greater than 70%, where it can even exceeds 90% for processes such as electrohydrodynamic jetting and acoustic and micro-valve bioprinting [58]. Advantages of inkjet bioprinting include low cost, high speed, high resolution, compatibility with many biological materials, and the potential to print different concentrations of biological materials by altering droplet densities or sizes. At the same time, disadvantages of inkjet printing usually include non-uniformity of droplet size and incidents of nozzle-clogging by high cell density bioinks. Droplet size (within the range of <1 pl to >300 pl in volume), patterns (single drops in which one or two cells are contained in lines $\sim$50 μm wide) and deposition rate (1–10,000 droplets per second) can be electronically controlled [58]. Although improvements have been made, simultaneous bioprinting of multiple cell and material types still remains a challenge.

3.1.2. Extrusion-based bioprinting (EBB)

Extrusion-based bioprinting technology is based on the extrusion of continuous filaments of bioinks and has been used extensively for bioprinting live cells [55,59]. It is a combination of material-handling and liquid-dispensing systems with an automated three-axis robotic system for extrusion (see Fig. 2B). The most common methods to extrude biological materials are based on pneumatic or mechanical (piston- or screw-assisted) dispensing techniques. A wide range of drive forces enables deposition of an array of biological materials with different rheological properties. Most cell support materials, in the form of hydrogels solutions with viscosities ranging from 30 mPa·s to $>6 \times 10^7$ mPa·s, have been shown to be compatible with this system [60]. Another advantage of EBB is the ability to deposit cells in very high densities such as spheroids. The most common method of bioprinting tissue spheroids is mechanical-driven extrusion systems. However, the high dispensing pressure and shear stresses inflicted on cells in viscous fluids decreases the viability of cells deposited by extrusion. This drawback can be mitigated somewhat but can result in a loss of bioprinting resolution and speed [61]. Use of bioinks with better biocompatibility, such as dynamically crosslinked hydrogels, and design of single-phase, dual-phase and functionally-graded tissue constructs have improved cell viability and function in EBB. Additionally, optimization and improvements in nozzle, syringe or motor-control systems are also pursued to reduce bioprinting times and allow deposition of multiple materials simultaneously [62].

3.1.3. Laser-based bioprinting (LBB)

Laser-based bioprinting modalities are also named as laser-assisted bioprinting (LAB) or laser direct-write (LDW). A laser-based system was first introduced in 1999 to process 2-D cell patterning [63]. Although less popular than DBB or EBB, LAB is now increasingly used for tissue engineering. Laser-assisted printers consist of a pulsed laser beam, a focusing system, a donor slide containing two layers (energy absorbing layer and biological material layer), and a collector substrate slide (see Fig. 2C). The laser is focused on absorbing substrates (e.g., gold or titanium) to create a bubble, subsequently generating shock waves that propel the cell-containing materials from the donor slide onto the collector slide. The resolution of LAB depends on many factors including laser energy, pulse frequency, thickness and viscosity of biological material layer, air gap between donor and collector slide, along with wettability of the substrate slide. Advantages of LAB include elimination of clogging issues since no nozzle is used, compatibility with viscosities ranging from 1–300 mPa·s, negligible effect on cell viability and function, and deposition of cells in densities of 10^8 cells/ml with a resolution of one cell per drop. LAB also has some disadvantages which include high cost, time-consuming preparation, and difficulty in accurate targeting and deposition cells. Some of these challenges are being addressed by developing cell-recognition scanning technology such as matrix-assisted pulsed laser evaporation-direct writing (MAPLE-DW) [64], and by applying a high concentration of cells and other means [65].

Advances in technical developments are continuously being investigated and applied to overcome the key hurdles of bioprinting technologies such as resolution, speed, cell viability, cell densities, and proper crosslinking methods. Based on the progress in the three major bioprinting methods, technological modification that facilitate simultaneous bioprinting of multiple cells and material types appear imminent to enable fabrication of the variously complicated 3D tissue models.

3.2. Advantages of bioprinting over conventional biofabrication method

Compared to conventional biofabrication methods (i.e., micromolding, freeze drying, solvent casting/particulate leaching), bioprinting has several advantages such as higher precision and accuracy, high resolution in cell deposition, high-throughput capability, feasibility of co-culturing cells in a spatial organization, and low chances of cross-contamination [66]. First, bioprinting enables fabrication of anatomically- correct 3D tissue constructs using medical image data from magnetic resonance imaging (MRI) or computed tomography (CT). Second, bioprinting allows fabrication of porous structures with controlled architecture, providing adequate/requisite space for cell proliferation, ensuring exchange of nutrients and oxygen for living cells and imposing proper mechanical requirements. Third, bioprinting is suitable for co-culturing of multiple cell types in a spatially organized manner [67]. One or more types of cells can be bioprinted separately or in combination with spatial control mimicking *in vivo* organization. Fourth, bioprinting facilitates the precise biomimetic patterning of cells and biological structures. Fifth, bioprinting has the ability to integrate vascularization within engineered tissues, which is necessary for maintaining cell viability in constructs of a size that exceeds critical limits [68,69]. Sixth, controlled delivery of growth factors and genes is easy to achieve through bioprinting, which is important when maintaining engineered constructs for long culture periods [70]. Seventh, bioprinting allows high-throughput fabrication of tissue models. Compared to other technologies (such as soft lithography, surface patterning, and microfluidic-based manipulation), this powerful technology is a promising method for advancing physiologically-relevant tissue models and microarrays for biome-

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dicine and pharmaceutical applications. Bioprinting technology has been used for the fabrication of a wide variety of 3D tissues including blood vessel [71,72], bone [73], tooth [74], lung [75], kidney [76], liver [77], cardiac [78], cartilage [79], skin [80], heart valve [81], brain [82], nervous [83], pancreas [84], retina [85], tendon [86], trachea vascular [87], composite tissues [88], and cervical tumor models [89]. Bioprinted tissue models for pharmaceutical use are not subject to the rigorous safety, ethical, and regulatory issues that are required for 3D bioprinted organ substitutes for transplantation. Commercial products such as bioprinted liver models and kidney arrays have therefore been viewed with interest by the pharmaceutical industries and these models have provided superior results in preliminary tests [90]. A detailed list of tissue models bioprinted for pharmaceutical applications is summarized in Table 1.

4. Designing the components of bioprinting for fabrication of tissue models

4.1. Bioink selection

Bioinks are usually comprised of cells, polymers and additives to form a cell-suspending solution. As mentioned, application of bioinks with or without polymers as the scaffold is the key factor that discriminates scaffold-based from scaffold-free bioprinting. The choice of scaffold-based or scaffold-free bioprinting is the first consideration of bioprinting in pharmaceutics. These two approaches provide different properties for specific applications. Scaffold-based bioprinting is preferred by many researchers due to better commercial availability, practicality and affordability. However, cells are immobilized within hydrogels and do not spread, stretch and migrate arbitrarily [79]. In addition, parenchymal cells in 3D bioprinted scaffolds exhibit reduced viability, phenotypic stability and functionality after long-term culture [54]. On the other hand, in scaffold-free bioprinting, cell seeded at high densities secrete their own ECM, mature and self-assemble as in native tissues. Without the confinement of hydrogel, cells can interact with each other to a greater extent compared to scaffold-based mobilization and interaction. These properties enable generation of tissues with close biomimicry and preserve the cell phenotype and functionality for longer times [54].

Depending upon the specific objective of the pharmaceutical (drug discovery/drug development/drug delivery etc.) studies, the most suitable bioprinting strategy should be selected. For example, in drug delivery research, scaffold-based bioprinting is preferable since drug release can be modulated by degradation of hydrogels; conversely, hydrogels can be considered for controlled delivery studies [91]. For efficacy testing of drugs, selection is determined by the biomechanical or biochemical cues needed for the tests. For example, hypoxic cores in microtissues fabricated by scaffold-free methods more closely reflect the tumor environment, so scaffold-free bioprinting has become increasingly relevant in cancer research and screening of novel *anti*-cancer drugs [10,92]. Stem cells have the ability to differentiate into multiple cell types. ECM compositions and properties including growth and cell signaling factors lead to different lineage commitments of stem cells [15]. Scaffold-based bioprinting is preferable for bioprinting of stem cells. Cell-based microarrays should also be microfabricated in high-throughput using scaffold-free bioprinting (usually by inkjet bioprinting) since the viscous hydrogel can easily clog nozzles. Scaffold-based bioprinting can be considered using lower viscosity hydrogels or with a mechanical controlled-valve extrusion method through which more viscous spheroids can be extruded [93]. LBB systems can also be used to deposit cell-embedded hydrogels within arrays in controlled manner [94]. When co-cultured cells are investigated, scaffold-free bioprinting rather than scaffold-based bioprinting is recommended since hydrogels may lead to immobilization of cells which confines cell–cell interplay.

For scaffold-based bioprinting, hydrogels should be selected based on cell specific requirements. The choice of hydrogel is determined by the bioprintability (viscosity, shear-thinning property), biocompatibility (cell binding potential, non-toxicity including degradation products), mechanical properties (stiffness, elasticity, strength), structure (pore size, permeability), and cross-linking mechanism (physical, chemical) [95,96]. Unlike tissue regeneration applications, the selection of hydrogels for pharmaceutics mainly considers the printability, biocompatibility, cross-linking mode and the target tissue characters. Natural hydrogels (such as alginate, agar, collagen, gelatin, fibrinogen, hyaluronic acid, Matrigel™) are more preferable than synthetic hydrogels such as poly (ethylene glycol) (PEG), poly(ethylene oxide)-poly (propylene oxide)- poly(ethylene oxide) (Pluronic), and protein hydrogels (collagen, gelatin, fibrin) are the most biocompatible. Modifying synthetic hydrogels with arginine-aspartic acid-glutamic acid (RGD) peptides or other molecules can further enhance biocompatibility. Chemical crosslinking tends to form stronger bonds than physical crosslinking, so the latter is preferable where high permeability in bioprinted constructs is necessary for oxygen and nutrition transport. Additionally, hydrogels should be selected based on characteristics of the target tissue. For example, bone cells may be embedded in collagen type I since it is the main component of ECM in bone, while chondrocytes may be bioprinted within collagen type II, and fibrin is preferable for printing of endothelial cells. Matrigel™ is derived from the ECM of Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and is preferable for growing many types of mammalian cells including cardiac and cancer cells [97].

4.2. Bioprinting process selection

Since scaffold-based bioprinting is conveniently performed using EBB, DBB and LBB, studies of controlled drug delivery can be accomplished with all bioprinting modalities. DBB is useful for studies involving drug administration at gradient concentrations [98,99]. Likewise, for delivery of genetic elements such as DNA or oligonucleotides, thermal-based inkjet bioprinting is preferable as this method ensures high efficiency of gene transfection by forming transient membrane pores without causing significant cell damage [100–102]. Regardless of efficiency or toxicity assays, drug testing with low-throughput can be employed using EBB approaches in order to bioprint tissue models encapsulating individual cells or tissue spheroids. HTSs were mostly scaffold-free and undertaken with DBB, however, HTS with hydrogel encapsulation of cells can be also realized with EBB [93] or LBB approaches [94]. Overall, for pharmaceutical applications, DBB has been the commonly used approach, and LBB is highly promising as an alternative as it does not pose any major problems related to nozzle clogging and stress-induced cell damage. However, LBB is an expensive and sophisticated method requiring further technological development before gaining popularity in the pharmaceutical industry.

Though large numbers of cells are needed for scale-up bioprinted constructs for tissue engineering, such constructs are not necessary for pharmaceutical applications. A detailed comparison of bioprinting used for tissue engineering and pharmaceutics applications is presented in Table 2. Minimal construct size should be considered wherever possible. HTS droplets can be bioprinted with the resolution of one cell per dot on microarray micro-wells or chips. Scaffold-free spheroids with a diameter of approximately 100 μm can be bioprinted spatially on cast molds, which can

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Table 1
Bioprinted tissue models for pharmaceutical applications.

Category	Bioprinting Modality	Cell Type	Bioink Type	Drug Type	Culture Time	Drug Effects	3D *vs* 2D	References
Drug testing	EBB	Hela cells (cervical tumor cells)	Gelatin/alginate/fibrinogen	Paclitaxel	Cultured for 5 days and 3 more days with drugs	Cell morphology	More chemo-resistance in 3D than in 2D	[89]
	EBB; printer consisting of a three-axis motion control stage	Human mesenchymal stem cells (hMSCs); Mouse endothelial cells (MS1); mouse fibroblast L929 cell line	HA-PEG-based hydrogel with cells was patterned into the shape of a microfiber in the 3D Matrigel casted in PDMS cylindrical mold	ROCK inhibitor Y27632 and *anti*-cadherin antibody	Constructs were cultured up to 16 days. Treated by drugs for 7 days	Cell patterning analysis	.	[119]
	EBB; printer consisting of a three-axis motion control stage	hMSCs	Cells-laden HA-MMP sensitive peptides solutions in cylinder-shape matrigel matrix	ROCK inhibitor Y27632 and *anti*-cadherin antibody	3 days *in vitro*; 1 week for implantation. Drugs mixed with medium	Morphology		[120]
	Thermal inkjet; Hewlett-Packard (HP) Deskjet 500	Mouse myoblasts C2C12	Suspending C2C12 cells in phosphate-buffered saline (PBS) solutions	Veratridine (VTD), an alkaloid neurotoxin	Cultivate 4 days; treated with drug and investigated immediately	Detect the myotube contractile by laser		[118]
	Thermal inkjet modified HP Deskjet 6500 with 50 firing chambers	Human articular chondrocytes	PEGDMA	FGF-2/TGF-β	4 weeks	Chondrogenic properties		[121]
Drug delivery	EBB integrated with multi-nozzle electrospinning		Bioprinting gelatin/sodium alginate struts as microstructures for scaffolds	Gentamycin sulfate (GS), desferoxamine (DFO)	25 days	Electrospinning polyvinyl alcohol (PVA)-GS or PVA-DFO nanofibers were deposited on hydrogel scaffolds	Drugs can be released temporally and spatially	[117]
	EBB	Human endothelial progenitor cells (EPCs)	Matrix/alginate scaffold for cell; gelatin microparticles (GMPs) for drugs	VEGF	The controlled release of VEGF from GMPs was continuous for 3 weeks	Potential of forming vascular network *in vitro* and *in vivo*	Controlled releasing of VEGF from GMPs are suitable for vascularization	[70]
	Customized micro-extrusion bioprinter	Primary rat bone mesenchymal stem cell (BMSC)	Methacrylamide gelatin scaffolds	BMP2-collagen binding domain (CBD) recombinant protein	14 days	CBD-BMP2 collagen microfibers were bioprinted within BMSCs-laden methacrylamide gelatin scaffolds	Controlled release of CBD-BMP2 but not BMP2 could be achieved, which promotes osteogenic differentiation of BMSCs	[116]
	Extrusion-based BioScaffolder system	Osteosarcoma MG-63 cells; primary MSCs from Dutch milk goats	Alginate with 3% concentration	Plasmid pcDNA3.1/rhBMP-2(pBMP-2)	7 or 14 days for *in vitro* culture; 6 weeks for implantation	pBMP-2-added alginate was bioprinted with cells seeded thereafter	High transfection efficiency of plasmid and BMP-2 release were shown with higher osteogenic differentiation	[113]
	DBB with a 2D inkjet printer with a piezoelectric nozzle (MicroFab, Technologies, Plano, TX)	Mouse mesenchymal fibroblasts C3H10T1/2	After coating fibrin or serum on polystyrene fibers and before seeding cells, where growth factors were overprinted at different doses	BMP-2, FGF-2	24 h	Overprinting single or combinatory heparin-growth factors droplets on substrates	Printed FGF-2 and BMP-2 patterns dose-dependently promoted tenocyte and osteoblast fates, respectively	[98,99,115]
		C2C12 myogenic precursor cells	Substrates coating with fibrin or nitrocellulose	BMP-2, IGF-II, FGF-2	Up to 10 days		Factors deposited spatially in linear or exponential gradient	
	DBB with a modified HP DeskJet 500 with HP 26 Black ink cartridge	3T3 fibroblasts	phosphate-buffered saline (PBS) solutions	Fluorescently labeled g-actin monomers	3 h after bioprinting		The thermal inkjet printing process was shown to temporarily disrupt the cell	[102]
	Modified Hewlett Packard (HP) DeskJet 692C and 550C printers aswell as HP 51626a and 51629a ink cartridges	Porcine aortic endothelial [PAE] cells	Suspension buffer	The plasmids pmaxGFP or pIRES-VEGF-GFP	2 days for *in vitro* and 1 week for *in vivo*		membranes in minutes to create transient pores allowing the DNA plasmid entry with no damage to cells	[100]

(*continued on next page*)

Table 1 (*continued*)

Category	Bioprinting Modality	Cell Type	Bioink Type	Drug Type	Culture Time	Drug Effects	3D *vs* 2D	References
	Modified HP Deskjet 500 thermal inkjet printer and HP 51626A black ink cartridge	Chinese hamster ovary (CHO) cells	DPBS	Green fluores-GFP DNA plasmid	24 h			[101]
Drug delivery, high throughput microarray	2D DBB. an array of 12 piezoelectric ejectors	Human cell line HEL92.1.7	Cell media	DMSO; human genomic DNA, BSA-conjugated oligonucleotides		Arrays of genomic DNA, BCA-conjugated oligonucleotides have been high throughput ejected	Efficiency, accuracy and throughput of array printing have been validated	[125]
High throughput screening model	The BioFactory bioprinter with CF300 N valve-based print head can jet or contact dispense with additionally mounted needle tip (regenHU Ltd., Switzerland)	Human alveolar epithelial type II cell line A549; EA.hy926 hybrid human cell line derived by fusing human umbilical vein endothelial cells (HUVEC) with A549	Matrigel™		3 days	ECM was printed by contact dispensing, while cells were printed with jetting. Human air-blood tissue barrier analogue composed layers of EA.hy926, Matrigel™ basement membrane and A549	Bioprinted barriers showed similar permeability or barrier function compared to those made using a manual approach. Bioprinting enabled reproducible thinner and homogeneous cell layers	[75]
	DBB with a 2D inkjet printer (Hewlett Packard, 8112A)	Mouse ESCs (mESCs, line E14)	Cell media		24–96 h	Droplets of cell-medium suspension were bioprinted onto the lid of a Petri dish and were hung up for 24 h to allow for EB aggregation	Bioprinted embryoid bodies (EBs) were shown uniformity in size and larger size EBs compared to EBs by manual pipetting approach	[130]
	EBB	Undifferentiated mES cell line	Gelatin and alginate		Up to 7 days	3d scaffold embedding cells.	Generating pluripotent, high-throughput, highly uniform and size controllable EBs	[93]
	DBB with a mechanical valve ejector	Primary bladder SMCs from Sprague Dawley rat	Type I bovine collagen		Up to 14 days	This high throughput system printed tissue constructs from microdroplets	Providing uniform cell seeding, 3D cell patterning layer by layer, and high viability over long-term culture	[128]
High throughput screening model for skin	DBB with dispenser controlled by eight electromechanical valves	Fibroblasts (HFF-1) and keratinocyte (HaCaT)	Collagen hydrogel precursor (rat tail, type I)		Up to 3 weeks	Layer-by-layer bioprinting of collagen matrix, keratinocytes, and fibroblasts to construct the dermal and epidermal compartments in skin	3D bioprinted skin tissue was more biologically and morphologically representative of *in vivo* human skin tissue than those made using conventional methods	[80,163]
High throughput and array model	LBB	Human adipose-derived stem cells (ASCs) and endothelial colony-forming cells (ECFCs)	Hyaluronic acid-fibrinogen crosslinked with thrombin		Up to 2 weeks		Bioprinted 3D array is freely scalable. Direct cell–cell contacts trigger the development of stable vascular-like networks in VEGF-free medium	[94]
	DBB with a custom-designed pressure assisted valve and a solenoid valve ejector	Periodontal ligament stem cells (PDLSCs)	Gelatin methacrylate (GelMA) and PEG dimethacrylate		Up to 5 days		Cell viability and spreading area decreased along with increasing the ratio of PEG to GelMA.	[132]
	DBB with a valve-based droplet ejector	Human MSCs	Methacrylated gelatin (GelMA)	BMP-2, TGF-B, BMP-2 + TGF	Up to 36 days		Multiphasic anisotropy of the incorporated biochemical factors was shown after patterning	[131]

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Table 2
Comparison of bioprinting for pharmaceutics vs tissue engineering.

	Bioprinting for pharmaceutics	Bioprinting for tissue engineering
Purpose	Screening of drug efficacies, toxicities or metabolisms, drug delivery	Regenerative medicine, transplantation
Approach	DBB > EBB > LBB	EBB > DBB > LBB
Strategy	Scaffold-free > scaffold-based	Scaffold-based > scaffold-free
Cells types and density	Primary cells > cell lines > stem cells, Low cell density	Stem cells > primary cells > cell lines, Medium to high cell density
Co-culture ability	Necessary	Necessary
Bioink type	Natural, synthetic (modified)	Natural, synthetic
Crosslinking	Physical	Physical, chemical, enzymatic
Substrate/platform	Chip/multi-wells plate	Slide glass or in-situ
Bioprinting Time	Short to Medium	Long for maturity and self-assembly
Throughput level	High to medium	Medium-to-low
Microarray Requirement	Necessary	Unnecessary
Product Type	Organoids	Tissue constructs
Required properties of the bioprinted constructs	Permeability Biocompatibility High-throughput Structural integrity	Mechanical and structural integrity Biocompatibility Permeability Biodegradable Low-immunogenicity Scalability
Scale (size)	Scale-down	Scale-up
Cross-contamination	Possible	N/A
Vascularization or innervation	Unnecessary	Necessary
Bioreactor requirements	Unnecessary for acute assays; necessary for chronic assays with individual separation	Single integrated system
Biosensors applied	Multiplex (optical, electric, etc)	Optical, harmless

mature into organoids for HTS or drug testing. Encapsulation of cells should be appropriately scaled for bioprinting within micro-wells. It may also be noted here that for quick assays, such as acute toxicity studies and evaluation of chemotherapeutic agents, manual pipetting is used for renewal of media or introduction of new drug/doses. However, for chronic studies, individual or paralleled bioreactors with automated perfusion system should often be necessary for the bioprinted constructs [103,104].

4.3. Co-culture of heterocellular models

The ability to perform controlled co-culturing of multiple cell types is an unparalleled advantage of bioprinting over other 3D fabrication methods. For *in vitro* bioprinting of tissue constructs, cell–cell interactions can significantly alter physiological functioning as well as responses to pharmaceuticals. For example, in a 3D chitosan nanofiber scaffold-based culture system, primary rat neonatal ventricular cardiomyocytes co-cultured with fibroblasts resulted in polarized cardiomyocyte morphology, synchronized contraction and retention of morphology and function in long-term cultures. Meanwhile, cardiomyocytes monocultures or co-cultures with endothelial cells resulted in loss of cardiomyocyte polarity and isolated contractions [105]. Drug-induced liver injury is often caused by interactions between multiple cell types (such as hepatocytes, Kupffers cells, stellate cells, etc.) and mediated by release of inflammatory mediators or reactive oxygen species [106,107]. To fabricate a 3D tissue construct, functional cells should be bioprinted with a proportion of supporting cells chosen based on the targeted pharmaceutical requirements. Bioprinted tumor cells are often co-cultured with endothelial cells to investigate the migration, metastasis and angiogenesis processes in tumor constructs [108]. Fibroblasts are usually used as surrogate of stromal cells for maintaining stable architecture of 3D constructs [109,110]. Bioprinted tumor cell-immune cell co-culture systems are used to represent the immunological responses of tumor cells which is important for investigating biological therapy or immunotolerance of *anti*-tumor drugs [111]. Different types of cells mixed in certain proportions can be bioprinted in conjunction with hydrogel matrices, if a specific matrix is deemed essential. Different cells can also be bioprinted separately using different nozzles in DBB or EBB [112].

5. Exploratory applications of bioprinting in pharmaceutics

5.1. Drug delivery

As mentioned before, thermal inkjet printing has been used for gene transfection. The thermal inkjet printing process temporarily disrupts the cell membranes to create transient pores allowing the entry of DNA plasmids. This technique is relatively benign as the pores close in time to maintain cell viability. Shear stress and heat causes temporary microdisruption of the cell membrane, allowing cells and gene plasmid to pass through the ink channels of the printhead during the bioprinting process. Plasmids were thus transferred into the cells and subsequently, droplets containing genetically-modified cells were spatially delivered to target sites within a 2D or 3D matrix [100–102]. Extrusion-based bioprinting constructs are also utilized for transfection of plasmid DNA into cells. Bioprinted constructs made of alginate loaded with multipotent stromal cells (MSCs) and calcium phosphate particles were extruded either in a porous or a solid shape [113]. The non-viral plasmid DNA encoding bone morphogenic protein-2 (BMP-2) was found to be efficiently transfected into cells. After *in vitro* culture for 14 days, bioprinted constructs containing BMP-2 plasmids showed higher osteogenic differentiation as demonstrated by higher ALP activity and osteocalcin (OCN) than the non-transfected control as shown in Fig. 3.

Campbell's group developed an inkjet-based overlapping methodology to create an immobilized "solid-phase" pattern of unmodified growth factors on natural biological material substrates [99,114,115]. For example, a piezoelectric drop-on-demand inkjet printhead was utilized to spatially deposit single or combination of heparin-binding growth factors like BMP-2 and insulin-like growth factor-II (IGF-II) in different concentration patterns on fibrin-coated coverslips. Different growth factor patterns were created by overprints either with linear gradient in different slopes or exponential gradient. Additionally, the overlapping bio-

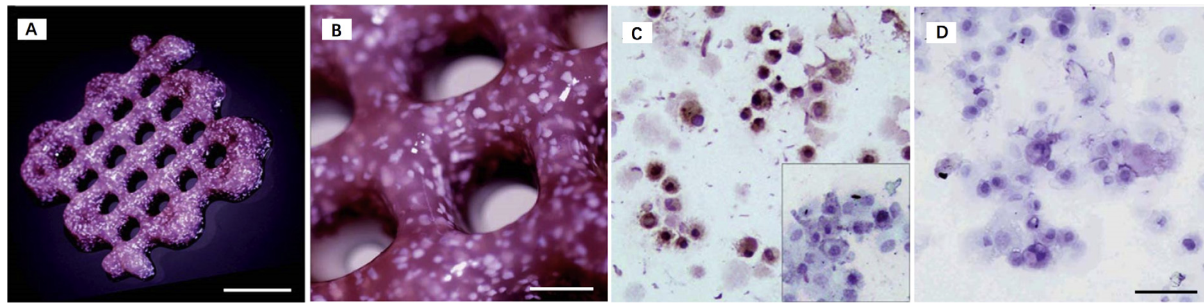

Fig. 3. (A and B) Bioprinted porous constructs containing MSCs, ceramic particles with plasmid DNA encoding BMP-2 (scale bar: A 500 μm, B 100 μm), (C) Osteogenic differentiation in bioprinted constructs was shown as osteocalcin immunocytochemistry on cytospins of cells after dissolution of the porous construct with pBMP-2 (70% positive cells), inset: isotype matched control antibody staining, (D) control, scale bar 100 μm (reproduced/adapted with permission from [113]).

printing method was used to create a combinatorial square pattern consisting of various surface concentrations of BMP-2 and fibroblast growth factor-2 (FGF-2), leading to cell differentiation. Since the method is programmable, the gradient shapes are easily created. This technique has also been applied for basic studies in cell biology as well as in studies with patterning and delivery of growth factors [98].

Controlled delivery of growth factors is considered to be an important factor in generating physiologically-relevant tissue models. Some investigations have presented convincing efforts towards addressing this challenge. For example, the means to prolong activity of vascular endothelial growth factor (VEGF) at the targeted location was realized when gelatin microparticles (GMPs) encapsulating VEGF were formulated and added to 3D bioprinted human endothelial progenitor cells (hEPCs)-Matrigel™/alginate scaffolds [70]. Continuous release of VEGF from GMPs was observed for three weeks *in vitro*. Bioprinted constructs were implanted subcutaneously in nude mice for *in vivo* analysis of vessel formation. Histological results revealed that slow release of VEGF from GMPs lead to much more vessels formation than fast release of VEGF by adding VEGF in media. However, it should also be noted that delivery of growth factors with specified differentiation potential could not be controlled very effectively via the reported vehicles of polymer-microspheres or water–oil emulsions [116]. Thus, TKKTLRT, a short collagen binding domain (CBD)-derived from mammalian collagenase, was used to make the growth factors which specifically bound to collagen. Dai's group used a custom-made bioprinter to print bone mesenchymal stem cell (BMSC)-laden methacrylamide gelatin scaffolds combined with CBD-BMP-2 collagen microfibers. Unlike the rapid release behavior of BMP-2, controlled release of CBD-BMP-2 was achieved using the collagen microfibers. CBD-BMP-2 collagen microfibers were also found to promote osteogenic differentiation of BMSCs-laden methacrylamide gelatin scaffolds as confirmed by the increased expression of osteogenic markers such as ALP/BSP/OCN, COLLA1 and Alizarin [116].

To deliver multiple biomolecules with diverse spatial–temporal release profiles, composite scaffolds were fabricated using bioprinting as an integrated system. Gelatin/sodium alginate strut microstructures were deposited by an extrusion-based bioprinter. Gentamycin sulfate (GS) was incorporated into electrospun polyvinyl alcohol (PVA) nanofibers, while desferoxamine (DFO) was incorporated into coaxial electrospun core (PVA-DFO)/shell (polycaprolactone) nanofibers [117]. For temporal release of drugs, it was seen that GS release was faster than DFO during the early period while the release of DFO was sustained for longer periods. Further, the vertically graded porous architecture in sodium alginate/gelatin scaffolds enabled the release of DFO in a gradient mode demonstrating that the developed method using composite scaffolds helped in achieving various release profiles independently for each drug by manipulating the struts and nanofibers [117].

5.2. Drug screening for efficacy or toxicity testing

Several bioprinted tissues with different cells, ECMs and architectures in low-to-high throughput have been fabricated to explore their potential to act as *in vitro* models for testing of drug efficacy, toxicity, chemotherapy or chemoresistance. Nevertheless, only a very few constructs have been commercially implemented for testing drug efficacy and toxicity. An ideal *in vitro* pharmacological model for drug testing should combine biomimetic architecture with measurable endpoints to quantify drug efficacy. A bioprinted integrated biological microelectro-mechanical system (Bio-MEMS) device has demonstrated promising potential to serve as functional biosensor for efficient analysis of drugs [118]. In that study, a Hewlett-Packard (HP) Deskjet 500 thermal inkjet printer was modified to precisely print and align C2C12 cells onto cantilevers (biopaper) at 300 dpi (85 lm) resolution. Cells aligned very close to each other and formed confluent myotubes on cantilevers on the fourth day post bioprinting, while non-bioprinted cells were randomly distributed on cantilevers without formation of myofibers after seven days. Further, myotubes also showed contraction upon excitation with an electrical pulse. Myotubes were then treated with veratridine (VTD), an alkaloid neurotoxin which acts on nerve and muscle membranes by sustained opening of the voltage-gated sodium channels rendering the cells unable to contract. The myofibers regained the ability of synchronous contraction upon electric stimulus after removal of VTD. The bioprinted Bio-MEMS devices with simultaneous, spontaneous chemical stimulation demonstrated that this technique had the potential to incorporate functional biosensors, motors and actuators as needed.

The utility of bioprinting to satisfy the diverse needs of pharmacological testing models is also reinforced by examining the versatility of the construct properties containing different growth factors or agents which trigger specific signaling pathways. For example, a bioprinter was used to fabricate cell-laden hyaluronic acid (HA)-PEG microfibers onto cylindrical 3D matrigel matrix [119]. Different patterns of cell aggregation and migrations were observed with different cell types. While mouse fibroblast L929 showed a tendency to spread in a single-cell distribution pattern, hMSCs aggregated and formed cell clusters. The aggregation of hMSCs was attenuated by treatment with a Rho-associated protein kinase (ROCK) inhibitor Y27632 and cadherin antibody. Angiogenic-specific gene CD105 activity was found to be down regulated when exposed to Y27632, a phenomenon not observed with treatment by *anti*-cadherin. These results show that cell patterns in a 3D matrix, as demonstrated by cell aggregation and migration over time, were dependent on the cell types and intercellular interactions. Additionally, hMSCs in 3D matrices showed higher expression of angiogenic markers such as CD31 or CD105 compared to cells in 2D [119]. Recently, the same group also showed that ROCK inhibition enhanced *in vitro* angiogenic sprouting and vascularization in rat tissue by enhancing the secretion of

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VEGF or epidermal growth factor (EGF) in bioprinted 3D constructs of matrix metalloproteinase (MMP)-sensitive peptide [120]. In order to test the applicability of bioprinting in cartilage tissue development and examine the influence of differentiation factors on chondrogenicity, primary human articular chondrocytes suspending in hydrogel poly(ethylene glycol) dimethacrylate (PEGDMA) were bioprinted with a modified HP Deskjet 500 thermal inkjet printer [121]. Superior chondrogenic characteristics were found with FGF-2/TGF-β (transforming growth factor-β) co-treatment comparing with single factor, which was attributed to synergistic stimulation of cell growth and differentiation.

3D neoplastic tissues have been bioprinted to test their sensitivity as well as resistance to chemotherapy (anticancer) drugs. Several bioprinted 3D tumor cell constructs have been shown to simulate *in vivo* tumor responses to drugs. For example, Sun et al. fabricated a 3D bioprinted *in vitro* cervical tumor model (see Fig. 4A1–A4). In their work, HeLa cells encapsulated alginate/gelatin/fibrinogen hydrogels were extruded with 90% viability. Cells within 3D scaffolds apparently formed spheroids. Compared to 2D planar samples, 3D printed constructs were found to be more chemoresistant to paclitaxel as evidenced by assessment of cell morphology, metabolic activity and MMP activity [89]. Scaffold-free human breast cancer cells were bioprinted to test the chemotherapeutic effects of tamoxifen with Organovo's NovoGen Bioprinting™ platform in which cancer cells were surrounded by a biomimetic ECM consisting of MSC-derived mammary fibroblasts, endothelial and adipose cells [122]. Histological analysis showed that bioprinted tissues formed a clear compartmentalization of adipose, stromal and epithelial components with formation of micro-capillaries. The tissues maintained viability for two weeks *in vitro*. The chemotherapeutic effects were assessed by adenosine triphosphate (ATP) luciferase assay and the results showed that isolated 2D cancer cells were more susceptible to tamoxifen-induced toxicity than the cells growing in 3D bioprinted constructs. Organovo also engineered a 3D-bioprinted 'exVive3D' liver tissue models to screen drugs for liver toxicity (see Fig. 4B1 and B2) [90,123]. A human liver cell pellet, consisting of primary hepatocytes, stellate cells and endothelial cells was bioprinted on a temporary mold structure with hexagonal shaped building units to form a scaffold-free liver tissue. After an incubation time of 60 h, microcapillaries were formed within tissue. The bioprinted tissues produced liver proteins such as albumin and fibrinogen while expressing hepatic enzyme markers and preserving cell viability for more than 42 days. Two drugs, Levofloxacin and Trovafloxacin [107], were used to validate the amenability of this bioprinted liver for toxicity assays (see Fig. 4B3 and B4); while one was a drug that was commercially available for years and thus considered safe, the second drug tested had earlier failed in phase III clinical trial due to liver toxicity. However, no hepatoxicity was evident for either of the two drugs throughout the development phase including preclinical *in vitro* and *in vivo* cellular toxicity assays. Organovo's bioprinted 3D system clearly demonstrated toxicity of the failed drug and safety of the commercial drug.

5.3. Microarrays and High-throughput screening

Amid earlier reports of low productivity, HTS technologies were adopted by the pharmaceutical industry in the 1980s in an effort to increase the number of lead molecules entering the discovery/development pipeline [124]. Bioprinting offers a fabrication technique, which is amenable to high-throughput manipulation with the advantages of high yield, less time consumption, and the convenience culture medium replenishment. Thus, a number of arrays have been bioprinted for HTS that enables parallel investigation of efficacy or toxicity of hundreds of drugs. Among all approaches, DBB is the earliest and the most utilized approach in fabricating microarrays for HTS [58]. In one of the first studies, BCA-conjugated oligonucleotide arrays were printed in high-throughput without compromising the bioprinting accuracy [125]. Recently, a HP model 5360 compact disc printer was modified to make an inkjet-based bioprinter with a resolution of picoliter per droplet and used to micro-engineer a high-throughput miniature drug screening platform [126]. A schematic of the experiment is depicted in Fig. 5A1. Three layers were printed successively onto the same location on a glass slide. The first layer consisted of a blend solution of agar and bacteria, the second layer 0.3% alginate, and the third layer $CaCl_2$ and three selected antibiotics. The results demonstrated that cell viability, functionality and *anti*-bacterial effects of antibiotics in the inkjet bioprinted samples were similar to those in the micro-pipetted samples (see Fig. 5A2 and A3).

In order to overcome some of the drawbacks of inkjet bioprinting including cell damage and ink clogging, droplets can be ejected by acoustic- or micro-valve-based methods. Demirci's group devel-

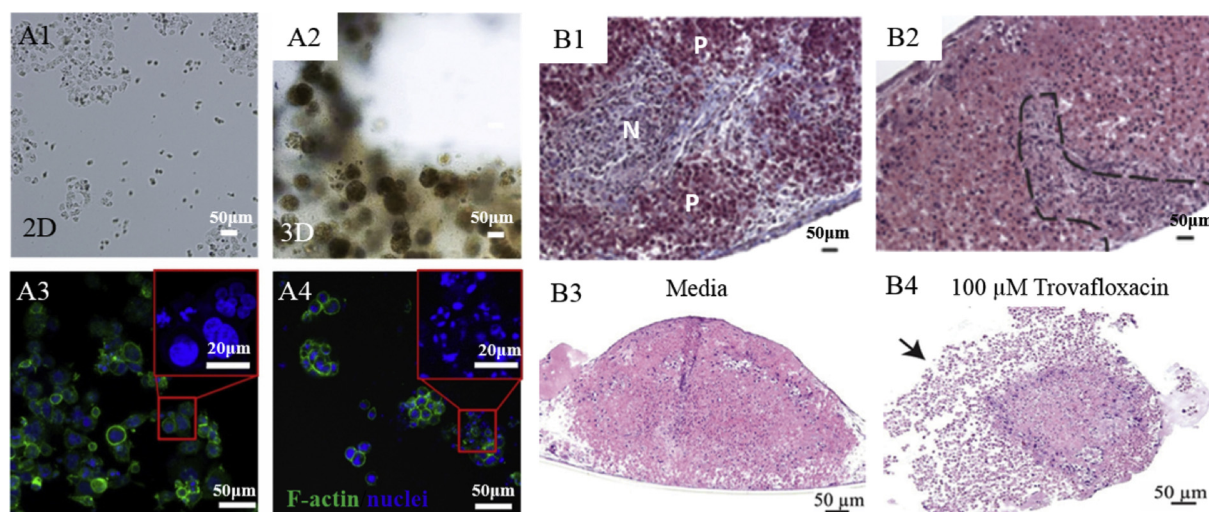

Fig. 4. Chemoresistance of Hela cells after paclitaxel treatment in (A1-A3) 2D planar culture and (A2–A4) 3D hydrogel construct (reproduced/adapted with permission from [89]; (B1) H&E staining showing parenchymal (P) and nonparenchymal (N) regions (image courtesy of Organovo) and H&E staining of a tissue cross-section; compartmentalization between the parenchymal and non-parenchymal fractions can be readily visualized (dashed line) (B3) H&E staining of an untreated (media) and (B4) 100 μM Trovafloxacin-treated 3D liver tissue (reproduced/adapted with permission from [107]).

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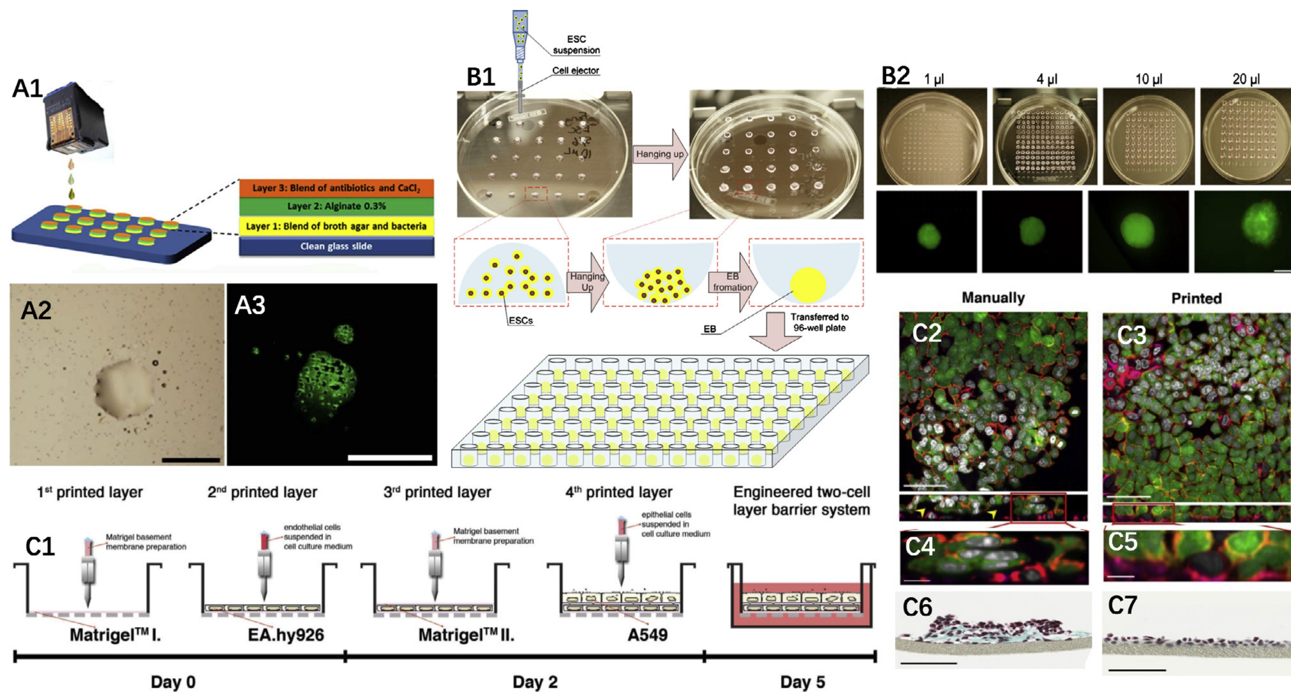

Fig. 5. (A1) A schematic showing inkjet-bioprinting of three-layer microarrays on glass slide (A2) light-microscopy and (A3) fluorescence imaging of the bioprinted samples; (reproduced/adapted with permission from [126]; (B1) Schematic of the embryoid body (EB) formation process using bioprinting approach, (B2) uniform-sized droplets encapsulating ESCs were bioprinted to form EBs with droplet sizes of 1, 4, 10, and 20 μm (upper column). Fluorescent images of GFP positive EBs at t = 96 h stained with ethidium homodimer (lower column) (reproduced/adapted with permission from [130]; (C1) Schematic of the timeline for bioprinting the two cell-layer barrier system, (C2, C4) manually seeded co-culture cells and (C3, C5) bioprinted four-layer lung tissue model with highly organized distribution of a549 cells (green) and endothelial cells (labeled with VE-cadherin in pink), where F-actin and nuclei are labeled in red and white, respectively. (C6, C7) Histological cross-section stained with Masson–Goldner trichrome coloration showing uniform thickness of a tissue sample. Cytoplasm, collagen fibers and cell nuclei are stained in red, green and dark brown, respectively. Bioprinted but not manually seeding tissue demonstrates uniform epithelial layer on the top and endothelial cell layer at the bottom (reproduced/adapted with permission from [75]).

oped an acoustic-based bioprinting with various cells (including mouse embryonic stem cells, fibroblasts, AML-12 hepatocytes, human Raji cells, and HL-1 cardiomyocytes) for HTS applications. Single cells were ejected from a nozzleless pool in picoliter droplets at rates ranging from 1 to 10,000 droplets per second; cell viability was maintained almost 90% across various cell types [127]. This group also developed a mechanical valve ejector for high-throughput bioprinting of a high viscosity collagen-encapsulated rat bladder smooth muscle cells [128,129]. Through this platform, constructs were bioprinted with uniform cell seeding yielding a layer-by-layer 3D cell pattern with controlled spatial resolution and maintaining high viability over long-term cell culture. The group has also integrated micro-valve bioprinting with hanging drop method to create controllable, uniform-sized embryoid bodies (EBs) from embryonic stem cells (ESCs) as shown in Fig. 5B1 and B2 [130]. The bioprinting approach resulted in formation of EBs with a high degree of size uniformity. The overall size of the EBs was also larger as compared to EBs formed by a manual pipetting process. Since the combined approach was simple, robust and rapid, the EBs fabricated by this approach were deemed appropriate for applications in high-throughput screening of drug candidates as well as for evaluation of drug toxicity to embryos. Recently, Sun's group reported a novel method in which ESCs-laden hydrogels composed of gelatin and alginate were extruded to form 3D scaffolds. Uniform, pluripotent, high-throughput and size-controlled EBs were formed through cell proliferation instead of aggregation after the EBB [93]. Additionally, Demirci's group also engineered an anisotropic biomimetic fibrocartilage microenvironment by bioprinting MSCs in nanoliter gel droplets [131]. A valve-based droplet ejector was used to bioprint an array of methacrylated gelatin encapsulated MSCs with one single phase of BMP-2, TGF-β, or composition of BMP-2 and TGF-β. Multiphasic anisotropy of the patterned biochemical factors was confirmed by genomic examination. As a functional *in vitro* 3D tissue model and platform, the bioprinted microscale anisotropic tissue constructs showed the potential to be utilized for high-throughput pharmaceutical testing and validation studies.

Heterogeneous cellular co-culture microenvironment can also be fabricated in a high throughput manner by DBB. Demirci's group introduced a high-throughput automated cell bioprinting system to bioprint a 3D coculture model using cancer cells (OVCAR-5) and normal fibroblasts (MRC-5) micropatterned on Matrigel™ [67]. A nanoliter dispensing valve (solenoid valve ejector) was controlled by a pulse generator to generate droplets with a wide nozzle (150 μm diameter) to minimize local shear forces. Two ejectors were used, one for each cell type. The two ejection systems were synchronized and two cell types were patterned within a spatially controlled microenvironment (e.g., cell density, cell–cell distance) in a high-throughput and reproducible manner. Both cell types remained viable during printing and continued to proliferate to form 3D acini, which were cultured up to 3 weeks. This bioprinting scaffold-based co-culture system provided a biomimetic tool for high-throughput drug screening.

An advanced 3D lung model for high-throughput screening for safety assessments and drug efficacy testing has been engineered by 3D bioprinting [75]. The main component of the bioprinter was the process unit comprised of a tool changer with three workstations and equipped with print heads that allowed for printing up to three different biomaterials/cells. Matrigel™ was printed by contact dispensing, whereas the human alveolar epithelial type II cell line A54956 and EA. hy926 hybrid human cell line (derived by fusing human umbilical vein endothelial cells (HUVEC) with

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A549 cells) were bioprinted by droplet jetting process. 3D air-blood barrier models were bioprinted into cell culture inserts in a layer-by-layer manner as shown in Fig. 5C1. The bioprinted constructs were shown to have similar permeability or barrier functions to those made with the manual method (see Fig. 5C2–C7). However, the bioprinting method accorded an automated and reproducible construct creating thinner and homogeneous cell layers essential for an optimal air-blood tissue barrier for the intended application.

A bioprinted microarray for screening cellular responses to ECM manipulations can also be used in high-throughput drug screening. Xu's group proposed a nanoliter-sized cell-laden hydrogel array with a custom-designed pressure assisted DBB system containing a solenoid valve ejector. Periodontal ligament stem cells (PDLSCs) were loaded within a gradient of gelatin methacrylate (GelMA)/PEG hydrogel matrix to observe human PDLSC response to ECM modifications. Cell behavior in GelMA/PEG array was shown to be dependent on the volume ratios of GelMA/PEG, with cell viability and spreading area decreasing with a corresponding increase in the ratio of PEG [132]. This array model can be extended for research in drug delivery, high-throughput screening or dose–effect relationship studies. A laser-based bioprinting approach was used to investigate interactions between different types of cells and their environment in a high-throughput manner. Human adipose-derived stem cells (ASCs) and endothelial colony-forming cells (ECFCs) encapsulated in hyaluronic acid/fibrinogen were patterned and arrayed. In these 3D arrays, cell spots can be arranged layer-by-layer. Cell–cell ratio, cell quantity (density), cell type combination, spacing and the height of the 3D array were successfully controlled. It was observed that direct cell–cell contacts triggered the development of stable vascular-like networks even in VEGF-free medium [94] emphasizing the potential of this method.

5.4. Absorption, distribution, metabolism and excretion (ADME) assays

Pharmacokinetic of drugs includes absorption, distribution, metabolism and excretion of drugs, which means the whole delivering processes of administrated drugs and their metabolites in body. ADME properties of drugs should be analyzed in preclinical discovery period. Although *in vivo* ADME assays are undertaken in animals, biomimetic *in vitro* models for ADME assays should be helpful for promoting to seek druggable compounds, especially exploring the role of the metabolites on the efficacy or toxicity of drugs. Sun's group has also developed an *in vitro* 3D microfluidic, micro-analytical, micro-organ device for *in vitro* pharmacokinetic analysis [133]. A bioprinted micro-liver was fabricated with an automated syringe-based direct cell writing process through which human hepatocyte (HepG2)-encapsulated alginate strands were directly extruded into a microfluidic tissue chamber composed of poly(dimethyl siloxane) (PDMS) elastomer which was fabricated by soft lithography. A syringe pump was connected to the integrated 3D tissue chamber unit to supply media and drugs through a convectional microchannel in a sinusoidal flow pattern. The ability of the fabricated micro-liver to simulate physiological function of liver to perform drug metabolism was demonstrated by measuring the extent of transformation of a non-fluorescent pro-drug, 7-ethoxy-4-trifluoromethyl coumarin (EFC), to a fluorescent products 7-hydroxy-4-trifluoromethyl coumarin (HFC). Additionally, the authors successively extruded HepG2 and human epithelial cells individually encapsulated in Matrigel™ in an indentation in the PDMS substrate [134]. The bioprinted constructs were sealed under glass covers on microfluidic chips which were connected to form dual micro-tissue microfluidic chips and were dynamically perfused by a syringe pump. In this study, hepatocytes were used as the target cells and epithelial cells were used to mimic drug transportation paths, as epithelial cells line the lumen through, which drugs have to be absorbed before they reach the target hepatocytes. Further, an *anti*-radiation drug, amifostine, was used to evaluate the metabolizing efficacy of epithelial cells since it is a pro-drug, which is converted to an active drug by the epithelial cells. The radiation damage to hepatocytes was measured by formation of binucleated cells with micronuclei by labeling with the fluorescent nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) as the probe. Through this integrated tandem dual micro-tissue system, multicellular interactions and downstream effects of metabolism on a target can be investigated.

6. Key considerations for bioprinting in drug discovery and development

Although bioprinted 3D tissue constructs have several advantages and exploratory experiments have shown encouraging results for pharmaceutical testing, industrial research applications of 3D bioprinting in drug discovery and development process is required to be deliberated along with the regulatory concerns [49]. The key questions to be considered are the necessity of using bioprinting in pharmaceutical discovery and development, the application areas to be targeted, the exact situations when it should be applied, which are discussed in the following sections.

6.1. Why is it necessary to use bioprinting in pharmaceutical discovery and development?

As previously discussed, decreasing the attrition rate remains a major challenge for the industry. The high attrition rate along the timeframe highlights the need for novel approaches to develop more predictive *in vitro* assays for efficacy and safety analysis. Several 3D models with high predictability have been introduced to address this challenge, however, only a few 3D models have been vetted for use in discovery and development due to the strict regulatory and validation requirements [39–41]. While evaluating an application of novel *in vitro* models, regulatory authorities evaluate the published scientific evidence and accept the submitted data only after joint cross-industry validation. As a recently introduced technique, bioprinting has the critical advantages of automation, stability, biomimetic among other 3D models; hence, it should be relatively easy and quick to get approval from regulatory agencies. Bioprinting techniques can be deployed to fabricate more predictable drug screening platforms, which will enable the 'quick-win, fast-fail' paradigm and reduce the attrition rate.

6.2. When and why should bioprinting be used in drug discovery and development?

Bioprinting should be used in the preclinical phase of the discovery and development process. Schemes of applications of bioprinting on drug discovery and development process are shown in Fig. 6. After validation and selection of a target, HTS is undertaken in target-to-hit stage in which a library of 10^5–10^6 individual compounds is tested for ability to bind to the target. The primary goal of HTS is to identify chemical hits. Depending on the target and assay, the output of HTS is typically a few thousand compounds that reproducibly produce the assay signal. Also, at this stage, confirmed hits are divided broadly into chemical series and each hit is evaluated with respect to potency, physiochemical properties, and other comprehensive properties such as cost, selectivity, scalability, etc. Those series that survive the triage process enter the hit-to-lead stage to be evaluated for *in vitro* efficacy and predictive *in vitro* toxicity. Focused libraries provide substrates for structure–activity relationships (SAR) work in target-binding

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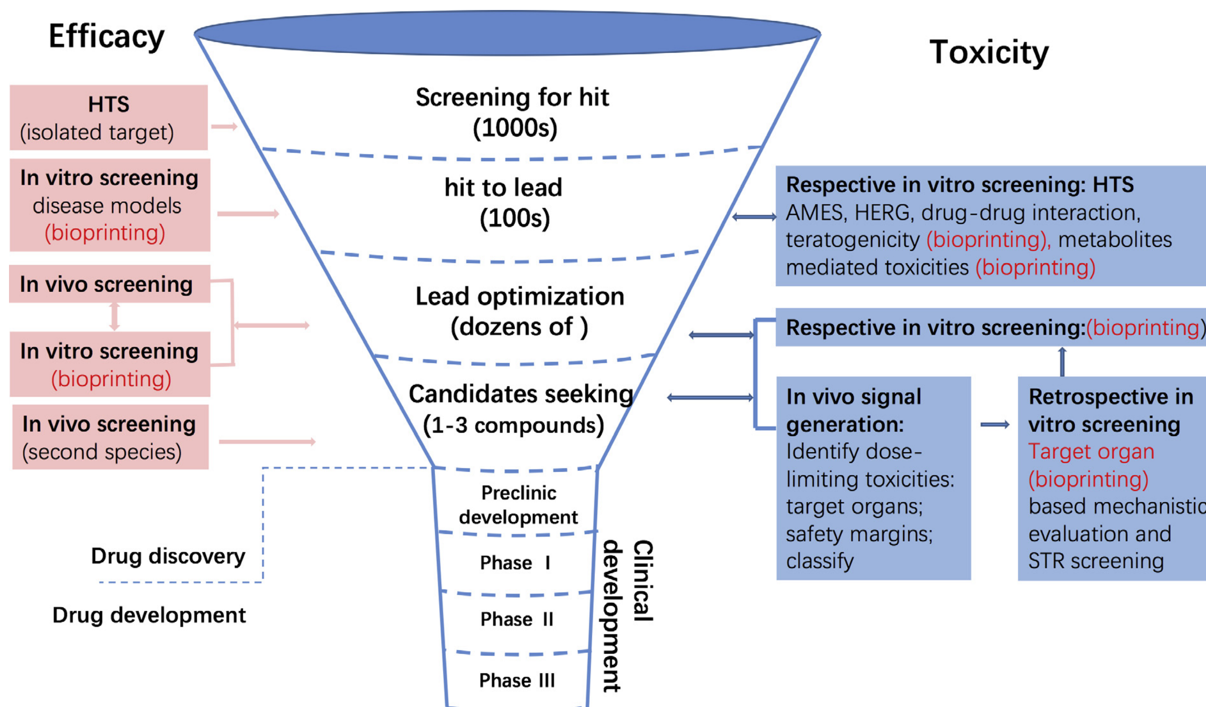

Fig. 6. Schemes of applications of bioprinting on drug discovery and development process. Bioprinting can be used for teratogenic screening based on differentiation of stem cells using different scaffold clues. Some human metabolism organoids can be bioprinted to produce components including active metabolites for following cytotoxicity screening, which may improve the predictivity for some *in vivo* toxicities. Based on *in vivo* signal generation, identified target organs can be bioprinted as organoids for mechanical evaluation and structure–toxicity relationship (SRT) to screen out development-limiting toxicities, improve safety margins and delivery superior lead candidates into drug development process. Bioprinted disease models with human cells can be adopted in *in vitro* screening of efficacy to improve the predictive potential for *in vivo* efficacies.

and cell-based target modulation assays. Hundreds of potent and selective leads are then evaluated in early *in vivo* ADME assays to measure relative physiochemical properties. In the succeeding lead optimization stage, proof-of-concept (POC) is achieved in a widely accepted preclinical model of disease (*in vivo* efficacy) and additional ADME assays (*in vivo*) are performed to get 10–12 optimized leads. Also in this stage, *in vivo* toxicity, structure–toxicity relationships (STR) and retrospective *in vitro* toxicity should be performed. In the candidate-seeking stage, the optimized leads are addressed with a second species PK/PD modelling, safety and formulation studies to identify one to three candidates for formal preclinical development. In the preclinical development stage, GLP toxicology studies including genetic toxicology, safety pharmacology and *in vivo* toxicology in two species are assessed for the candidates. Usually, this testing paradigm typically delivers drug-like compounds that have promising pharmacokinetic parameters and efficacy in preclinical models within a 1–2 year cycle time [135]. Although bioprinting is a versatile process and can be applied for every stage of drug discovery and development, bioprinting is not required for all of stages; rather, its application should be carefully deliberated to determine if bioprinting can be beneficial or commercially viable in the drug discovery and development process.

6.2.1. Target selection

In order to improve R&D productivity and reduce the costs of drug development, reductions in Phase II and III attrition are crucial. The first step is the selection of the most validated and treatable targets; the second step is to establish POC as early as possible in the development cycle (preferably in Phase I). This stage also requires an essential drug target whose association with the disease can be validated by biomarkers, clinically relevant endpoints or surrogate endpoints for making the early “go/no-go” decisions. Such targets and biomarkers are of special interest in some areas such as the central nervous system or oncology. As described in a review study, clinical attrition rates were higher for these areas with more than 70% of compounds failing in Phase II and 59% failing in Phase III, in part due to the unpredictable nature of the drug targets and to the lack of models with the capability of reliably predicting human physiology [136].

Target identification and validation leading to target selection needs to be confirmed by genetic evidence from humans with gain-of-function or loss-of-function mutations and knockout mouse models, as well as the biological effects observed after pharmacological modulation in animal models. Pharmacological modulation of targets in animal models frequently generates effects which differ from human responses due to variability between species. Common *in vitro* experiments using human cell lines also do not recapitulate the human *in vivo* response [137]. Bioprinting has the capability of mimicking human pathophysiological states with native complexities and clinical relevance, which can aid in identification and validation of potential targets along with their mechanisms. Additionally, the correlation of target with biomarkers or surrogate endpoints can also be directly investigated. As previously mentioned, two studies have shown that 3D bioprinted models are more efficient platforms to identify ROCK as a molecular target than 2D models for angiogenesis [119,120]. Nevertheless, extensive applications of 3D bioprinted platforms on physiological or pathophysiological models are needed for target identification and validation, and corroboration with genetic evidences and *in vivo* assays is essential.

6.2.2. Efficacy screening

Bioprinting can be employed to fabricate 3D models for *in vitro* efficacy screening in the hit-to-lead stage. Bioprinted constructs can also be considered as supplements or alternatives for *in vivo*

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efficacy for lead optimization. Human cell lines, stem cells or primary cells can be employed for these purposes and an appropriate ECM can be selected to mimic *in vivo* conditions. Bioprinted physiological tissues or pathological models are required to possess adequate stability to produce repeatable and robust data. The goal for bioprinting a successful efficacy study model is to create a model that expresses the key characteristics of a particular disease. For example, chemotherapy models are preferably created by bioprinting since cell–cell and cell–ECM interactions play important roles in tumor development and metastasis. Complicated co-culture models have been bioprinted to investigate angiogenesis and local immunological responses, which essentially improves the prediction of chemotherapeutic or chemo-resistance properties of test compounds [108]. However, except for a few tumor models with monotype or heterogeneous cells fabricated with scaffold-based [89] or scaffold-free [122] bioprinting methodologies, reports of bioprinting of other disease models are rare. On the other hand, for lead optimization stage, bioprinted constructs are emerging as an extremely economical and highly efficient complement to the classical *in vivo* disease models. The principal advantages include a reduction in costs associated with animals and compounds, elimination of ethical concerns arising from of animal use, shorter times required for expression disease properties that are not faithfully replicated by the animal models and lower data variation. However, it is necessary to obtain relevant regulatory approvals for bioprinted 3D *in vitro* models to be used as substitutes for *in vivo* animal models and large number of exploratory and confirmatory studies should yet be conducted [137].

6.2.3. Toxicity analysis

Historically, a limited preclinical safety assessment beyond basic *in vitro* toxicity assays was performed on lead molecules as they advanced through the process of discovery to development. However, the high rate of preclinical and clinical attrition has emphasized the importance of early application of toxicology assessments. Many companies have thus increased incorporation of preclinical safety assessment in the early phases of the drug discovery process; therefore, more systemic toxicity assays are undertaken prior to the application of standard preclinical GLP toxicity studies for the candidate molecules. Early toxicology includes prospective *in vitro* toxicity assays (predictive *in vitro* assays), *in vivo* signal pathway and retrospective *in vitro* toxicity assays (mechanistic *in vitro* assays) [135,138].

Prospective *in vitro* toxicity assays attempt to predict toxicities that are development-limiting and are likely to be overlooked (no histopathological correlation in short-term *in vivo* studies). These assays include general or cell-specific cytotoxicity, genotoxicity, human Ether-a-go-go-Related Gene (hERG) channel block, drug-drug interaction or metabolites-mediated toxicity [135]. *In vivo* signal pathway analysis aims to identify dose-limiting toxicity of target organs. A well-executed short-term (1 week) repeat-dose *in vivo* toxicity study may predict most of the dose-limiting target organ toxicities [135]. Once dose-limiting toxicities are identified, the safety margin is determined and conclusions on whether or not the findings are development limiting are drawn. After the identification of target organs, the target organ specific retrospective *in vitro* toxicity assays are implemented to screen out development-limiting toxicities, understand STRs, optimize leads with minimized adverse effects, and finally deliver a superior lead candidate to the development phase. Although a tremendous investment in *in vitro* toxicology screening has been made within the industry, the data from four major pharmaceutics companies between 2000–2010 has shown a similarity in rates of preclinical toxicology failure of the candidates in the pre- and post-2005 periods. This implies that predicting organ toxicity at an *in vitro* level still remains a challenge [3]. In the last two decades, the activities to improve early prediction of *in vitro* assays was primarily driven by the notion that improving quality and increasing the number of endpoints related to cellular events at a systems biology level rather than just at the single endpoint level. These efforts were characterized by the introduction of technologies such as genomics, proteomics, transcriptomics, metabolomics, and high content imaging to *in vitro* assays [139]. Although genomics—sometimes referred to as "toxicogenomics"—has proved very useful and highly predictive at *in vivo* level [140], "-omics" technologies for predicting organ efficacy or toxicity at an *in vitro* level has remained a challenge. The improvement of drug toxicity predictions based on *in vitro* data depends not only system and better readouts, but also the models used to generate those data. *In vitro* 3D models have revealed more biomimetic toxicities to drugs than traditional 2D models [105,141]. Thus, highly biomimetic 3D bioprinted models should be considered for *in vitro* toxicity assays over 2D or other 3D models.

Not all *in vitro* toxicity assays need a 3D model to improve the predictivity. Early predictive *in vitro* toxicology screening significantly improves the ability for early rejection of compounds owing to adverse general toxicity (such as phospholipidosis) or to development-limiting toxicity (such as genotoxicity, hERG inhibition). The genotoxicity assays include Ames testing, micronucleus testing (MNT), teratogenic potential (embryonic stem cell test (EST)), and the Comet assay. These classic *in vitro* assays use a single cell type model with a well-established specific endpoint, allowing researchers to make more informative decisions [138]. Most of the predictive *in vitro* toxicity assays are based on specific well-established endpoints on designated cells or other carriers rather than native tissues, e.g., the hERG-binding assay to detect compounds with a potential risk of inducing cardiac arrhythmias uses the human KCNH2 potassium channel gene stably cloned into HEK 293 cells combined with patch clamp analysis. The long-term history and the availability of large data sets allow researchers to make decisions on potential of drug development. Since the bioprinting technique produces highly biomimetic constructs, it can be stated that bioprinting is unnecessary to the *in vitro* predictive toxicity screenings. However, for the teratogenic potential screening, embryonic stem cell texting (EST) are undertaken. Briefly, 3D EBs fabricated by hanging drop of mouse embryonic stem cell line (mESC line D3) is used to investigate the cardiomyocyte differentiation and embryonic toxicity. The 3D spheroids-like EBs are spontaneously formed and manipulated in a high-throughput manner using bioprinting producing more uniform and controlled sizes of EB-properties, which are undoubtedly beneficial for EST screening of compounds [93,130].

After identification of target organs and the safety margin by *in vivo* signal assays, the target-organ specific retrospective *in vitro* toxicity assays are important for optimization of leads and identification of candidates. Unlike predictive *in vitro* toxicity assay models, the key to retrospective *in vitro* toxicity assay model is a higher degree of biomimetic traits that is most similar to the native organs. Longevity, real-time demonstration indexes and low-to-medium throughput are also considered. The limitation of current models for retrospective *in vitro* toxicity assay in drug safety is primarily that the short culture time of simple monolayer cell culture system does not accurately reflect the complex physiology of a target organ. The lack of *in vitro* systems that efficiently identify organ toxicity is still problematic in the pharmaceutical industry. It is well known that the most frequent cause for drug rejection is liver toxicity. However, most of the published data utilizes monolayer and monotype cultures of transformed cell lines like HepG2 or primary hepatocytes as liver surrogates in *in vitro* hepatotoxicity assays. It is well known that drug-induced liver injury (DILI), including idiosyncratic toxicity and hepatocarcinogenesis, are mediated by interplay among the different cell types

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residing in the liver; thus, accurate liver toxicity cannot be predicted using the monolayer and monotype cell culture. At present, a few human 3D co-culture liver surrogate systems are available for use in drug safety assays and all show better predictability than 2D monolayer systems [30,39,142]. 3D models of the heart, kidneys and the skin, the major target organs of drug-induced side-effects have also been developed [143–145]. Besides validated biological relevance, the technical set-up of 3D models is need to be compatible with industrial-level testing, incorporating automation, ease of use, and reproducibility of the models. Bioprinted 3D models may fully address these challenges, and hence should be utilized by the companies in retrospective *in vitro* toxicity assays of compounds. So far, some bioprinting companies have developed bioprinted tissue models for toxicity studies [90,107].

6.2.4. High-throughput screening (HTS)

Since there are different definitions of HTS, a clarification is needed. In a drug discovery process, HTS for hits means that a large number of compounds are directly screened in parallel on recombinant and purified targets or targets expressed in supports; assessment is made based on binding potential and functional activities of enzyme, receptors and ion channels. This screening is usually high-throughput and based on a specific target molecular rather than global cellular function endpoint, rendering bioprinting inappropriate for this objective. However, as a screening strategy, HTS means high-throughput parallel screenings of efficacy or toxicity on a miniaturized platform such as micro-tissue or micro-organ array within micro-well plates or chips. HTS performs simultaneous assays for different targets, compounds, doses and indices without cross-contamination. HTS improves efficiency by executing parallel assays under the same controlled conditions, saving time and money. Additionally, miniaturized systems require only a small amount of the test drug which are sometimes very costly and difficult to obtain. Efficiency of HTS may be influenced by the throughput, analysis indices and fidelity. Fidelity means that the miniature screening system should recapitulate the reaction *in vivo*; low fidelity of a model could result in misdirection of the development process. Microarrays based on 2D cell culture system are currently the most common HTS platform used for drug screening [146]. However, the 3D microarray is expected to replace 2D microarrays due to higher fidelity of the 3D systems. 3D microarrays can be fabricated by processes such as microwell, surface patterning, and microfluidic techniques, etc. Although high resolutions is no longer a problem with these fabrication techniques, several aspects of 3D microarray still need improvement, such as uncontrolled spatial deposition and densities of cells. Bioprinted microarrays have the potential to overcome these limitations.

For HTS, bioprinted 3D micro-tissue spots can be collected on a chip or a substrate. Bioprinted organ-like functional units, called organoids, can also be collected for micro-engineering in a microarray or micro-organ array. In microarrays, media or drugs should be added to every unit under investigation without any cross-contamination. Various methods including drug patterning, drug stamping, aerosol spraying, and microfluidic drug loading have been developed for drug delivery onto cell microarrays [147]. In order to screen the chronic effects of different drugs or toxins in a high-throughput manner, every unit in a microarray or micro-organ array should be connected to an independent flow based on the bioreactor.

In drug discovery, HTS with bioprinted 3D microarrays can be used for *in vitro* efficacy or toxicity screening at corresponsive phase. *In vitro* efficacy assays are needed in the hits-to-leads stage. To speed up lead certification or optimization, HTS can be used for *in vitro* efficacy assays with cell-based functional phenotype screening. Bioprinted 3D microarray HTS using human cells should be undertaken as early as possible to quickly screen the hits with higher potential. In the retrospective *in vitro* assays, bioprinted 3D microarrays provide high predictive platforms allowing "fast fail" for leads, optimized leads and candidates.

6.2.5. Absorption, distribution, metabolism and excretion (ADME)

Poor pharmacokinetic properties and oral bioavailability that are not predicted by preclinical ADME studies result in overlapping of effective and toxic doses, which account for the high number of Phase I and Phase II failures [4]. In fact, the attrition due to poor pharmacokinetic profiles seems to have reduced significantly in the recent years due, in part, to the improved preclinical ADME characterization which includes early evaluation of pharmacokinetics and drug metabolism along with increased throughput and sensitivity [4,148]. Although adverse pharmacokinetics and bioavailability are cited as the third most common cause of attrition in Phase I ($\leq$10–20%), the predictability of human pharmacokinetic parameters can be improved further by animal models or, to a certain extent, by the use of *in vitro* models with human cells [3]. Nevertheless, closely mimetic *in vitro* models become more valuable for evaluation of pharmacokinetic properties if these models are integrated with efficacy or toxicity assays to investigate the influence of human metabolism on drug efficacy or toxicity. With the aid of fabricated *in vitro* tissues simulating human native tissues, pharmacologist can quickly predict the efficacy or toxicity of the compound and its metabolites. As seen in some studies, 3D bioprinting of different metabolism-related cells in their native topology efficiently predict the effects of pharmacokinetic activities on target organs [103]. Additionally, bioprinted organoids can be connected on a chip creating an "organ-on-a-chip" or "human-on-a-chip" that can be designed to better predict the global effects of compounds and their metabolites [149,150]. This *in vitro* platform is preferable for implementation in the lead optimization stage for as a complementary *in vivo* efficacy assay as well as in retrospective *in vitro* assays of toxicities.

6.2.6. Phenotypic screening

Phenotypic screening is a type of screening used in biological research and drug discovery to identify substances that alter the phenotype of a cell or an organism in a desired manner. The pharmaceutical industry is in transition from an era of 'me-too' or 'slightly me-better' drugs to one of highly innovative medicines that deliver markedly improved therapeutic outcomes [4], making it necessary to re-focus on discovery and research. Target-based drug discovery requires identification of therapeutic targets and *in vitro* screening of drug candidates based on the targets. The goal of phenotypic screening is to identify active compounds that ameliorate disease phenotypes without concern for the targets and molecular mechanisms of actions of the compounds. The phenotypic screening of drugs is mainly based on endpoint responses in cell-based assays or animal models. Employment of *in vitro* models that capture key characteristics of diseases, while remaining amenable to high-throughput is essential for an effective *in vitro* phenotypic screening. One group analyzed the discovery strategies for new drugs approved by the Food and Drug Administration between 1999 and 2008, and found that phenotypic screening was the most successful approach for approved first-in-class drugs, whereas target-based screening was the most successful for follower drugs during that period [151]. Additionally, repositioning or reconsideration of some approved drugs or failed drugs for other treatment can also be preferable to phenotypic screening through predictive preclinical models as a more efficient and cost-saving strategy [152]. With its inherent beneficial properties, the immense potential of bioprinting should be considered for conducting phenotypic screening of new drug compounds or the repositioning of existing drugs.

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7. Future outlook

7.1. Organ-on-a-chip and micro-physiological systems (MPS)

Bioprinting technologies also offer the possibilities of miniaturizing tissue arrays. By designing and fabricating functional cells and/or supporting cells, 3D bioprinting can accurately engineer multi-organoids to create micro-organs, which can be combined with a microfluidic chip to form "organs-on-a-chip" [137,149]. The miniaturized *in vitro* "micro-organ" or "organ-on-a-chip" device can be used to investigate the pharmacological and toxicological effects of drugs; the microfluidic flow component can provide long term and constant delivery of drug and simulate physiologically relevant mechanical forces such as fluid shear stress [153]. The combined technique of bioprinting with microfluidic provides a promising platform for *in vitro* testing in drug discovery with the comprehensive capability of automated manipulation, long-term culture, HTS, and real-time monitoring.

In order to produce a more global assessment of drug responses of tested compounds, multiple organ models such as liver-on-a-chip, heart-on-a-chip and kidney-on-a-chip, should be linked with each other to create more predictive human-on-chip platforms. Details on these systems can be found in a recent review article [143]. For miniaturizing the whole human body, bioprinting can be used to simultaneously deposit multiple types of organoids at different locations on a chip to form a human-on-a-chip or a body-on-a-chip. Connection of organoids can be realized with interconnected sprouted vascularization in the area or by direct bioprinting of vessels [111]. The generated vascular network with branches in multiple scales has the capacity to provide a platform to facilitate physiologically-relevant flow conditions for maintaining systemic functions and testing of whole body responses to administered drugs. Vascularization in 3D models also plays a vital role in tissue growth, survival, and drug delivery. Bioprinting enables high-resolution fabrication of tissue microenvironments also containing vascularization [154]. Vascular network connections can be generated using existing techniques, such as biological sprouting of capillaries by co-culturing with endothelial cells and creating anastomosis between organoids [111], or by using fugitive ink for perfusion channel fabrication in an indirect bioprinting mode [155].

Effects of drugs on bioprinted constructs can be observed with fluorescence, colorimetry, and enzyme reporter methods. Fluorescence method is usually adopted for dynamic *in situ* imaging especially in micro-organs or micro-arrays. As different organoids have different physiological responses over time, bioprinted human-on-a-chip devices should be monitored spatially in high resolution in real time. Advanced biosensors such as lens-less charge-coupled devices (CCD) and micro-electrodes are developed and are compatible with bioprinting technologies [156]. Two or more biosensing techniques have also been combined, such as in a dual-parameter cell analysis system that integrates intracellular granularity with an impedance spectroscopy technique to monitor cell-to-cell and cell-to-matrix adhesion and a light scattering technique that is used to determine the number of cells [157].

While whole parts of an organ such as micro-tissue, micro-organ, organ-on-a-chip, human-on-chips or human-on-a-chip, have been recognized MPS, they require more detailed study with regard to the advantages of miniaturization of the human system. Efficient micro-fabrication of MPS cannot be realized by simply scaling-down the macro system. MPS requires specialized expertise which is beyond this review but detailed elsewhere [158]. Nevertheless, with its versatility, bioprinting appears to be a highly promising technique independently or in combination with other techniques to fabricate sophisticated MPS.

7.2. Personalized pharmaceutics

To get maximum benefit and the least toxicity from a pharmaceutical treatment, a personalized medicine therapy based on individualized metabolism potential and response to a particular drug is gaining increased attention [1]. Ideally, a personalized pharmaceutics platform should be a body-on-chip encapsulating organoids comprised of important drug-sensitive organs such as heart, liver, kidney and target organs. Bioprinting of patient-derived primary cells has the potential to develop personalized medicine models to screen for the most effective treatment with minimum safety issues. Induced pluripotent stem cells (iPS) and multipotent stem cells can be used for differentiate cells of different lineages from an individual patient. As a promising personalized medicine tool for drug screening, iPS technology could also be implemented in personalized medicine. Somatic cells from the patient could be induced into iPS and the latter can be bioprinted within specialized microenvironments to differentiate into different organ cells. Differentiated cells or organs can also be bioprinted for optimization of the most effective treatments. In GSK, ordinary human skin cells have been converted into iPS cells with the capacity to differentiate into any cell type in the body. These iPS cells can be differentiated into heart muscle cells for predicting the cardiac toxicity of investigational drugs [159]. Additionally, iPS from humans can be bioprinted for commitment to different organs in MPS for selection of the most efficacious lead compounds.

7.3. Commercial considerations

At present, bioprinted 3D constructs, microarrays, and micro-organs are used in drug screening as a supplement to standard processes involved in drug discovery and development; however, it is too early to conclude that the bioprinted models can replace animal models. For a 3D bioprinted construct representing a target organ for drug testing, acceptance of the system will need to be validated using reference drugs based on large sets of well documented exploratory experiments. An important example of a novel human-relevant *in vitro* model gaining acceptance is the 3D skin models used for testing cosmetics [160]. After validation of the model using a series of test compounds with known mode of action on the skin, the *in vitro* skin approach is now considered as an acceptable test for skin deterioration and irritation, and has proven to be a better predictor of drug effects in humans, thereby reducing the need for animal testing. For wider applications, 3D skin models can be suitably modified for the creation of disease templates and HTS [160]. Progress in terms of designing flexible and versatile platforms, where cells of different types and origins can be bioprinted and allowed to grow in a more physiologically-relevant environment are now progressing at a rapid pace. The key advantage of a platform-based approach is to provide a basic system which is biocompatible with different organ requirements. Efficient cell seeding in pre-defined areas might be achieved by use of bioprinting technology whereas nutrients and oxygen supplementation can be facilitated by integration with microfluidics systems.

8. Concluding remarks

Exploratory studies of bioprinting in pharmaceutics have shown promising applications of this technique in the field, with the unparalleled advantages of automation, high-throughput, precise spatial control, and potential for co-culture and fabrication of hierarchical structures. During the process of drug discovery and development, 3D bioprinting can be used for target identification and validation. For the hits-to-leads step, 3D bioprinted disease

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models can be used for HTS for *in vitro* efficacy. For the optimization of leads, 3D bioprinted constructs can be used for *in vitro* efficacy assessment, as well as *in vitro* retrospective toxicity assays after confirmation of target organs from *in vivo* toxicity assays. After continuously improving loops of structure–activity relationship, synthesis of new compounds, *in vitro* and *in vivo* assays, candidates are screened from optimized leads following the regulatory development periods.

Bioprinting is expected to reduce the cost and time of preclinical discovery. However, the principal consideration in application of bioprinting in drug R&D should be the balance between the cost and the value of bioprinting in discovery and development, since 3D models have not been popular in the industry due to their complexity and cost over 2D counterparts [10]. Although the advantages of bioprinting in pharmaceutics have emerged, more persuasive evidence and commercial level customized bioprinted products are still lacking. The advantages of bioprinting should be demonstrated before regulatory agencies accept bioprinted constructs as quality control and regulatory tools for pharmaceutical applications. More evidence is also needed to confirm the superiority of bioprinting constructs over conventional models and other 3D constructs. Further investigations are also required to show similar efficacy or toxicity of drugs in 3D bioprinted constructs as *in vivo*. Meanwhile, cost-effective, high-throughput, automated and stable bioprinting techniques and devices should be developed for the industry use. More interactive models and disease models expressing major pathological characters should be bioprinted for drug testing. Additionally, the 3D bioprinted tissue constructs coupled with high-content readout such as comprehensive genomic or proteomic expression analysis of biomarkers via bioinformatics data mining tools will provide massive amounts of valuable data and a promising new avenue for drug testing and mechanistic analysis.

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