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REVIEW ARTICLE

Toward better drug development: Three-dimensional bioprinting in toxicological research

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Abstract

The importance of three-dimensional (3D) models in pharmacological tests and personalized therapies is significant. These models allow us to gain insight into the cell response during drug absorption, distribution, metabolism, and elimination in an organ-like system and are suitable for toxicological testing. In personalized and regenerative medicine, the precise characterization of artificial tissues or drug metabolism processes is more than crucial to gain the safest and the most effective treatment for the patients. Using these 3D cell cultures derived directly from patient, such as spheroids, organoids, and bioprinted structures, allows for testing drugs before administration to the patient. These methods allow us to select the most appropriate drug for the patient. Moreover, they provide chance for better recovery of patients, since time is not wasted during therapy switching. These models could be used in applied and basic research as well, because their response to treatments is quite similar to that of the native tissue. Furthermore, they may replace animal models in the future because these methods are cheaper and can avoid interspecies differences. This review puts a spotlight on this dynamically evolving area and its application in toxicological testing.

Keywords: Liver; Drug development; Three-dimensional printing; ADME test; Organoid; Spheroid

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1. Introduction

In drug development, the goal of a scientist is to develop a novel drug that alleviates a particular disease, thereby improving or even preventing a particular biological

process. New chemicals, called xenobiotics, that contain any active moiety in certain biochemical pathways should be safe to consume and able to be excreted from the body after exerting their pharmacologic effect by the rules of pharmacodynamics and pharmacokinetics. When a new chemical, which is deemed to be foreign and potentially toxic, enters the body, the body limits the toxic effects by activating a number of functions, such as resistance to absorption, so as to limit its distribution, and metabolize it to a form that can be easily removed from the body through urine or feces. The absorption, distribution, metabolism, excretion, and toxicology (ADME-Tox) processes covers a wide variety of mechanisms. The assessment of ADME-Tox is essential in drug development and related research disciplines in which it may play a role in pharmacodynamic, pharmacokinetic, or toxicokinetic studies[1].

Drug development can be divided into two major phases: drug safety evaluation and development. Drug development is very often called "the valley of death" because to achieve the ultimate goal – getting the drug to market – a huge amount of money, time and work is necessary, while the work is often associated with a very high degree of failure. 96% of tested drugs fail during clinical trials, and the success rate is even lower if the new drugs target new mechanisms of action, the diseases are not well studied, and their cure has not been developed yet[2,3]. Identifying targets, performing basic research, and conducting comprehensive bioinformatics analyses consume large amounts of money and take years to screen out a few thousand potential molecules. Most of the molecules will then be subjected to *in vitro* and *in vivo* toxicological and ADME tests in the following years, with only about ten molecules reaching the preclinical development phase and clinical trials. The efficacy of clinical trials is around 10%. This means that a single drug is born from nearly 10 years of research, and nearly US$ 800 million is invested initially to support the research of about 10,000 potential molecules. Focusing on the specific activity, there are four main steps in drug development: (i) a period of research and development that takes 3 – 6 years with the primary goal of determining drug target molecules, followed by testing of drug molecules that can safely act only on the given target; (ii) a preclinical study, which usually lasts for one year, accompanied by *in vitro* and *in vivo* studies to elucidate the behavior of the drug molecule in a biological environment, and testing of the drug's efficacy and toxicity in at least two mammalian species; (iii) the clinical trial (phases 1 – 3), which lasts for 4 – 7 years, and the testing of the drug on voluntary human participants; and (iv) the review and approval, which takes 1 – 2 years. The first phase usually involves 20 – 80 people, and the main goal is to determine the side effects, as well as how quickly the drug is processed and when it is eliminated from the body. The second phase involves 100 – 300 people, and its main goal is to assess how effective the drug is in the patients with the disease compared to the placebo group. The third phase involves 1000 – 3000 people, where the effective but safe dose is ascertained compared to other existing therapies. In the last step, after obtaining approval by the appropriate committees, production is regulated, and post-release monitoring takes place (Figure 1)[1,4-10].

The drug discovery process usually takes 10 – 15 years and has several difficulties until a drug could be released to the market. The success rate is extremely low because most of the drugs fail on these tests because of unmanageable toxicity, lack of clinical efficacy, poor drug properties, or unappropriated strategies during development. Finding the safe and effective dosage of drugs is necessary for bioavailability prediction and successful development[11]. Furthermore, knowing of precise process of hepatic clearance – uptake, metabolism, efflux – is indispensable in effective drug discovery. Liver models are the golden references for drug testing, as studies on them are essential before the drug is released to the market[12]. In addition to its many functions, the liver is responsible for the breakdown and elimination of various drug molecules, so it is particularly important to examine how toxic a drug is to liver cells. Many models and services are currently available for drug testing, and many diseases have even been modeled using artificial tissues. It is a huge benefit for the drug developing companies to test potential drugs on three-dimensional (3D) models before treatment. As a result, potential drugs can even be tested on samples from patients so that the most optimal treatment for the patient can be selected. The liver models on the market are extremely reliable, because they have native tissue-like functions and structures, as well as viable and long-lasting co-cultures that can be safely used for disease modeling and drug testing (Table 1)[4,13,14].

2. The organ in focus: The liver

The liver plays a crucial role in upkeeping homeostasis in the body, as most of its constituent cells, such as hepatocytes, cholangiocytes, and other non-parenchymal cells, play a prominent role in performing metabolic, exocrine, and endocrine functions in collaboration with stromal, endothelial, and mesenchymal cells. During organogenesis, the embryonic progenitor cells of the liver, that is, hepatoblasts, originate from the posterior foregut endoderm. Hepatoblasts undergo morphological changes, proliferate, and migrate into the adjacent mesoderms to form the liver bud as a response to the signaling molecules secreted by the surrounding mesenchyme, such as fibroblast growth factor, bone morphogenetic

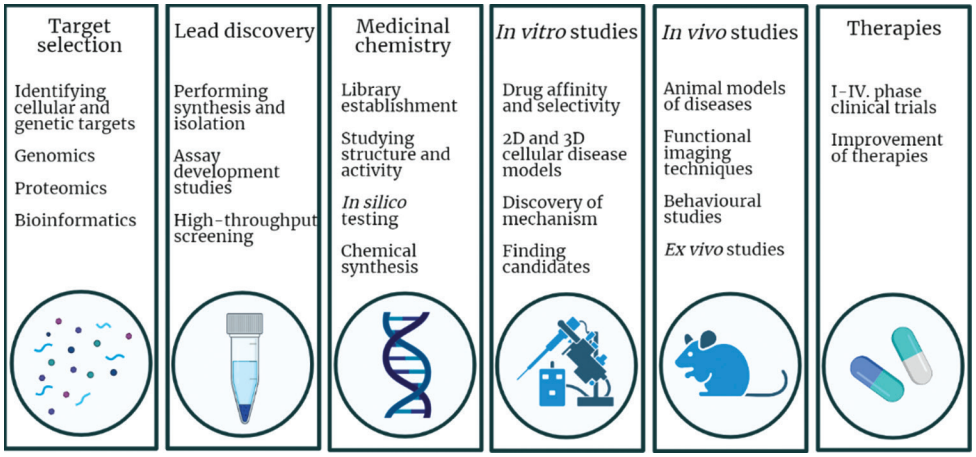

Figure 1. The main steps of the drug development process. Image created with BioRender.com.

Table 1. CYP enzymes and their substrates investigated in ADME testing

Substrates of CYP enzymes				
CYP1A2	**CYP2A6**	**CYP2B6**	**CYP2C8**	**CYP2C9**
4-Aminobiphenyl	4-Nitroanisole	7-Benzyloxyresorufin	Amiodarone	Diclofenac
7-Ethoxyresorufin	Coumarin	7-Ethoxy-4-trifluoro-methylcoumarin	Amodiaquine	Lauric acid
7-Methoxyresorufin	Diethyl-nitrosamine	7-Ethoxy-coumarin	Arachidonic acid	Lornoxicam
Caffeine	Indole	Bupropion	Dibenzylfluorescein	Mefenamic acid
Coumarin	Losigamone	Cinnarizine	DMZ	Naproxen
MeIQ	Methyl t-butyl ether	Deprenyl	Fluvastatin	S-Flurbiprofen
Melatonin	Nicotine	Loperamide	Retinoic acid	S-Ibuprofen
Naproxen	NNK	Propofol	Rosiglitazone	S-Warfarin
Phenacetin	Quinoline	S-Mephenytoin	Zidovudine (AZT)	Tienilic acid
Tacrine	SM-12052	Verapamil	Zopiclone	Tolbutamide
CYP2C19	**CYP2D6**	**CYP2E1**	**CYP3A4**	
Clobazam	4-Methoxy-amphetamine	1,2-Dichloroethene	1-Nitropyrene	
Diazepam	Bufurolo	4-Nitrophenol	7-Benzyloxyresorufin	
DMZ	Bunitrolol	Chlorzoxazone	Coumarin	
Imipramine	Debrisoquine	Dapsone	Erythromycin	
Omeprazole	Dextromethorphan	Dimethylnitrosamine	Felopidine	
Phenytoin	Imipramine	Ethanol	Ketamine	
Proguanil	Metoprolol	Ethosuximide	Midazolam	
R-Mephobarbital	MPTP	Isoprene	Nifedipine	
S-Mephenytoin	Propranolol	Paracetamol	Ondansetron	
Ticlopidine	Thioridazine	Salicylic acid	Verapamil	

protein, hepatocyte growth factor (HGF), and Wnt ligands. During the formation of lobes and establishment of liver bud, hepatoblasts can differentiate into hepatocytes or cholangiocytes after lineage-commitment[15-17]. Recent research has shown that a single Lgr5-positive hepatoblast can generate both hepatocytes and cholangiocytes *in vivo*. The fate of the hepatoblast is affected by signal transduction: subsets of hepatoblasts exposed to signals near the portal mesenchyme generate cholangiocytes, while hepatoblasts located farther from the portal mesenchyme are closely related to the hematopoietic cell. To support physiological functions, the adult liver should be maintained to support homeostasis. The ability of the liver to self-renew is typically 60 – 150 days in mice, slower than,

for example, the intestinal epithelium. Liver epithelial cell maintenance is primarily mediated through self-duplication of terminally differentiated cells. Despite the slower rate of cell proliferation in the liver tissue, the liver is still capable of extraordinary self-renewal and regeneration, although it is exposed to many damaging effects. If these effects are chronic, they may however lead to liver function defects and irreversible fibrotic damage. After partial surgical resection of the liver, a regeneration process takes place by the remaining healthy mature hepatocytes that respond to injury-induced signals (e.g., tumor necrosis factor alpha and interleukin [IL]-6) and restore physiological liver mass within a week[16-19]. This phenomenon has greatly improved the method of organ transplantation and liver resection in patients suffering from cancer. Nevertheless, toxin-mediated damage or chronic liver disease, such as non-alcoholic fatty liver disease, results in impaired liver cell function, so the regeneration process that restores liver mass cannot take place, as can be observed after partial hepatectomy. In this case, an alternative pathway is activated, during which the ductal cells become activated and begin to proliferate, thus restoring the liver tissue to its original state. Due to its large and remarkable ability to regenerate and self-renew, the liver-derived cells are extremely important *in vitro* tools in applied research, as they provide insights into development, function, and various diseases of liver (Figure 2A)[15-22].

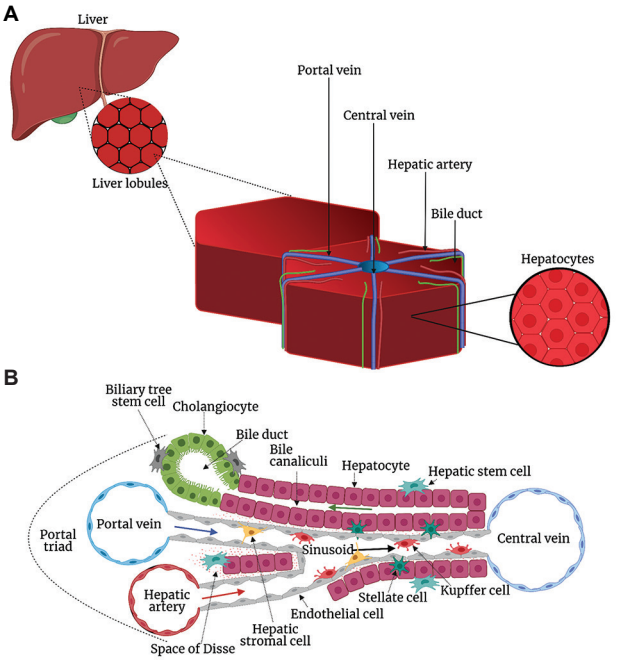

Figure 2. Structure and cell types of the liver. (A) The hexagonal building blocks determine the metabolic activity of the cells. (B) Cells that play key roles in drug metabolism, niche and homeostasis of the liver. Image created with BioRender.com.

3. Liver structure and function

In the case of the liver, it is especially true that function and structure define each other, and this 3D structure allows: (i) the control of carbohydrate, protein, lipid, and hormone metabolism; (ii) a number of detoxification mechanisms; (iii) the storage of glycogen, Vitamins A, D, and B12, ferritin, and blood; and (iv) the production of bile, bile acid, and bile dye, bilirubin metabolism and excretion, and selenium cofactor production essential for the function of various enzymes. Histologically, the liver is made up of the right and left lobes, which consist of three types of hexagonal lobules (classical, portal, and hepatic acinar). In the lobes, there are islands of hepatocytes in direct contact with the sinusoids, where the metabolic exchange between the blood and the hepatocytes takes place. Branches of the central vena cava are in the center of the hexagonal classical lobules, and the edges are connected by the branches of the hepatica arteria, the portal vein, and the bile duct forming the portal triad. The liver is made up of different cell types, such as the parenchyma (hepatocytes arranged in a single-cell-thick disk), connective tissue stroma in direct contact with Glisson's sheaths covering the outer surface, sinusoidal capillaries (which are capillaries covered with discontinuous and highly fenestrated endothelial cells between hepatocyte discs), and perisinusoidal or space of Disse located between the sinusoidal endothelium and hepatocytes containing Kupffer cells and stellate cells[15,16,20,23-29].

Due to their functions, hepatocytes are highly involved in the drug metabolism and transport, thus they are well-polarized and carry functionally different membrane domains to perform drug processing. Each side has a distinct function: the sinusoidal membrane part exchange solutes with blood, the lateral side shapes junctions between cells (tight junctions, desmosomes, and gap junction), while the canalicular membrane secretes bile through its efflux transporters. Circulation and drug uptake take place through the fenestrated sinusoidal membrane into the perisinusoidal space. Small lipophilic molecules can pass through the sinusoidal membrane by diffusion, while less lipophilic, amphipathic, and polar drugs are transported by sinusoidal uptake transporters. The canalicular membrane contains many efflux transporter proteins mainly from the ATP-binding cassette superfamily, which are responsible for bile salt export, multidrug, and toxin elimination. Since the basolateral membrane also includes some efflux proteins, where hepatocytes extrude the metabolites into the bloodstream, the direction of the drug transport pathway can be controlled by the liver. These facts corroborate that proper 3D structure and diversity of the liver are necessary for its function (Figure 2B)[15,16,20,23,28-31].

4. Recently used two-dimensional (2D) test systems

The liver plays a key role in drug processing, so investigating its response to different drugs is critical in pharmacokinetics. *In vivo* animal models are a usable and convenient tool for drug testing, but due to animal welfare considerations and their disadvantages, such as costly experiments and the fact that the physiology of animal cannot be recapitulate that of humans, animal models may not be the perfect tool for drug testing. There are many 2Dand 3D *in vitro* liver models, which are useful for ADME-Tox tests, but each has its own limitations[23]. Frequently applied 2D *in vitro* methods are using human primary hepatocytes (hNHEPS) and cell lines, immortalized hepatoma lines, stem cells-derived hepatocyte-like cells, liver slices, and microsomes. Modeling the liver architecture is an extremely difficult task because the cells have to be polarized, owning functionally distinct membrane domains, expressing the right type and number of enzymes, transporters and other proteins, and in contact with blood and bile flow to perform the proper and native tissue-like liver function. Finding the best 3D liver model is still a challenge; the current methods are mainly suitable for studying the intrinsic hepatic clearance in human in the presence of drugs[23-27].

Hepatocyte fractions as well as primary hepatocytes in two dimensions and in sandwich cultures are the most widely used models for mechanistic ADME-Tox studies at the early stage of drug development. 2D models cannot provide a proper structure, but the maintenance cost is relatively low; and the models are easy to handle, allows unlimited growth; and can be used with cell lines, immortalized hepatoma lines (HepG2, Hep3B, Huh7, anf HepaRG) and stem cell-derived hepatocyte-like cells. Other methods are liver slices, but they have major limitations, such as short-term maintenance and rapid loss of activity. These slices are able to produce albumin and urea, but they are not suitable for longer tests[32,33]. These models are often used for drug processing studies, but do not show long-term metabolizing activity and/or cannot express all metabolic enzymes. Furthermore, gene expression in cell lines would change during passaging, showing that their similarity to native tissue would lose with time. Applying immortalized hepatocyte cell lines, such as HepG2, HepaRG, Huh7, and Fa2N-4, might provide a solution for these limitations, as these cell lines are well characterized and less variable, but many of their hepatic functions are underexpressed or even totally lost in these cells[4,23,34].

Primary hepatocytes may be the best option among 2D models because they contain endogenous enzymes and transporters and are polarized in sandwich configuration, but their expression level and functional activity reduce in just few days in culture. Moreover, primary cells derived from different donors can be used to investigate interindividual differences but they have lower reproducibility. 2D primary hepatocytes and sandwich cultures dedifferentiate in a couple of days and lose many of their hepatocyte-specific functions, such as metabolic capacity, transporter expression, and sensitivity to toxic effects. Freshly isolated hepatocytes are known as the best cell type in resembling the intact liver, though recently, cryopreserved cells have been found to maintain hepatocyte characteristics quite well, so they might be a surrogate to freshly prepared hepatocytes and provide a solution for addressing the limited availability of fresh liver tissue. The interindividual variability of human hepatocytes often makes the interpretation of preclinical drug testing challenging[23,35] In pharmacokinetic research, using primary hepatocyte cultures from humans and other species is the standard practice, which facilitates the identification of unique human metabolites and the exploration of proper liver models that can be used in the testing of drugs for human diseases. Important steps in drug development are metabolite prediction, biological barrier modeling, prediction of *in vivo* pharmacokinetic processes, mitochondrial toxicological testing, and quantitative *in vitro-in vivo* extrapolation[1,23,36-39].

5. Micropatterned hepatocyte cultures

In the micropatterned co-culture technique, 24- or 96-well plates are used. Collagen islands with a diameter of 500 μm are fabricated on these plates at a distance of 900 – 1200 μm from each other. Collagen islands provide a 3D extracellular matrix for primary human hepatocytes, so the cells are able to form organ-like morphology and polarity. In this case, the inter-island space is filled with supporting J2-3T3 murine fibroblast. The co-culture system of primary human liver cells and J2-3T3 mouse embryonic fibroblasts is sustainable for 4 – 6 weeks and is suitable for drug testing, representing an easy-to-use, robust and reproducible system formed simultaneously from fresh or cryopreserved hepatocytes derived from multiple donors. Once the co-culture is established, the islands can be infected with a virus involved in various liver diseases, making it an excellent model for mimicking individual liver diseases (Figure 3)[40-43].

Khetani *et al.* found that HepaRG/3T3-J2 co-cultures produced higher albumin than mono-cultures after 4 weeks of culturing and showed increased sensitivity to drug-mediated CYP induction and hepatotoxicity since they have more stable albumin and CYP activity[40]. The micropatterned hepatocyte cultures are expensive and not suitable for high-throughput screening, which are some of the drawbacks of this technique.

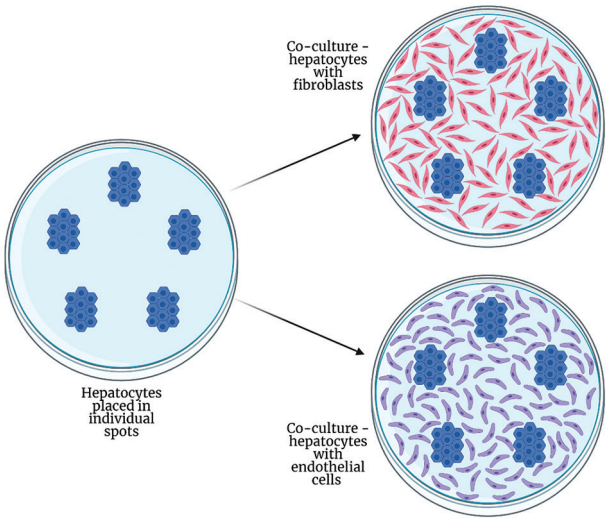

Figure 3. The micropatterned co-cultures. In these co-cultures, the hepatocytes placed in individual islets on a plate, and then fibroblasts or endothelial cells were seeded around them. Image created with BioRender.com.

6. Spheroids

Spheroids are 3D spherical cellular aggregates in which a cell-cell interaction is formed, permitting the observation of their morphology and polarity. It is known that in spheroids made of the HepG2/C3A cell line, a canaliculi-like structure can be observed and bile acid production is detectable too[44,45]. Spheroids could be generated in both scaffold-based and scaffold-free ways, but the structure is divided into three shells in each case. The outer shell is the proliferation zone where the cell proliferation potential is the highest; the quiescent zone is located at the inner side where the cells are at rest; and the innermost side, the core, has a zone of necrotizing cells because nutrients and oxygen cannot reach there. Their maintenance of spheroids is simple, and they can be generated from many cell types. Spheroids are involved in many drug development methods, especially tumor spheroids used in cancer research, which offer more insights into tumor biology due to their biosimilarity to solid tumors *in vivo* with regard to cell morphology, proliferation, oxygenation, nutrient uptake, waste excretion, and drug uptake. Since the geometric localization of cells is crucial for proper function, the spheroid models can be applied in toxicology tests, so they can serve as suitable pharmacological tools[38,46-55].

Spheroids can be produced in different ways, such as using of ultra-low attachment plates, hanging drop technique, magnetic levitation and magnetic 3D printing, spinner flasks, matrix encapsulation, matrix-on top and matrix embedding, microcarriers beads, and microfluidic devices. Several studies declare that these techniques could produce spheroid within 24 – 72 h [56].

Several papers accentuate the important role of spheroids in toxicology studies. Penzes *et al.* examined the toxic effect of primycin-sulfate in 2D and 3D HepG2 and hNHEPSco-cultured with fibroblasts in 1:4 ratio. They used V-shaped plates to form aggregates within 24 h and observed that the level of primycin-sulfate induced cell death was lower than in 2D cultures. Furthermore, the non-toxic level of primycin-sulfate treatment resulted in an increased expression of IL-11 and IL-24[57]. Bell *et al.* used ultra-low attachment plates to create spheroids from primary human hepatocytes and observed that the spheroids are suitable for modeling of drug-induced liver injury (DILI). They found that bile acid accumulated on chlorpromazine treatment and viral infection enhanced trovafloxacin-induced toxicity[37].

7. Organoid

An organoid is "a collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to *in vivo*"[58,59].

Studying the development and the diseases of human organs using *in vivo* experiments is challenging due to the significant interspecies differences between animal models and humans. A better understanding of the biological role of extracellular matrix and mapping of the regulation of signaling pathways in the microenvironment of stem cells have been made possible in the development of organoid culture systems. The terms of organoid include a wide range of different systems, but Fatehullah *et al.* set forth the most accurate definition of the term so far; they define "an organoid as an *in vitro* 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells (iPSC), capable of self-renewal and self-organization and exhibiting similar organ functionality as the tissue of origin"[60].

In the human body, cells are present in a highly complex microenvironment, and many synchronized signaling interactions result in a proper development, regulation, and maintenance of cell phenotype and function. Furthermore, cells in tissues are affected by signaling effector molecules, nutrients, and waste products along different concentration gradients. This phenomenon is mimicked by some 3D culture systems where cells are at the center of an aggregate or organoid and they have less access to media factors, as opposed to 2D systems. The development of physiological, biochemical, and biomechanical microenvironments by 3D techniques can affect cell proliferation, differentiation, cell survival, morphogenesis, cell migration, and mechano-reactions.

Based on the previous research, mouse and human organoid systems are promising models for therapeutic applications and are more physiologically relevant than previous models, and they allow genome engineering and manipulation of signaling pathways[7-9,34,48,58,60-77].

Organoids generated from tissue-resident stem cells or progenitor cells derived from adult or embryonic human tissue require special maintenance conditions. The cultivation of organoids must ensure that the *in vitro* microenvironment is similar to the physiological attributions of the tissue; therefore, medium composition, physical environment, and hydrogel should be carefully selected. By creating these conditions, multipotent progenitors will be able to follow their own built-in program and self-organize into 3D organoid structures through proliferation, and they must be similar to its origin tissue. Recent data show that primary cell-derived organoids are suitable for screening drug toxicity and studying molecular mechanisms of organ-specific functions, while stem cell-derived organoids can be applied as an effective model for organogenesis and development. Organoids are appropriate for reproductive toxicology studies and more suitable than animals in preclinical studies, because of interspecies differences[7-9,34,48,58,60-77]. To form organoids, progenitor cells and an extracellular matrix-like scaffold are needed to create a cell-cell and cell-matrix interactions. These interactions are essential for the cells to gain polarity and proper cell function, and after that, the concentration gradient can be observed too (Figure 4)[78].

Chesne *et al.* created an organoid system using cryopreserved differentiated HepaRGs and human liver non-parenchymal fractions (NHS) in 1:2 ratio. They exposed the cells with acetaminophen to simulate drug- DILI. They used fibrotic compounds, allyl alcohol and methotrexate, and observed that NHS is only activated in 3D co-cultures[77].

8. 3D bioprinting

3D tissue printing is a dynamically evolving, computer-controlled microarchitectural technology that uses living cells, molecules, and biomaterials, called hydrogels, to create complex and functional tissue structures. 3D tissue printing offers many opportunities for researchers in the field of regenerative medicine, as complete organs can be built layer by layer using raw biomaterials and living cells directly from the patient. The complexity of different tissues can also be excellently modeled using human-derived cells and biomaterials, and the resultant model can yield much more realistic responses in drug testing. Thus, 3D biofabrication undoubtedly outperforms the traditional 2D cultures[36,79-84]. A further advantage, in addition to its structural features, is that different types of tissue-specific cells can be incorporated into an artificial organ that function together, but can be in the same or separate modular units, as in the case of the original organ. In addition to the presence of tissue-specific cell types, the role of the extracellular matrix can also be easily mimicked, so that the cells are in the correct 3D position

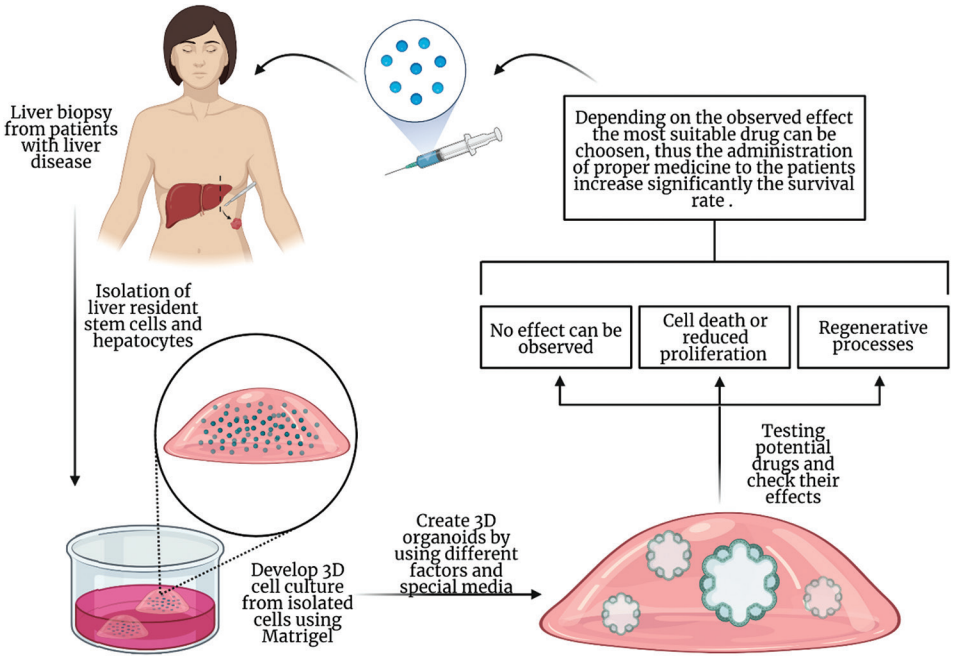

Figure 4. Drug discovery and personalized medicine using organoids. Created with BioRender.com.

and their native microenvironment can also be mimicked. Thanks to the porous structures, blood flow can also be modeled, and even vascularized tissues can be built. The success of implantation depends on many factors, but the most important include functionality, proper remodeling, and a satisfactory host-graft response. *In vivo* research on the effects of transplantable 3D biofabricated tissues on an individual is still ongoing, but by improving the method, it will be possible in the future to replace damaged or missing human organs with new ones that are not only functional, but also able to cooperate with the human innate organs too[5,9,10,83,85-92]. The development of preclinical models suitable for toxicological tests is of particular importance (Figure 5). Finding the ideal model is essential for quickly identifying novel medications with excessive toxicity at an early phase[10,82]. Since organs have complex structure, spatial location and presence of cell types characteristic of organs are essential for appropriate function. A lack of proper 3D structure prevents the measurement of toxicity because different medications may cause different reactions in different cell types[82,93]. The liver and kidneys play a central role in the elimination of drugs, but the skin is also exposed to many toxic agents[10,82,94,95]. Characterizing their response to various drugs is essential for drug development since systemic drug toxicity has a significant impact on these organs. Modeling presents numerous challenges, including the selection of appropriate spatial arrangement and cell type as well as the selection of a suitable hydrogel. In many cases, the hydrogel affects the cell viability, reproduction rate, morphology, and spatial location. Therefore, the selection of appropriate hydrogel is critical, because the cells need to be encased in a tissue-like, biocompatible extracellular matrix that must be soft enough to allow cells

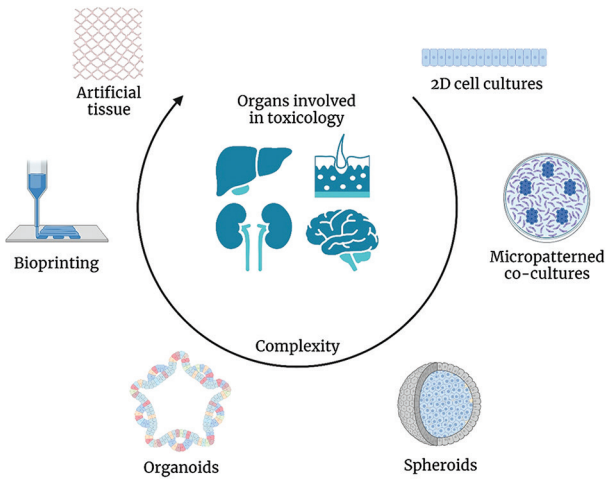

Figure 5. 3D models in toxicology testing. Created with BioRender.com. 3D cell cultures can be developed for drug testing so as to allow the selection of suitable medicine for patients.

to expand and proliferate, while remaining stiff enough to adhere during migration[10,47,83,85,86,88-90,96]. The blood vessels, tubules and lumens found in organs are extremely difficult to bioprint, but tissue mimicry will fail without these structures. In the absence of channels, nutrients and oxygen could diffuse to a maximum of 200 μm, so it is not allowed to build larger structures. Bioprinting of organs with tubular structure, such as the kidney, is also challenging for researchers. The tubular structure of the kidney allows it to function properly, so the 3D structure has to resemble the original structure. Applying sacrificial hydrogels makes it possible for printing lumens and tubules, by filling the interior of the lumen with liquefiable material that can be washed out at the end of the printing process so as to leave an empty tube[79,96,97].

According to ISO/ASTM 52900:2015-12 Standard Terminology for Additive Manufacturing, there are three categories of bioprinting, namely material jetting, material extrusion, and vat polymerization. This review provides a brief introduction of widely used bioprinting methods (Figure 6)[10,47,83,85,86,88-90].

8.1. Material extrusion

Extrusion techniques apply pneumatic or mechanic pressure to eject bioink through a nozzle. The pneumatic approach uses air flow to compress bioink, while the mechanic one works with axial piston to jet or form droplet. Both variants have one or multiple cartridges fixed on a moveable XYZ platform and a printing surface. The design of parameters and printing process are computerized, allowing printing of defined structure. Extrusion-based bioprinting is able to manage a variety of hydrogels, high cell density, and constructs with complex structure and composition. These features allow the printing of heterogenic biomimetic structures; therefore, this kind of technique is suitable for tissue/organ printing. Further advantages, such as affordability, easy handling, and commercial availability, make this technique the most common printing method. The drawback of this technique is that the high pressure causes shear stress effects on cells, so the cell viability rate of this technique is lower than that of the jetting techniques. Issues in nozzle clogging, printing resolution, and speed could be a problem in several cases[80,94,98,99].

8.2. Material jetting

8.2.1. Inkjet bioprinting

Inkjet bioprinting could be divided into continuous, drop-on-demand, and non-drop-on-demand inkjet bioprinting. Since the continuous method is not used for bioprinting, it is omitted from discussion. One of the main advantages is

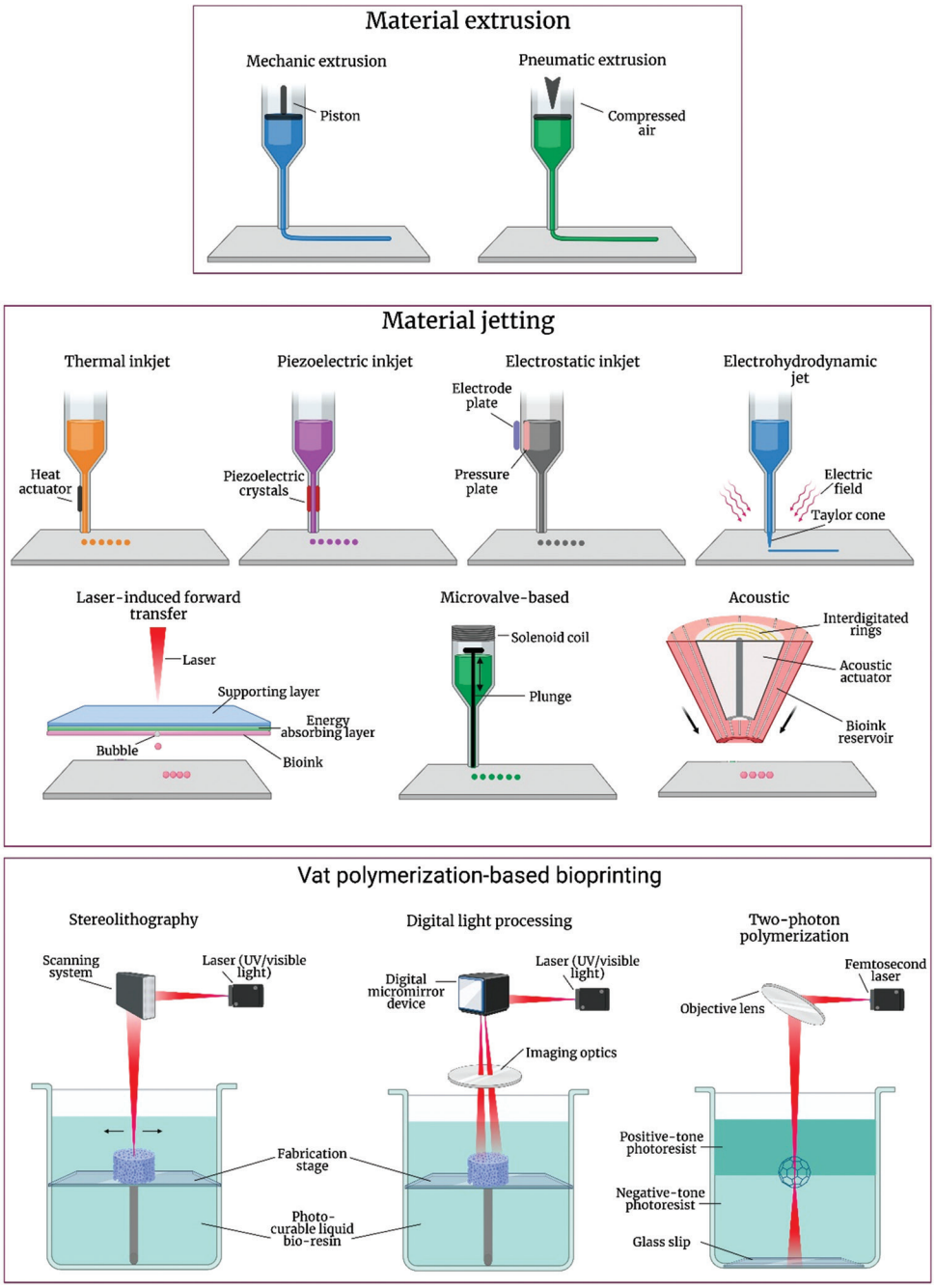

Figure 6. The main types of 3D bioprinting. Despite the fact that different technological solutions are used, the basis and principle of printing are similar in each case. Image created with BioRender.com.

that the nozzle creates droplets only when emitting signal presents, thus drop-on-demand bioprinting is a very accurate, high-throughput and efficient printing method, which is highly controllable. Since the droplets could be positioned precisely into desired spot and even without pressure force, the surface tension inhibits the leakage of ink, and the droplet formation occurs only when pulse energy transcends the threshold[80,100]. Its attribution gives an opportunity to avoid the droplet charging, ink circulation, and electrostatic field generation. Altering the densities or volumes of droplets establishes gradient concentration between distinct areas of printed material. Several hydrogels, such as agarose, alginate, collagen, fibrinogen, gelatin methacrylate (GelMA), and thrombin, could be utilized for this technique, which also allows scaffold-free printing. Material jetting can be categorized into four

different subtypes: thermal inkjet bioprinting, piezoelectric inkjet bioprinting, electrostatic inkjet bioprinting, and electrohydrodynamic jet bioprinting[10,80,90,94,100-106].

(A) Drop-on-demand methods

Thermal inkjet bioprinting is based on a heat actuator located in chamber. It develops heat bubbles during printing by heating the ink locally for several microseconds with high temperature (250 – 350°C). The brief but very high heating phase causes the vaporization of bioink, and then this heat bubble bursts, forming the driving force of droplet ejection, since ink is forced toward the nozzle outlet to emit the droplet. This method is preferred because it is relatively cheap and has fast printing speed as well as its extremely brief heating phase only increases the material temperature by 4 – 10°C above room temperature, so most of the cells remain viable. Since droplet formation depends on explosion of heat bubble, the optimization and maintenance of standard printing protocol is difficult to approach, and shear stress could also affect the cells[10,80,90,94,100,102-107].

Piezoelectric inkjet bioprinting takes advantage of piezoelectric crystals located on the inner wall of chamber. Piezoelectric actuator converts voltage into mechanical energy. The pulse crystals undergo deformation in the presence of voltage, thus squeezing the ink and promoting droplet formation. High viability rate was observed after printing although the mechanical shape formation caused the generation of ultrasonic waves and the shear stress could be harmful to the cells. Drop-on-demand method is a popular technique because of a few features, such as easy control of droplet production, availability of a wide variety of nozzle sizes, and easy cleaning[10,80,90,94,100,102-107].

Electrostatic inkjet bioprinting applies a pressure plate placed in chamber, which allows the squeezing of ink during droplet formation. The pressure plate could move between two positions because of static electricity to influence the chamber volume. In circuit, the pressure plate is attracted to the electrode plate, thus increasing the holding capacity of chamber. When the circuit is disconnected, the attraction is immediately ceased and the plate returns to its original position. This action reduces the chamber volume, and the ink leaves the nozzle as droplet. It is a very safe method for printing cells, since the bioink is not exposed to heat or sonication, but the only concerns are small nozzle diameter and shear stress[10,80,90,94,100,102-107].

(B) Non-drop-on-demand methods

Electrohydrodynamic jet bioprinting is different from previous methods because it uses electric fields for droplet emission. The bioink is mechanically pulled to the aperture of nozzle, creating a meniscus between ink and substrate ground. The application of electric force causes the formation of Taylor cone, which is the result of a state of equilibrium between the Maxwell forces present in the molten liquid and the surface tension that maintains the meniscus. The electric fields promote the accumulation of charges that gives rise to the formation of sharper cone, and when the surface charges exceed surface tension, the ink could be ejected from the nozzle. The main characteristic is that the droplet is pulled out by electric field between the ink and the substrate plate and it cannot eject single droplet. A great advantage of this technique is that the droplet size could be variable. Due to the Taylor cone-dependent droplet emission, the droplet could be smaller than the diameter of nozzle, thus increasing printing resolution and allowing printing of viscous hydrogels[10,80,90,94,100,102-108].

8.2.2. Laser-induced forward transfer (LIFT)

The laser-LIFT method applies a laser generator, a laser path-adjustment module consisting of mirrors and focusing lenses, and a cell transfer module for bioprinting. In general, the cell transfer module contains a ribbon and a substrate layer. Two setups of LIFT exist and require different ribbon constructs: matrix-assisted pulse laser evaporation direct writing without energy absorbing layer and absorbing layer-assisted LIFT (AFA-LIFT). For bioprinting, the AFA-LIFT method is more suitable, since it has energy-absorbing layer to protect the cells. In this setup, the ribbon consists of three layers: a transparent and thick supporting layer, a nano-scale laser-absorbing sacrifice layer, and a bioink layer containing the transferrable material. During printing, the laser generator launches the laser beam throughout the laser path-adjustment module, which directs and focuses the beam to the desired spot on the upper side of sacrifice layer. When the laser beam reaches the bioink layer at the irradiation point, a bubble is spawned and grows until the bioink elongates. The expansion drops the inner pressure results in bubble collapse, and the high pressure in bubble pole drives the jet or droplet formation. Then, the substrate layer receives the apex of stretched bioink, thus a small portion of ink is printed. LIFT has excellent features, including: (i) nozzle-free and very fast (5,000 droplets/sec) printing speed; (ii) micro-scale resolution; (iii) ability to handle high cell density within bioink; and (iv) highest viability rate among all bioprinting methods. Other than that, LIFT gives the opportunity to achieve in situ bioprinting and can be combined with other printing techniques. Despite its advantages, there are several flaws needs to be improved, especially in the aspects of efficiency, productivity and building cost[10,80,84,90,94,102-105].

8.2.3. Microvalve-based bioprinting

The process of droplet formation applies electromechanical micro-valves made up of plunge and solenoid coil, which is

located on the aperture of nozzle. The gas regulator keeps the bioink under pneumatic pressure, thus contributing to the ink ejection. The valve opening depends on micro-valve parts and back-pressured bioink. On receiving voltage pulse, solenoid coil generates magnetic field, pulling the plunge upwards to unblock the orifice and eject the bioink. This technique applies variable nozzle sizes and low pneumatic pressure, which is favorable for avoiding cell damage, but it could not achieve high resolution printing because of the larger size of droplets[10,80,90,94,101-105,109].

8.2.4. Acoustic bioprinting

Acoustic bioprinting is a nozzle-free method, and the droplet formation is based on acoustic waves during printing. The acoustic actuator is made up of interdigitated gold rings placed on a piezoelectric substrate located in a pool. The actuator is surrounded by the bioink in microfluidic channels, with exits at the bottom. The actuator generates gentle circular acoustic waves, which create acoustic focal points on the interface between bioink and air. The acoustic radiation-impinged force overcomes the surface tension at the exit of channel, so that the bioink could be ejected. Since it does not use nozzles and mechanical forces to form droplet, the cells avoid shear stress, heat shock, high pressure and voltage, preserving the high rate of viability. However, viscosity of hydrogel and cell density could be an obstacle of printing[10,80,90,94,101-105,110].

8.3. Vat polymerization-based bioprinting

Vat polymerization-based bioprinting can be divided into three categories: stereolithography, digital light processing, and two-photon polymerization. Building process occurs when the photo-curable/photo-activable liquid bio-resin is radiated by a laser source and the cross-linking through photo-polymerization solidifies the material. Biocompatibility of vat polymerization is significantly lower than the above-mentioned techniques. Since the printing circumstances do not promote cell survival, it is mainly used for creating tissue scaffolds made for traditional cell seeding. In most of the cases, post-curing is necessary and printing requires support material, which has to be removed at the end of fabrication process, as well as non-polymerized resin. However, these setups are commercially available and suitable for rapid printing of large-volume, highly detailed structures[10,80,90,94,102-105,111,112].

8.3.1. Stereolithography

Printing process is based on photo-polymerization of photo-curable liquid bio-resin. A UV/visible light laser radiates bio-resin with its specific curing wavelength; therefore, polymers could be formed to print desired scaffold. There are two possible ways to polymerize bio-resin: top-down and bottom-up methods. In both cases, the elevator moved fabrication platform is sunk into a photo-curable bio-resin filled vat, and a scanning system coordinates the laser beam. Top-down printing approach applies a laser right above the bio-resin vat, and the printing stage is descended after every cured layer to build 3D structure. In contrast, the bottom-up method uses a laser source located below the vat and printing stage is raised above each cured layer through a peeling step. This technique significantly slower than the top-down approach if peeling step is included. Both methods require manually removable supports to print 3D structures, which are built from the same material as the printed construct. The appropriate cross-linking between fabrication stage and printed structure is crucial, and finding the right cross-sectional area of each printed layer is necessary to avoid damages during peeling step. To perform photo-polymerization, the density of radiation has to overcome the threshold to initiate curing process, but excessive radiation could shrink bio-resin. Stereolithography is a fast, flexible, and accurate printing method, but this technique requires an expensive setup and the fragility of constructs printed by this technique may cause a problem[10,80,90,94,102-105,111,112].

8.3.2. Digital light processing

The set-up of digital light processing is very similar to that of stereolithography, and it utilizes properties of photo-curable bio-resins but applies a digital micromirror device instead of a scanning system. Digital micromirror device contains high number of rotatable micromirrors and allows projecting an image on printing stage. This feature facilitates the immediate solidification of an entire layer, achieving rapid printing speed. The layer thickness and exposure duration need to be controlled, depending on the applied bio-resin, to ensure strong interface bonding. Rapid printing speed and accuracy make this technique popular, but mechanical properties of built structure have to be improved[10,80,90,94,102-105,111,112].

8.3.3. Two-photon polymerization

Two-photon polymerization is suitable for printing complex high-resolution 3D micro- and nano-structures. In polymerization based on two-photon absorption, a molecule absorbs two photons within an extremely brief time interval, converting its ground state to excited state. The printing process occurs in a bio-resin-filled vat with a glass slide at the bottom, and near-infrared femtosecond laser radiates oil-immersion objective lens. The transparent liquid bio-resin acts as a photoresist material that contains negative- and positive-tone photoresists. Through objective lens, the laser beam is focused precisely onto the photoresist above the glass cover slip, thereby cross-

linking bio-resin by moving the beam. The polymerization through two-photon absorption allows direct printing into negative-tone photoresist, while reverse imprint is formed in positive-tone photoresist[111-115]. During printing, the laser power has to be kept in a range between polymerization threshold and burning threshold to achieve fine structure formation without material damage. The printed microstructure could be extracted from bio-resin by ethanol washing to remove unpolymerized negative photoresist. Its features facilitate high-spatial resolution, since two-photon absorption only occurs in the focal point, termed submicron-size voxel. Another great property of this technique is that the near-infrared laser is able to penetrate deeply into the photoresist and to print in three dimensions. Its drawbacks include long printing time and lack of capability to print scaffolds for soft tissues[10,80,90,94,102-105,111-115].

8.4. Recent achievements in 3D tissue bioprinting

At present, the 3D structure of the nephron is too complex to be bioprinted as a complete unit. Therefore, researchers must select the segment most affected by renal toxicity, and the proximal tubule segment is commonly selected for this purpose[116-118] Homan *et al.* developed a tubule-like structure with proximal tube epithelial cells and sacrificial hydrogel for forming a tube that is suitable for investigating the mechanism of drug-induced tubule damage[119]. They bioprinted a tubular structure with gelatin-fibrin hydrogel on the outside and liquefiable Pluronic F147 on the inside, which was removed at the end of the process. Thus, they created a vascularized construct with a fully epithelialized, perfusable channel, and albumin uptake, cyclosporin A-induced nephrotoxicity and polarized epithelium could be observed. A few years later, Lin *et al.* developed a model with two perfusable channels using sacrificial hydrogel[120]. In this kidney construct, they seeded proximal tubule epithelial cells into one of the two tubules and glomerular microvascular endothelial cells into the other one. They observed an active tubular-vascular exchange, albumin uptake, and glucose reabsorption. Lawlor *et al.* showed a high-throughput, self-organized kidney organoid system made up of pluripotent stem cells, that is suitable for nephrotoxicity testing using extrusion-based bioprinting[121]. This method makes it possible to produce organoids 15 – 20 times faster, with high reproducibility, than the manually made organoids. King *et al.* created a co-culture of renal fibroblast and human umbilical vein endothelial cells (HUVEC) in 50:50 ratio, dispensed Novogel and bioprinted onto a transwell insert[122]. They created an epithelial layer capable of producing large amount of extracellular matrix and maintaining a functional renin-angiotensin system and barrier.

The skin is also responsible for defense mechanisms, and as the first barrier of the body, it is impacted by many harmful agents that cause irritation, corrosion, or sensitization. Thus, it could not be excluded from toxicity studies. Because skin has multiple layers, cell types and appendages, creating native tissue-like models is difficult[123-127]. Abaci *et al.* demonstrated a human skin model with a perfusable vascular network using primary and iPSC-derived endothelial cells[128]. They built a micropatterned vasculature layer using sacrificial hydrogel, and dermal fibroblasts suspended in collagen type I were seeded around the sacrificial layer. Following the formation of the dermal region, keratinocytes were added to form an epithelial layer. This construct was used for *in vitro* perfusion experiments or *in vivo* grafting. Their results demonstrated that micropatterned vascularization enabled the development of complex human skin equivalents that are graftable and suitable for drug toxicity testing. Min *et al.* used human dermal fibroblasts, epidermal keratinocytes, and epidermal melanocytes to create a full-thickness pigmented skin model[129]. The printed tissue showed pigmented clusters consisting of melanocytes and keratinocytes and active melanin production confirmed by histological staining. Ng *et al.* developed a two-step bioprinting method to produce a pigmented human skin construct and produced a biomimetic dermal region out of different densities of human fibroblasts and collagen, resulting in a hierarchical porous structure[130]. To achieve native tissue-like structure, they printed human keratinocytes and melanocytes in a well-designed pattern onto the dermal layer. This technique allows the presence of melanin units as well as the creation of suitable microenvironment. The epidermal region resembled native skin in terms of melanin granules distribution and presence of biomarkers, such as HMB-45, K1, K6, and collagen type VII.

Hong and Song developed a gelatin-alginate based HepG2 3D model[131]. They used gelatin and sodium alginate in phosphate-buffered saline at a concentration of 40% (w/v) sterilized by UV light. The hydrogel-cell mixture, consisting of 10% gelatin, 4% alginate, and HepG2 cells, was printed as spheroid structures in mini-well dishes. They observed that this 3D model could mimic the organ complexity better than the 2D models and is suitable for hepatotoxicity tests[131]. Kizawa *et al.* created a bioprinted liver tissue, which consists of primary hepatocytes, using scaffold-free technology of Cyfuse Biomedical. This model is suitable for drug testing since it retains drug transporter proteins and metabolic enzymes expression[132]. Ma *et al.* reported their 3D-bioprinted tri-culture, developed by digital light processing-based method. Their liver model,

which consists of encapsulated human induced pluripotent stem cell (hiPSC)-originated hepatocytes, HUVECs and adipose-derived stem cells (ADSCs), was printed in a pattern that mimics the liver lobule structure. They used 1%, 2.5%, and 5% (w/v) GelMA to encapsulate endothelial cells and mesenchymal stem cells. After printing, they maintained the tri-culture and observed the expression of fetal hepatic marker α-fetoprotein, albumin (ALB), hepatocyte nuclear factor 4α (HNF4α), and transthyretin. This model showed the expression of different CYP450 enzymes too, such as the, CYP2B6, CYP2C9, and CYP2C19, and on the addition of rifampicin, the CYP3A4, CYP2C9, and CYP2C19 were induced[133]. Faulkner-Jones *et al.* developed a 3D model by valve-based inkjet bioprinting[134]. They printed hiPSCs and human embryonic stem cells and the cells were differentiated into hepatocyte-like cells. Differentiated cells expressed HNF4α and albumin so this model is suitable for drug testing, and the bioprinting process did not affect the viability and pluripotency of the cells[88,134]. Lei and Wang created a model using ADSCs and primary hepatocytes to form a complex mini organ with vascular systems[135]. With this four-nozzle low-temperature technique, the printing of liver organoid and other complex tissue can be performed (Table 2)[88,135].

Due to the complexity and coordinated functioning of human organs, 3D printing faces an extremely difficult challenge. In recent years, research has proven that we are getting closer to printing artificial tissues that function largely similar to the original organ. As soon as it becomes possible to print tissues that are identical in structure and function, the fields of toxicology, personalized medicine, and regenerative medicine will usher in the era of tremendous development. Despite the many difficulties in the printing of artificial 3D tissues, it has been proven that 3D printed tissues could ensure fast and efficient drug testing in the future[82,93].

9. Clinical use

Non-biological and biological liver support is available for the treatment of patients with acute liver failure. Biological methods take advantage of the functional capacity of xenogeneic or human-derived liver cells, thus supporting the function of the patient's liver. These functions include detoxification, metabolic functions, and synthesis of proteins and other molecules. One of the most effective clinically used bioartificial liver devices is the AMC-Bio-Artificial liver (AMC-BAL) system, a product developed by a research group in Netherlands. This product is a hollow fiber, polysulfone-coated bioreactor and plasmapheresis system. At least 1×10^{10} viable human (previously porcine) liver cells in a 3D configuration are attached to a nonwoven material in a hydrophilic polyester matrix. The matrix is 4 mm thick and its total surface is 5610 cm^2, which are helically wound around a huge core. Between the layers of the matrix, the on-site gas exchange takes place through hollow fibers in a longitudinal direction. During the treatment, blood of the patient is subjected to plasma filtering; the filtered plasma is received by the bioreactor that perfuses the blood cells. One of the most important qualities of AMC-BAL is the direct relationship between the small islets of liver cells and the incoming plasma, and its structure ensures optimal mass for liver cell transfer and direct oxygen supply[21,136-140].

3D tissue printing may be particularly suitable for the regeneration and/or replacement of diseased or damaged tissues. In such a case, it is important to design a proper structure so that the cells can have the correct polarity and function. When using non-synthetic scaffolds, decellularized liver tissue is considered an extracellular matrix. The technique involves decellularizing the target organ and removing all living cells and debris to leave behind the intact extracellular skeleton. The quality of the matrix is then checked and recellularized with healthy, tissue-specific cells.

Table 2. Tissue engineered liver models for drug testing or clinical use

Cell type	Bioink	Results
Hepatocytes	Gelatin	Hepatocytes showed high viability for more than 2 months and their biological function remained intact
Primary human hepatocytes, hepatic stellates, HUVEC cells, and non-parenchymal cells	NovoGelR 2.0 hydrogel	The cells were viable for 28 days
Primary mouse hepatocytes	Galactosylated alginate	The viability was >85% after 2 days
HUVEC	-	Multi-layered model for testing hepatotoxicity
Primary mouse hepatocytes	Alginate	The cells were viable for 14 days
HepG2, BM-MSCs	Decellularized extracellular matrix	Liver tissue model
hiPSCs, hESCs	RC-6 and alginate	The viability of cells decreased to >55% after 1 day
Primary hepatocytes	-	The cells were viable for 60 days

HUVEC, Human umbilical vein endothelial cells; BM-MSCs, Bone marrow mesenchymal stem cells; hiPSCs, human induced pluripotent stem cell; hESCs, human embryonic stem cells.

These are the first steps in creating transplantable liver grafts that can be implanted back into the patient.

Another artificial liver tissue graft, the mini-liver, may also be suitable for tissue regeneration or replacement. During the creation of the mini-liver, a 3D structure was created using a hydrogel and then implanted into nude mice after partial liver resection and radiation-induced liver damage. Mice were divided into four groups: control, hydrogel, hydrogel with HL-7702, and hydrogel with HGF. It was found that the hydrogel did not reduce viability and did not interfere with cellular functions, and after implantation of the hydrogel, which also contained hepatocytes, liver regeneration was enhanced and the individuals showed longer survival times.

Personalized therapies are facilitated by the creation of various artificial liver tissues, such as the Organovo ExVive™ system, which is suitable for studying DILI and related mechanisms at the tissue level. Several manufacturers provide artificial and bioprinted 3D liver tissues composed of liver tissue-specific cells, such as hepatocytes, hepatic stellate cells, Kupffer cells, and endothelial cells (Table 3).

10. Challenges and perspectives

Stem cells derived from the embryonic connective tissue of mesoderm origin, that is, mesenchyme, do not have the ability to form gametes, but being multipotent cells, they are able to differentiate into many different cell types within the body. In humans, they are found in the bone marrow and non-marrow tissues such as placenta, umbilical cord, adipose tissue, muscle tissue, corneal stroma, and dental pulp of deciduous teeth. In addition to tissue formation, these stem cells are involved in tissue regeneration and play a significant regulatory role in inflammation and carcinogenesis. Taking advantage of this ability, they are suitable to be used for therapeutic purposes and in the field of regenerative medicine.

During biofabrication or tissue engineering, a complex biological structure is created, which is almost identical in morphology and function to the native organ. Using raw biomaterials, cells, matrices, and engineering precision, these organ modules are printed, which, due to their similarity and function, can be used for regeneration or replacement

Table 3. Bioprinted and artificial liver models for transplantation

Device	Manufacturer	Description
ExVive™	Organovo®	3D bioprinted human liver tissue or functioning artificial liver tissue, which is suitable for *in vitro* investigation of DILI
Hepa-Mate™/HepatAssist	HepaLife	Biological artificial liver, cryopreserved porcine hepatocytes
Scaffold-free liver structure	Cyfuse	Bioprinted scaffold-free liver spheroids, which can be used for *in vitro* testing
3D liver tissue	Pandorum Technologies	3D hepatocytes spheroids, which are capable of long-term albumin secretion and CYP expression and suitable for DMPK and hepatotoxicity studies
3D liver tissue	Pandorum Technologies	3D liver organoids, which contain parenchymal hepatocytes, Kupffer cells, hepatic stellate cells and liver endothelial cells and are suitable for NASH drug discovery
Mini liver	Pandorum Technologies	Prototype of implantable vascularized organoids for bioartificial liver with a potential of engrafting by transplantation
HμRELhuman™/HμRELhumanPool™	HμREL® Corporation	Self-assembling primary hepatocyte-based microlivers co-cultured with non-parenchymal stromal cells in microtiter plates
HepaPredict system	HepaPredict	3D spheroid system based on freshly isolated primary hepatocytes co-cultured with non-parenchymal cells
3D InSight™ Human Liver Microtissues	InSphero	Pharma-validated *in vitro* co-culture model of primary human hepatocyte, Kupffer cell and liver endothelial cell, which originate from 5 female and 5 male donors
3D InSight™ Human Liver Steatosis Model	InSphero	Disease model of first stage of non-alcoholic fatty liver disease, which consists of a microtissue produced from a co-culture of healthy human primary hepatocytes, Kupffer cells and liver endothelial cells combined with special media and lipids
3D InSight™ Human Liver NASH Model	InSphero	Advanced tuneable microtissue co-culture of healthy primary human liver cells, which is suitable for drug testing
HepatoPearl	Cyprio	3D *in vitro* model of primary human hepatocytes with high predictability and size-controlled spheroids, which is developed using the BioPearl technology
3D human cell culture platform	Invitrocue®	3D co-culture model of primary human hepatocytes and Kupffer cells and tri-culture of primary human hepatocytes, Kupffer cells and stellate cells, which are suitable for drug testing and cytotoxicity studies
3D HepaRG spheroids	Cyprotex	Scaffold-free multicellular native tissue-like models

DMPK, drug metabolism and pharmacokinetics; NASH, nonalcoholic steatohepatitis; DILI, drug-induced liver injury.

Table 4. 3D liver models and the tested CYP enzymes

3D liver models	Observed CYP expression
Spheroids of primary hepatocytes	CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP2C8
Spheroid co-culture from endothelial cells and primary hepatocytes formed by bioprinting	CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP3A4
Spheroids of primary hepatocytes in stirred tank bioreactor	CYP1A2, CYP2C9, CYP3A4
Spheroids of HepG2 cells	CYP1A1/2, CYP3A4
Bioprinted HepG2 spheroids	CYP1A2
HepaRG	CYP1A2, CYP2B6, CYP3A4
Spheroids from iPSC-derived hepatocytes	CYP1A2, CYP2C9, CYP3A4, CYP2C19, CYP2D6
Bioprinted spheroids from iPSC derived hepatocytes	CYP1A2, CYP3A4
Organoids from primary hepatocytes and iPSC-derived hepatocytes	CYP3A4
Perfusion bioreactor and Liver-on-a-Chip models	-
Primary hepatocytes	CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP1A2, CYP3A4, CYP2C8, CYP2C9, CYP2C19
HepG2	CYP1A2, CYP3A4
HepaRG	CYP3A4
Upcyte hepatocytes	CYP1A2, CYP3A4

iPSC: Induced pluripotent stem cells

of different organs. Therefore, these rapidly evolving technologies deserve a place in regenerative medicine as their application in therapeutic arena is promising. Principal component analysis of expression profiles has shown that the pluripotent stem cells differentiated into hepatocyte-like cells. The biopharmaceutical market offers many artificial 3D liver models for drug toxicology testing as a service. These are well-tested and improved systems theat are suitable for pharmacological studies. These systems, which are owned and developed by different companies and universities, are excellent tools for ADME testing. Several 3D models are available, which can be developed in laboratories for research purposes and are suitable for longer studies. These models and cells are well characterized, and their role in hepatotoxicity has been proven in many studies (Table 4)[4,35,38,141].

11. Conclusions

3D cell cultures offer huge advantages in research, specifically in the field of drug therapy research. Due to their 3D structure, the cells have polarity as well as can perform different functions and form spaces that mimic lumens. Thus, the model is able to respond to different treatments in very high degree of similarity to the original organ. These models allow us to investigate different organs and their diseases at molecular level as well as the response to drug treatments in the case of diseases. In the future, some of these models can be used to predict the effect of drugs in individual patients, thereby facilitating personalized therapy. Some of these models can also be used in tissue regeneration. 3D models offer a chance to better understand drug therapies and help increase the efficacy of patient-specific treatments[8,142-146].

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Conflict of interest

The authors declare no conflict of interest.

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Availability of data

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