



# Recent advances in the development of in vitro liver models for hepatotoxicity testing

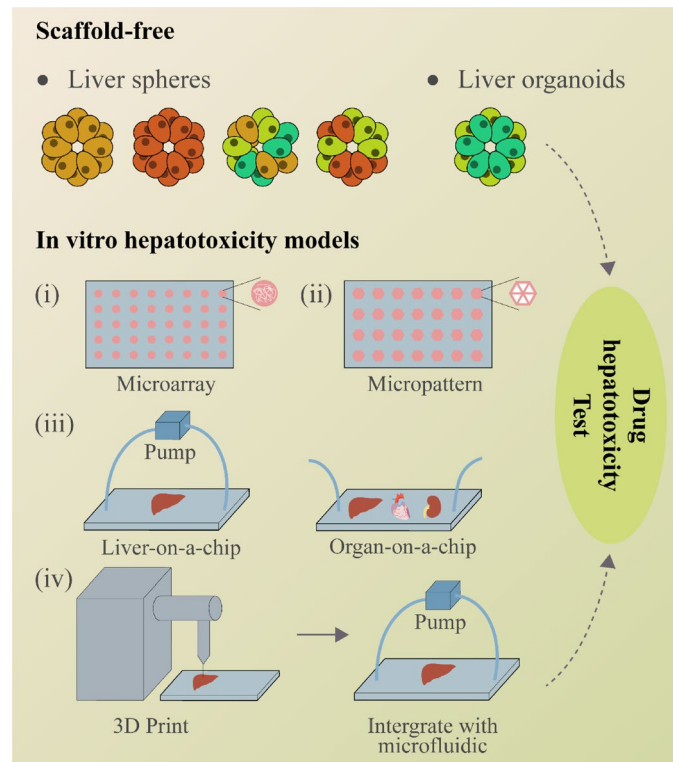
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## Abstract

Liver injury is a common cause of drug approval withdrawal during drug development, pre-clinical research, and clinical treatment. If not properly treated, patients with severe liver injury can suffer from acute liver failure or even death. Thus, utilization of the convenient in vitro hepatotoxicity assessment model for early detection of drug-induced hepatotoxicity is vital for drug development and safe personalized medication. Biomaterials (e.g., hydrogels, nanofibers, decellularized liver matrix) and bioengineering technologies (e.g., microarrays, micropatterns, 3D printing, and microfluidics) have been applied for in vitro hepatotoxicity assessment models. This review summarizes the structure and functions of the liver as well as the components of in vitro hepatotoxicity assessment models. In addition, it highlights the latest advances in developing hepatotoxicity models with the ultimate goal of further clinical translation.

## Graphic abstract



**Keywords** Liver injury · Drug toxicity · In vitro model · 3D culture

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## Introduction

Drug-induced liver injury (DILI) remains a common cause of drug withdrawal in drug development, pre-clinical research, and clinical treatment [1]. Patients with severe liver injury may suffer from acute liver failure or even death. The annual incidence of DILI is reported to be between 1 in 10,000 and 1 in 1,000,000 people worldwide [1]. In addition, Chinese medicine and dietary supplements are believed to be important factors causing DILI [2–6]. Rarely, medications can induce hepatotoxicity in individuals with the toxicity remaining undetected until the late clinical stage [7]. Moreover, the response of different individuals to the same dose of the same drug and the final progress of the disease are different [8, 9]. Therefore, there is an urgent need to develop an effective and reliable method to investigate liver toxicity in the drug development stage, before clinical usage.

The importance of *in vitro* hepatotoxicity assessment models is widely recognized, since they can help prevent hepatotoxicity and are vital for drug development and safe personalized medication [10, 11]. Current methods for drug screening in liver disease mainly depend on *in vitro* cell culture and *in vivo* animal models [10, 12]. Among these methods, human primary hepatocyte culture is considered the gold standard for *in vitro* hepatotoxicity assessment models. However, human primary hepatocytes de-differentiate and rapidly lose liver-specific functions when maintained in conventional two-dimensional (2D) monolayer culture [10]. As such, conventional 2D human primary hepatocyte cultures for long-term studies of liver biology and assays that require liver-specific functions. Although animal models can mimic the human *in vivo* environment, they cannot always predict toxicity in human due to fundamental biological differences [13, 14]. Moreover, these animal models have a high cost, a long experimental period, and doubtful ethics and thus are not suitable for rapid hepatotoxicity testing and drug discovery. More recently, the shortcomings of traditional *in vitro* drug testing models have been overcome with the advent of the three-dimensional (3D) *in vitro* cell culture models. Likewise, the 3D liver drug testing models provide rapid and simple detection of drug hepatotoxicity, offering great advantages over 2D cell models and animal models for drug development and safe personalized medication [11, 15]. *In vitro* liver models, such as liver spheroids [16], scaffold-based 3D tissue construction [17, 18], 3D printing [19, 20], and livers-on-a-chip [21–24], have been widely explored for the detection of drug hepatotoxicity. The 3D cell culture system can establish a 3D growth state similar to tissues and organs *in vivo*, accurately simulating the *in vivo* microenvironment [25–29]. Drug screening

models based on 3D *in vitro* cell culture more accurately demonstrate toxic responses and can also be used in high-throughput screening and individualized assays.

The *in vitro* drug hepatotoxicity model is a promising and rapidly emerging branch of liver tissue engineering. In this review, we provide an overview of 3D hepatotoxicity testing models. First, we give a detailed introduction to liver structure and liver function. Second, we summarize the components of the liver model and review the latest advances in *in vitro* hepatotoxicity assessment models. Finally, the common obstacles and future prospects for developing *in vitro* hepatotoxicity assessment models are discussed.

## Liver structure and function

The liver is the largest solid organ of the human body, with more than 1500 functions such as metabolism, secretion, excretion, and biological transformation [30, 31]. Liver damage causes corresponding functional abnormalities and metabolic disorders in the human body, and sometimes even death. The liver tissue is composed of numerous hepatic lobules with the same structure, and similar size and shape, and its main functions are detoxification and drug metabolism (Fig. 1).

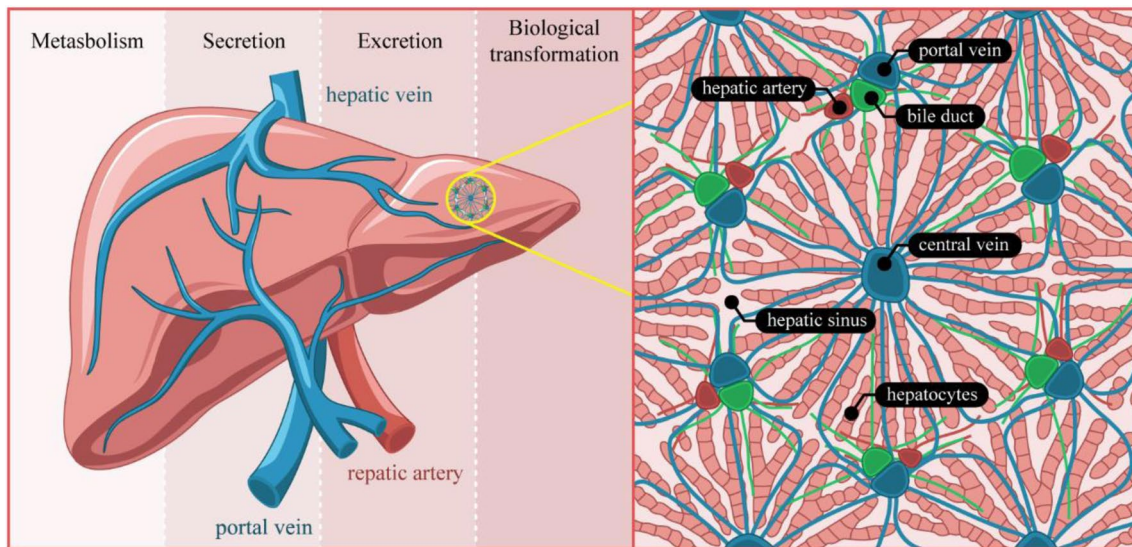
### Hepatic lobule structure

The hepatic lobule is a hexagonal structure composed of a central vein, a liver plate, a hepatic sinus, and a capillary bile duct [32]. The liver plate and hepatic sinus are distributed radially around the central vein, and the capillaries are distributed among hepatocytes. The irregular cavity formed between the liver plates is called the hepatic sinusoid and allows for blood flow between the liver plates. The hepatic sinus consists of a sinus wall and a sinus cavity, which participate in the immune response [30]. Capillary bile ducts constitute the hepatobiliary system, which participates in material metabolism and drug conversion.

The microenvironment (extracellular matrix (ECM)) is a diverse collection of proteins and carbohydrates around the hepatocytes and plays an important role in cell growth [33, 34]. In summary, hepatocytes, endothelial cells, liver macrophages, and their extracellular microenvironment form the basic unit of hepatic lobules, and they work together to make the liver function.

### Liver function

Liver tissue is the most important detoxifying and drug-metabolizing organ of the human body, and it can transform



**Fig. 1** Schematic of liver function and structure

many non-nutritional substances inside and outside the body. Biotransformation in the liver is carried out in two ways, namely protein binding and enzyme binding. Protein binding occurs when various foreign substances (e.g., poisons, drugs, carcinogens) or substances produced by the body's own metabolism (e.g., bilirubin or ammonia) enter the liver [5, 32]. These substances reversibly bind to plasma protein. On the other hand, enzyme binding refers to the chemical modification of liver-related enzymes, which increases their effects [35]. The polarity or water solubility of these modified substances makes it easy to excrete them from the body in bile or urine, which gives the liver an inactivating and detoxifying role in maintaining and regulating the stability of internal environment in human body [36].

Liver injury causes changes in the liver's structure and function, which may lead to related diseases such as hepatocyte necrosis, fatty liver, cholestasis, biliary tract injury, liver cirrhosis, and tumors. For instance, the Hawaii Department of Health reported an acute hepatitis outbreak that was caused by taking OxyELITE Pro [5]. Seven healthy young men and women developed jaundice, and their serum transaminase levels were significantly elevated. The researchers described 36 cases of liver injury caused by taking the supplement; one of the patients died, two underwent an emergency liver transplant, and the other patients had a longer course of illness. Nijima et al. [37] reported the case of an elderly patient with type 2 diabetes who took ipragliflozin, a sodium-glucose cotransporter-2 inhibitor, and then developed cholestatic DILI. The patient's condition improved after discontinuation of ipragliflozin. Incidents like this show that addressing liver toxicity as early as possible is crucial.

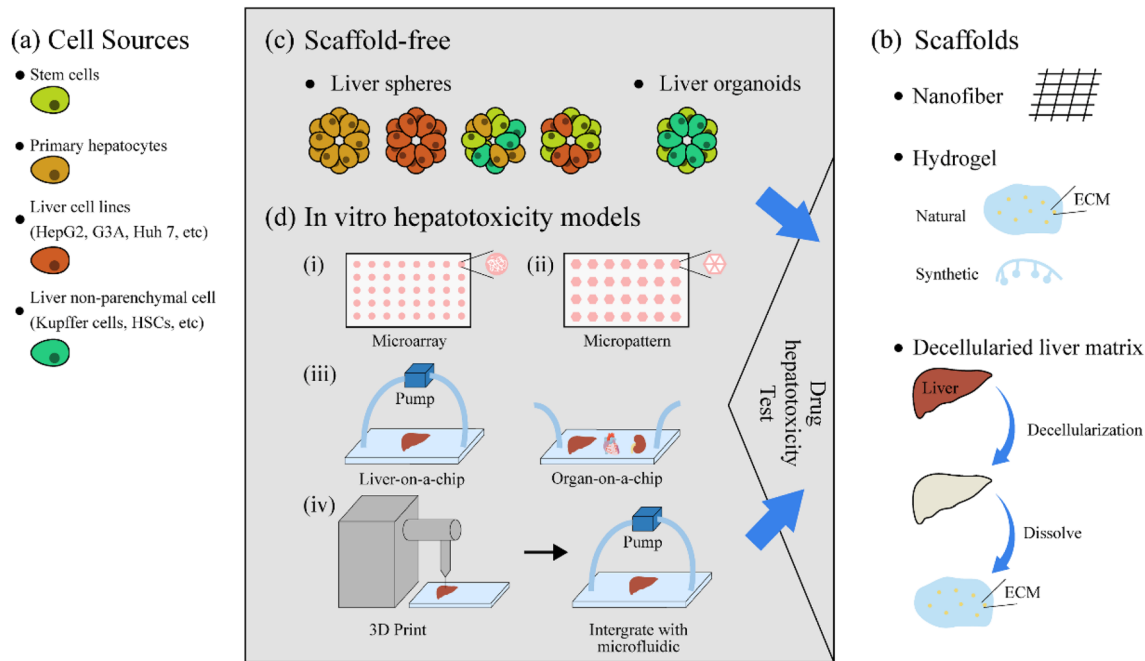
## Components of the in vitro hepatotoxicity assessment model

In vitro hepatotoxicity assessment models play an important role in hepatotoxicity research and are constructed with cells and biomaterials (Figs. 2a and 2b).

### Cell sources

Currently, cell sources utilized in liver hepatotoxicity testing include primary hepatocytes [38], stem cells [39, 40], hepatocyte lines [41], and liver non-parenchymal cells [42, 43]. We will discuss the characteristics of these cells for in vitro liver hepatotoxicity in this section.

Primary hepatocyte culture is considered the gold standard for metabolic and toxicity studies [44, 45], and primary hepatocytes maintain the functionality of drug metabolism and liver-specific functions, such as urea and albumin secretion, liver-specific transporter expression, and cytochrome P450 (CYP450) enzyme activity [46]. However, primary hepatocytes lose their liver functions after long-term culture in vitro, including protein synthesis and CYP450 activity. Another major obstacle in primary hepatocyte culture is that the cells gradually lose their replication capacity, original morphology, structure, polarity, and liver function (especially the rapid loss of drug metabolism ability), allowing only short-term effective hepatotoxicity detection [10, 47]. The next generation of long-term culture requires the application of engineering techniques to construct in vitro models. For instance, Sandra et al. applied primary hepatocytes to develop a



**Fig. 2** Model system for hepatotoxicity assessment. **a** The cells used in the in vitro hepatotoxicity assessment test model are stem cells, primary hepatocytes, hepatocyte lines, and liver NPCs. **b** The scaffold materials used in the in vitro liver hepatotoxicity detection model are nanofibers, hydrogels, and decellularized LEM. **c** Liver spheroids and organoids provide a unique model for hepatotoxicity assessment,

which, through cellular interactions, simulates the internal environment of liver tissues and spontaneously forms blood vessel-like structures. **d** Four kinds of in vitro hepatotoxicity assessment models achieved toxicity testing in vitro and provide new platforms for clinical diagnosis and drug development. NPC: non-parenchymal cell; LEM: liver extracellular matrix

micropatterned co-culture (MPCC) for liver disease research [38]. They found that hepatocytes in the MPCC model maintained high levels and stable liver function and polarity during several weeks of culture. In addition, they found that this use of hepatocytes could be extended to multiple liver-related infectious disease models and showed potential for drug development. Another study utilized MPCC to develop a micropatterned tri-culture (MPTC) platform for drug development [48]. Here, MPTC was composed of hepatic stellate cells (HSCs) around MPCC. This study found that the secretion of albumin and urea in MPTC and MPCC was similar, indicating that primary human hepatocytes (PHHs) differentiate well. However, the increased number of HSCs down-regulated the activity of hepatic CYP450 and transporters in MPTC and caused steatosis in two weeks. In addition, inhibition of farnesol X receptor (FXR) and NADPH oxidase (NOX) activation with drugs can reduce liver dysfunction in MPTC. Despite the advances in culture conditions and technologies, there are still considerable technical obstacles to the isolation and culture of primary hepatocytes in liver tissue engineering.

More recently, hepatocytes derived from stem cells have provided an opportunity for patient-specific hepatotoxicity testing, since stem cells (e.g., embryonic stem cells (ESCs),

mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs)) have a pluripotent nature and the ability to proliferate [39, 40, 49, 50]. For instance, human-induced pluripotent stem cell (hiPSC) derived hepatic progenitor cells (hiPSC-HPCs) with adipose-derived stem cells and endothelial cells were embedded in a triculture to form an in vitro liver model [51]. After several weeks of culture, it was found that hiPSC-HPCs expressed higher levels of liver-specific genes and enhanced CYP450 and metabolites which could be used for drug screening. These studies demonstrated the feasibility of differentiating hepatocytes from stem cells. Nevertheless, differentiated hepatocytes still have lower enzyme activity and gene expression profiles compared with cells in vivo.

To surmount the limitations of hepatocytes and stem cells in the most common applications, cell lines derived from hepatocellular carcinoma are widely used [52]. A few commonly used cell lines are HepG2, C3A, HepaRG, and Huh7. These cell lines can proliferate indefinitely, have a relatively stable phenotype, are easy to manipulate, and overcome the problem of inter-individual variation. In addition, transcription factor (nuclear factor erythroid 2-related factor 2 [Nrf2]) is still expressed in these cells, which is essential for drug metabolism and toxicity [41]. For example, encapsulating HepG2 cells on alginate provided an in vitro 3D liver model

for drug hepatotoxicity testing [53]. In this study, 10 mM 7-ethoxy-4-trifluoromethyl coumarin (EFC) stock solution mixed with HepG2 medium demonstrated the effectiveness of drug metabolism in the liver chamber. The drug metabolism conversion rate increased by 26%, which reproduced liver function *in vivo*. Thus, this model can be used to support drug toxicity screening. In another study, Deng et al. used four cell lines (HepG2, LX-2, EAhy926, and U937) to fabricate a microfluidic liver model for hepatotoxicity detection [54]. The 3D cell cultures maintained liver function (e.g., albumin and urea secretion). In addition, compared with the gold standard primary hepatocyte plate model, the response of this platform to acetaminophen (APAP) treatment was similar. However, the main disadvantage is the lack of expression of key metabolic enzymes and the low metabolic capacity compared with other cell types.

In order to further circumvent the challenges of stem cell-delineated cultures, hepatocytes can be co-cultured with non-parenchymal cells (NPCs) (e.g., Kupffer cells and HSCs) to preserve certain cellular mechanisms and metabolic pathways *in vitro* for drug toxicity testing. NPCs make up roughly 20% of liver mass and exhibit mediated immune/pro-inflammatory responses and functional 3D tissue structure [10, 46]. As liver cytotoxicity involves cascading pro-inflammatory pathways and complex interactions between NPCs and ECM, the presence of NPCs is largely considered to be a necessary condition for accurate modeling of toxicity assays *in vivo* [10, 42, 43, 46, 55]. Hence, the presence of NPCs not only helps to prolong the survival and function of hepatocytes in culture, but also increases their sensitivity to DILI detection involving inflammatory mediators and hepatocyte NPC “communication.” Therefore, NPCs and ECM components, which can be also co-cultured with 3D liver buds to further improve physiological relevance, are important to develop accurate *in vitro* liver models for metabolic and hepatotoxicity testing.

### Scaffolds for *in vitro* liver models

A 3D bioscaffold is an important element in the construction of 3D liver tissue structures and drug testing models. Scaffold materials generally should have the following characteristics: (1) a 3D porous structure that can facilitate cell growth and timely supply of nutrients and then discharge metabolic waste; (2) good biocompatibility that can promote cells to grow, proliferate, and adhere, and easily degrade in the body; (3) plasticity; (4) the ability to bind to the ECM naturally and better reshape the body [56]. For liver tissue construction, nanofibers [57–59], hydrogels [60–62], and a decellularized liver matrix [63–66] have been used as suitable ECMs for hepatocyte culture, maintenance of hepatocyte function, or promotion of liver differentiation of stem cells for drug hepatotoxicity testing (Fig. 3).

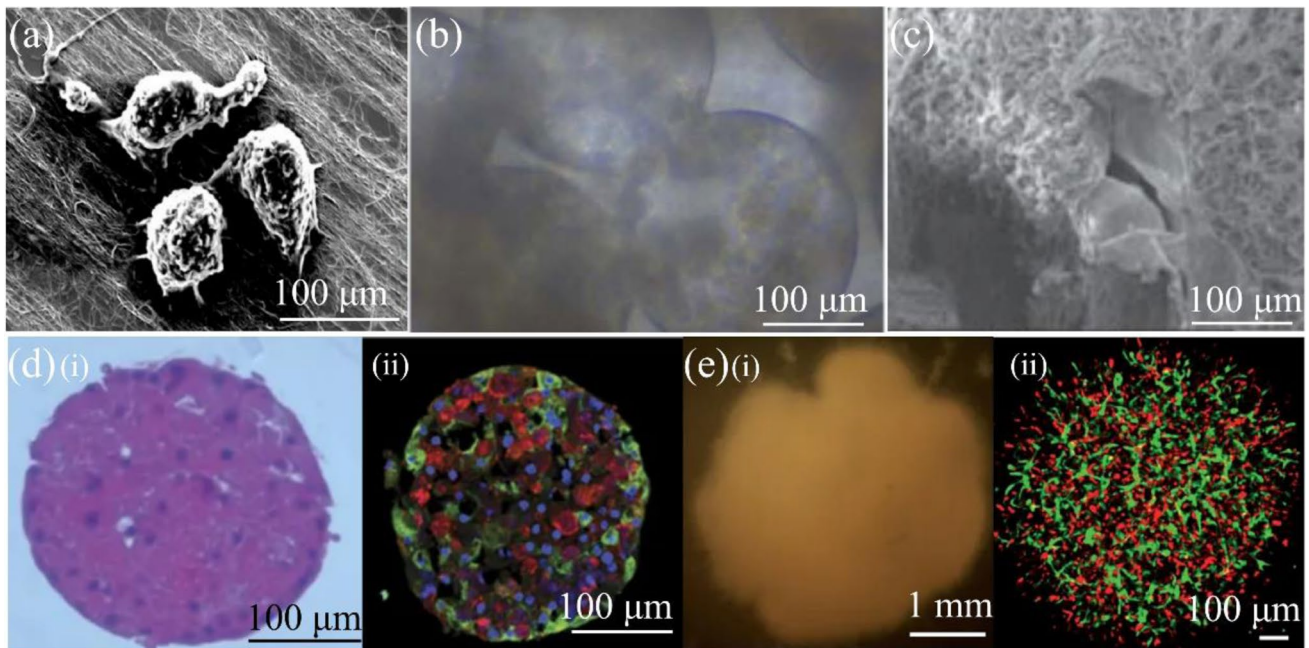
### Nanofiber scaffold materials

In recent years, nanomaterials have been developed rapidly in the field of liver tissue engineering. Nanofibrous scaffolds in particular not only provide structures similar to natural tissues, but have mechanical properties and high surface area/volume which promote cell proliferation *in vitro*. Nanofibers for *in vitro* liver models also have the potential to predict drug toxicity, since this nanomaterial can enhance liver function and upgrade critical drug-metabolizing enzyme activity (CYP) [57, 58, 67, 68]. For example, Brophy et al. demonstrated that hepatocytes cultured on nanofibers maintain high CYP450 activity [58]. In addition, the amount of albumin produced by hepatocytes on nanofibers was twice that of hepatocytes produced on laminin. In another study, a 3D scaffold of multi-walled carbon nanotubes (MWCNTs) was fabricated for liver tissue engineering and drug discovery (Fig. 3a). Primary rat hepatocytes were cultured using aligned nanogrooved MWCNT sheets, MWCNT yarns, or 2D standard culture. The cell viability between these three culture conditions was comparable, but in cells cultured on MWCNTs, the production of albumin, a hepatocyte-specific marker, increased. In addition, the activity of CYP1A2 was enhanced on MWCNTs. These results showed that carbon nanotubes can be used in *in vitro* liver models with enhanced liver-specific function. To further promote the adhesion properties of nanomaterials for hepatocyte culture, researchers used chitosan to prepare polycaprolactone (PCL)/chitosan nanofibers using electrospinning technology [57]. The pores of the scaffolds were suitable for infiltration of mouse hepatocytes. In addition, the *in vitro* cell culture results confirmed the biocompatibility of the scaffold for later construction of an *in vitro* liver model. The increased liver function of this model showed potential for hepatotoxicity detection. Thus, the biocompatibility of nanomaterials and their retention of affinity and enzyme activity in hepatocyte culture make them useful in both liver tissue construction and hepatotoxicity testing.

### Hydrogel scaffold materials

To construct liver extracellular matrix *in vitro*, biomimetic properties and soft materials are required, and thus hydrogels are a good option [60, 61, 69]. Moreover, the cells in hydrogels have a 3D environment that is similar to natural tissues. Utilizing hydrogels to fabricate an *in vitro* liver model reproduces the true state of the liver in the body, and the model can be used for hepatotoxicity drug detection.

Hydrogels are of two types, natural and synthetic. Synthetic hydrogels, such as polyethylene glycol (PEG) [60, 69] and polypropylene amine (PAAm) [61], are artificial hydrogels with a defined composition and a strong mechanical structure which are widely used. For example,



**Fig. 3** Scaffold-based liver models and scaffold-free liver models for hepatotoxicity testing. **a** SEM image of hepatocytes cultured in MWCNTs (reproduced with permission [68], Copyright 2014, American Chemical Society). **b** Optical microscopy images of colloidal crystal scaffolds seeded with hepatocytes after 1 day (reproduced with permission [60], Copyright 2019, Royal Society of Chemistry). **c** SEM images of recellularized liver graft after 2 days of culture (reproduced with permission [66], Copyright 2010, Springer

Nature). **d** H&E staining (i) and immunofluorescence staining (ii) of liver spheroids (reproduced with permission [78], Copyright 2016, Springer Nature). **e** Gross observation of a liver organoid (i) and nascent endothelial networks inside a liver organoid (ii) (reproduced with permission [83], Copyright 2013, Springer Nature). SEM: scanning electron microscopy; MWCNT: multi-walled carbon nanotube; H&E: hematoxylin and eosin

a biofunctionalized 3D inverted colloidal crystal (ICC) hydrogel platform was used to establish an *in vitro* liver model for assessment of drug hepatotoxicity (Fig. 3b) [60]. Here, poly(ethylene glycol)diacrylate (PEGDA) ICCs were modified with either collagen type I or fibronectin, and then hepatocytes were cultured on this scaffold. After two weeks, hepatocyte functions (i.e., albumin, urea, alpha fetoprotein (AFP), hepatocyte nuclear factor four alpha (HNF4A), and CYP450) were evaluated and were found to be higher in the experimental group than in the control group. In addition, a non-steroidal anti-inflammatory drug (NSAID) was investigated for drug hepatotoxicity and showed potential for toxicity studies. Another report mentioned three polymers (i.e., PEG, polypropylene acid (PAAc), and PAAm) for fabricating *in vitro* liver models [61]. In this study, insulin-like growth factor 1 (IGF-1) plus tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was photo-immobilized on the surface of these three polymers to promote mesenchymal stem cell (MSC) adhesion and differentiation. The hepatocytes cultured on the synthesized biomaterial exhibited liver function and potential for hepatotoxicity testing. However, currently, there are few applications of synthetic hydrogels *in vitro* hepatotoxicity assessment models.

Natural hydrogels are ECM-derived components or ECM analogs, and the fibrous structure and chemical composites are similar to native ECM [62, 70]. At present, natural hydrogels are used to form the liver structure for *in vitro* hepatotoxicity assessment. For example, a 3D environment that included transglutaminase-cross-linked gelatin, growth factor-depleted Matrigel, and collagen type I, was synthesized for nanoparticle toxicity testing [62]. In this study, Matrigel enhanced the adhesion and proliferation of liver cell line HepG2. Moreover, secretion of albumin and urea demonstrated that this system re-establishes normal hepatocyte-like properties in HepG2 cells, which cannot be achieved with conventional 2D culture. Additionally, the time- and dose-dependent cytotoxicity was higher when the cells were embedded in hydrogels. This system provided a tool for examining the dose effect. Moreover, Lee et al. utilized gelatin methacryloyl (GelMA) to fabricate ICC scaffold for liver tissue engineering [70]. Compared to 2D systems, this GelMA ICC scaffold provided cell attachment sites, which promoted intercellular interaction of hepatocytes and thus improved cell functions. Meanwhile, the results of liver-specific gene expression demonstrated the potential of GelMA ICC scaffolds to fabricate liver

engineering platforms for drug toxicity testing and regenerative medicine.

### Decellularized liver matrix

Decellularization refers to the removal of all cells from the original tissues/organs by physical and chemical methods to obtain cell-free, non-immunogenic/low immunogenic tissue scaffolds. Decellularized scaffolds have a unique value in the field of tissue engineering [71, 72]. Decellularization retains the ECM components of the original tissues and organs and some unique growth factors. Therefore, recellularization using a decellularized scaffold can closely conform to the characteristics of the original tissue for organ transplantation, tissue repair, drug testing, and other applications.

Although decellularized scaffolds present challenges for in situ colonization, they are rich in natural ECM components and essential growth factors for cells, which allows accurate construction of drug screening models [66, 72–74]. A classic study showed that an acellular liver matrix can be used for liver tissue engineering via characterization of its components (Fig. 3c) [66]. In this study, decellularization retained the structural and functional characteristics of the natural microvascular network, allowing hepatocytes to effectively recellularize the liver matrix. The recellularized grafts supported liver-specific functions such as albumin secretion, urea synthesis, and CYP450 expression, which were similar to in vivo conditions. Hussein et al. utilized a decellularized rat liver scaffold to create a hepatocellular carcinoma model for pharmacological studies [72]. In this work, a simplified closed-perfusion system was developed as a bioreactor and HepG2 cells were infused into the decellularized liver scaffold, which maintained their function and oncogenicity. Through anti-cancer drug testing, it was demonstrated that this in vitro system could provide a precious 3D cell culture environment and thus evaluate the efficacy of new antitumor drugs. Another team developed a decellularized liver matrix solution for 3D cell culture [73]. Here, 3D printing was used to create the in vitro 3D structure, and the decellularized liver matrix bioink improved the functionality of printed structures.

It has also been found that combining acellular matrix with a hybrid scaffold not only preserves its unique biological factors but also increases the performance of the hybrid scaffold. For example, Lee et al. enzymatically decomposed decellularized liver scaffold and combined it with type I collagen to form a composite hydrogel; they then cultured primary hepatocytes using the composite hydrogel [74]. Mass spectrometry revealed a variety of ECM components and characteristic growth factors in the acellular matrix. Acellular matrix composite hydrogels had improved biophysical and mechanical properties compared to single hydrogels. They also promoted cell viability and liver function, and the

animal experiments have confirmed the superiority of composite hydrogels [74]. In summary, acellular matrix composite hydrogels are a promising biological material and play a unique role in the in vitro culture of hepatocytes for drug hepatotoxicity evaluation.

### In vitro models for hepatotoxicity testing

The different components of in vitro hepatotoxicity assessment models, including cell sources, scaffold materials, and liver spheroids, were described in the previous sections. Now, we will introduce the technology behind these models. At present, the foremost models are liver spheroids, organoids, microarrays, micropatterns, livers-on-a-chip, and 3D-printed livers (Figs. 2c and 2d).

#### Scaffold-free construction of liver models

##### Liver spheroids

Liver spheroids are a rising model for 3D culture of hepatocytes. These spheroids can mimic the ECM and improve hepatic function in vitro [11, 16, 75]. The principle of formation of hepatic spheroids is that monodispersed cells are able to change the 3D configuration by self-repolymerizing, with the unavailability of a suitable biomaterial for attachment causing cell–cell attachment into 3D spheroid structures. The production of spheres increases proliferative activity and metabolic function, leading to enhanced cell viability, stable morphology, and polarization [76]. Thus, compared to 2D cultures, liver spheroids have more powerful functions and provide a valuable tool for drug development and pre-clinical hepatotoxicity testing.

For instance, liver spheroids produced by HepaRG2 cells showed the morphological characteristics of hepatocyte-like cells and the formation of bile duct-like structures [77]. In this study, researchers used a rotating bioreactor to construct spheroids with diameters of < 200  $\mu\text{m}$ . This system maintained stable albumin secretion, CYP3A4 induction, and uridine diphosphate glucuronic acid transferase (UGT) activity for seven weeks, and immunofluorescence staining revealed the distribution of bile duct-like structures and transporters. In addition, computer modeling analysis of acetaminophen (APAP) for hepatotoxicity demonstrated the utility of this system in assessing the biological activation of APAP and its associated cytotoxicity. In another study, Bell et al. used ultra-low attachment plates to produce monoculture PHH spheroids (Fig. 3d) [78]. During the 35-day culture period, a bile duct-like structure was formed in the spheroids, indicating stable functional polarization of hepatocytes. Subsequently, the spheroids were dosed every two days with several drugs (i.e., amiodarone, fialuridine, diclofenac,

bosentan, and tolcapone) and the viability was determined to investigate hepatotoxicity. Hepatotoxicity was not identified after short-term (48 h) exposure but was clearly detected after long-term (8 and 28 days) exposure that approached clinically relevant concentrations. Another team pre-laid 1.5% agarose to construct a low surface adsorption environment to culture C3A cell spheres [79]. The C3A spheres maintained culture for 32 days and showed similar structural features and liver-specific functions to the *in vivo* environment, such as the ability to synthesize and secrete urea and albumin, and functional tubule transporter and CYP2E1 expression. Additionally, analysis of four hepatotoxins (APAP, diclofenac, trovafloxacin, and floxuridine) indicated that the spheroid model was able to predict hepatotoxicity with higher sensitivity than conventional 2D monolayer cultures. Therefore, liver spheroids have great application potential for *in vitro* hepatotoxicity assessment.

### Liver organoids

Generally, current spheroid model systems exhibit a large gap between the cellular level and organ level. Stem cell-derived organoids are being used to develop 3D self-organizing tissue models and provide a compelling new biological model to act as both tissue and organ agents [80–82]. Organoids recapitulate a large number of biological parameters, including the spatial organization of heterogeneous tissue-specific cells, intercellular interaction, cell–matrix interactions, and certain physiological functions produced by tissue-specific cells in the organoid [83]. By controlling self-renewal and differentiation, specific tissues and organs can be formed. Liver organoids bridge a gap in existing model systems via providing a stable and scalable system to extend cultivation and manipulation, which better represents the *in vivo* physiology. Therefore, construction of liver organoids can not only be used in regeneration engineering, but also has irreplaceable value in drug development and toxicity evaluation.

A major investigation into liver organoids was previously reported, mainly using the self-assembly of three types of cells (i.e., endothelial cells, human iPSC-HEs, and MSCs) (Fig. 3e) [83]. In this work, gene expression and immunostaining analyses revealed sufficient similarity between *in vitro* and *in vivo* liver buds to permit liver regenerative therapy and drug toxicity testing. For drug metabolism investigation, liver organoids from stem cells have been used [84]. In this study, the CYP subfamilies (CYPs) in liver organoids were expressed. Then, the activity of docetaxel in a mouse liver organoid and human pancreatic carcinoid co-culture platform was measured. The results indicated that the cell survival rates of pancreatic tumors co-cultured with undifferentiated, differentiated, and CYPs-induced differentiated organoids treated with docetaxel were

(66.05 ± 2.14)%, (89.20 ± 2.67)%, and (101.90 ± 0.94)%, respectively. Thus, the organoids performed drug metabolism assessment and gene expression modification. *In vivo*-like *in vitro* investigation on drug toxicity may potentially be done with organoids as a stepping-stone to the clinical trial stage. It seems that although liver organoids have better function than liver spheroids, in practice, they are more commonly used in liver regeneration studies and are rarely used for hepatotoxicity testing.

### Liver cell microarrays

In the early phase of drug development, it is necessary to screen the metabolism of thousands of compounds. As the main detoxification organ, the liver is critical for clearing drugs. Assessing the ability of the liver to clear drugs requires a high-throughput artificial liver platform that provides operational data rapidly at a relatively low cost and that can be miniaturized. Next, we describe a state-of-the-art microarray platform for drug metabolism studies, which offers support for drug hepatotoxicity research.

Cell microarrays are used to seed cells on materials or biomolecules for high-throughput studies of microenvironmental signals on cells [85–87]. Kwon et al. reported a Transfected Enzyme and Metabolism Chip (TeamChip) that was developed for high-throughput investigation of drug metabolism toxicity screening in the human liver (Fig. 4a) [86]. This TeamChip was prepared by using a recombinant adenovirus in a complementary microwell chip to transfer genes into a miniature 3D cell microarray on a micro-pillar chip. This platform was able to transduce viral genes such as CYP3A4, CYP2C9, CYP2D6, CYP2E1, CYP1A2, and UGT1A4 for high-throughput screening in 3D cell culture microarrays. To verify the hepatotoxicity of this TeamChip platform, the effects of ketoconazole (a CYP3A4 inhibitor) and buthionine sulfoximine (BSO) were investigated in the TeamChip. The enhanced toxicity of APAP on the CYP3A4-expressing chip exposed to BSO was due to the lack of sufficient glutathione to detoxify the reactive intermediate. Accordingly, this TeamChip platform provided critical information for high-throughput assessment of metabolism-induced toxicity. In another work, a miniaturized 3D cell culture array (DataChip 2.0) and a metabolizing enzyme microarray (MetaChip 2.0) were developed by Yu et al. to predict metabolism-induced hepatotoxicity with high throughput [87]. This DataChip supported the Hep3B cells in a 3D microarray format, and then 22 compounds were distributed into the MetaChip and sandwiched with the DataChip. The drug toxicity testing results demonstrated that the platform had 100% sensitivity, 86% specificity, and 93% overall predictivity at optimum cutoffs of median inhibitory concentration (IC<sub>50</sub>) and C<sub>max</sub> values. As a multi-well platform, the DataChip/MetaChip was useful for

high-throughput drug screening. The researchers concluded that cell microarrays could be utilized as high-throughput platforms and rapid, inexpensive, and microscale alternative assessment of *in vitro* toxicity at early phases of drug development and clinical treatment.

### Micropatterned hepatocytes

Micropatterns, another kind of high-throughput platform, use accurate techniques such as lithography to construct an *in vitro* liver model in a size range of a few microns to a few nanometers [88–91]. Micropatterning was first reported in 1994 [88], when an elastomeric stamp was used to imprint a gold surface and create islands of a defined shape. With this technique, cells were placed in predetermined locations and arrays separated by a defined distance, assigned their shape, and then provided control over cell growth and protein secretion. Subsequently, Bhatia et al. used lithography to design the micropatterned co-cultures (MPCCs) platform, which was widely used in various *in vitro* models in subsequent studies. For this particular platform, hepatocytes and fibroblasts were seeded in a co-culture system by micropatterning [89]. This co-culture technique allowed control of the level of homotypic interaction in cultures of a single cell type and of the degree of heterotypic contact in co-cultures over a wide range, which allowed manipulation of the initial cellular microenvironment without variation of cell number. Later, Lin et al. utilized MPCCs to predict clearance of 26 drugs and drug–drug interactions (Fig. 4b) [90]. In this study, the long-term activity of cytochrome P450 enzymes produced by unpooled cryopreserved PHH donors was characterized in a 96-well plate and then MPCCs were used to predict metabolism of 26 drugs with a wide range of *in vivo* turnover rates. Meanwhile, the team detected the effects of drug-mediated P450 modulation on drug clearance rates to find out whether they could mimic *in vivo* drug–drug interaction (DDI) situations. More recently, this MPCC model was also employed to assess the safety and efficacy of RNA interference (RNAi)-based therapeutics (Fig. 4c) [91]. Here, the endogenous RNAi pathway was utilized to post-transcriptionally silence central drug metabolism genes and the team evaluated the impact of these changes on a natural substrate, as well as on DILI assessment of known hepatotoxins. This new capability could be utilized to open the door for structure–activity relationship detection of compounds in the setting of both high- and low-metabolizing genotypes. Therefore, this methodology has great potential for drug development and hepatotoxicity testing.

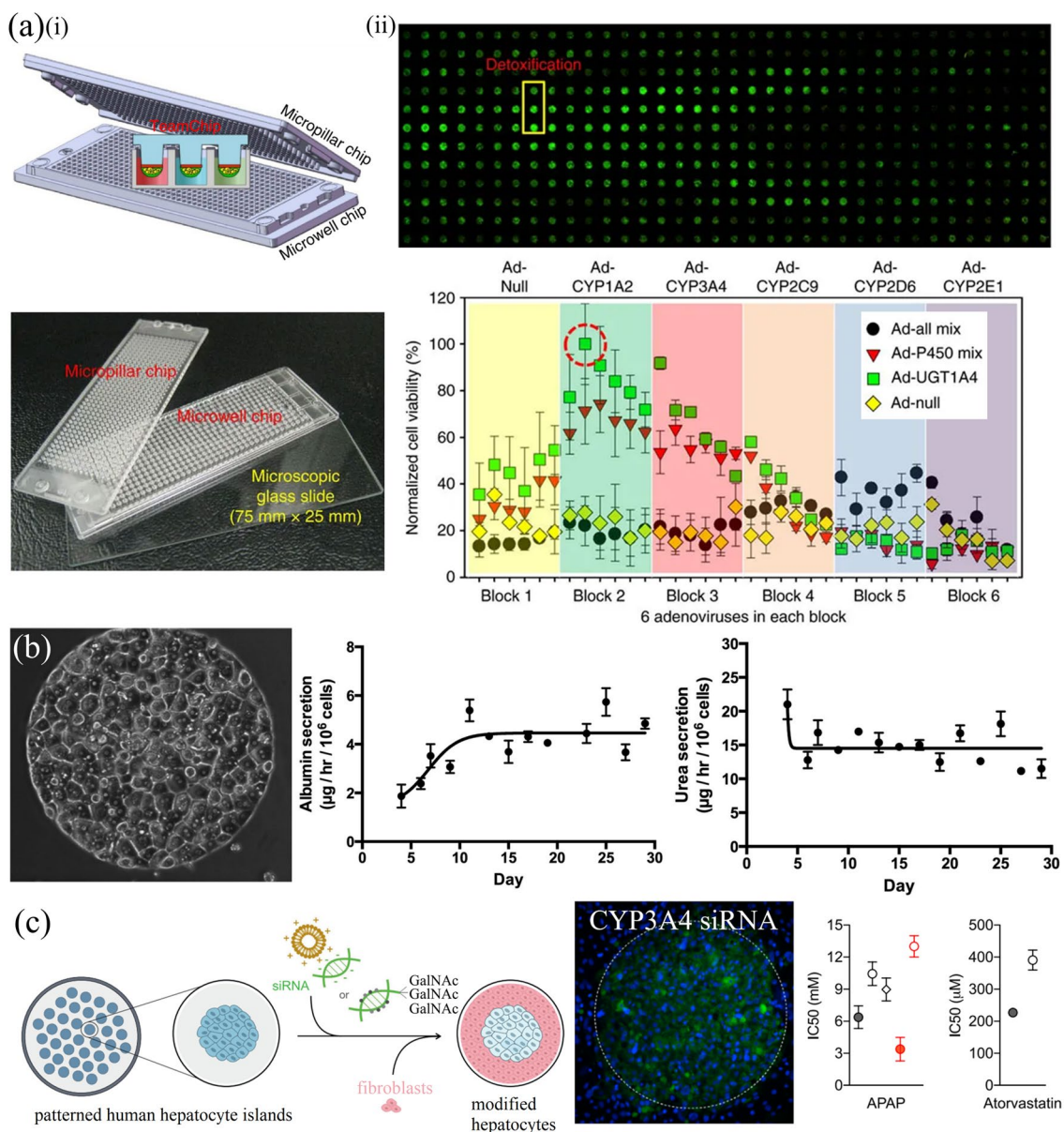
### Livers-on-a-chip

At present, livers-on-a-chip have become a research hot spot. They can simulate the structure of the liver *in vitro*, which

provides a flow environment to simulate blood flow. Livers-on-a-chip create a long-term culture environment that maintains the cell phenotype and function to fine-tune physical, chemical, and biological signals [92–96].

Since perfusion-based 3D culture mimics the growth of hepatocytes *in vivo*, hepatocytes on a chip can be used for drug detection [97–99]. For instance, a digital drug hepatotoxicity screening platform based on microfluidic technology was developed for detection of the dose effect of APAP on hepatocyte apoptosis [97]. In the microfluidic device, HepG2 cells and NIH-3T3 fibroblasts were encapsulated in a hydrogel to form a liver-like organ. Compared to a 2D culture system, hepatocyte function was enhanced. A 5 mM APAP dose did not have a significant toxic effect, while 10 and 20 mM of APAP demonstrated dose-dependent toxicity in the liver compared to non-treated controls. In another study, drug interaction testing was performed on a liver-on-a-chip platform with hepatic lobular micro-tissues (Fig. 5a) [98]. Drug hepatotoxicity testing showed that omeprazole or probenecid treatment for 48 h before APAP treatment aggravates APAP toxicity, while rifampicin or ciprofloxacin pretreatment relieves APAP toxicity. This work supports livers-on-a-chip as a potential drug development platform. To construct complex liver organs, iHep cells were cultured in a decellularized liver extracellular matrix (LEM)-coated microfluidic system to form a 3D vascularized liver organ (Fig. 5b) [99]. iHep cells, which transfected liver transcription factors, were co-cultured with endothelial cells in 3D LEM hydrogels. In the physiologically relevant culture microenvironment, the resulting 3D vascularized liver-like organs exhibited improved liver function. Finally, the feasibility of iHep-based 3D liver organs as a high-throughput drug screening platform was validated and applied to a multi-organ model consisting of multiple internal organoids. One recent work presented a microfluidic multi-tissue platform named metaEST that seamlessly integrates liver metabolism into embryonic stem cell tests (ESTs) [22]. In this “metaEST” platform, embryoid bodies (EBs) were formed on a chip using hanging-drop network technology as a micro-physiological system [39] and were co-cultured with the main 3D human liver micro-tissues (hLiMT). A metaEST assay was performed in a single microfluidic device, which was operated by gravity-driven flow to ensure constant intertissue communication and rapid and efficient exchange of metabolites. A test with the pro-drug cyclophosphamide revealed a fourfold reduction in the median infective dose (ID<sub>50</sub>) after biotransformation, which demonstrated the potential adverse effects of metabolites in embryotoxicity.

Livers-on-a-chip can be applied as a stand-alone unit or combined with other organ chips (e.g., heart, kidney, intestine) for investigating drug toxicity [100–102]. Because most drug failures are associated with liver or heart toxicity



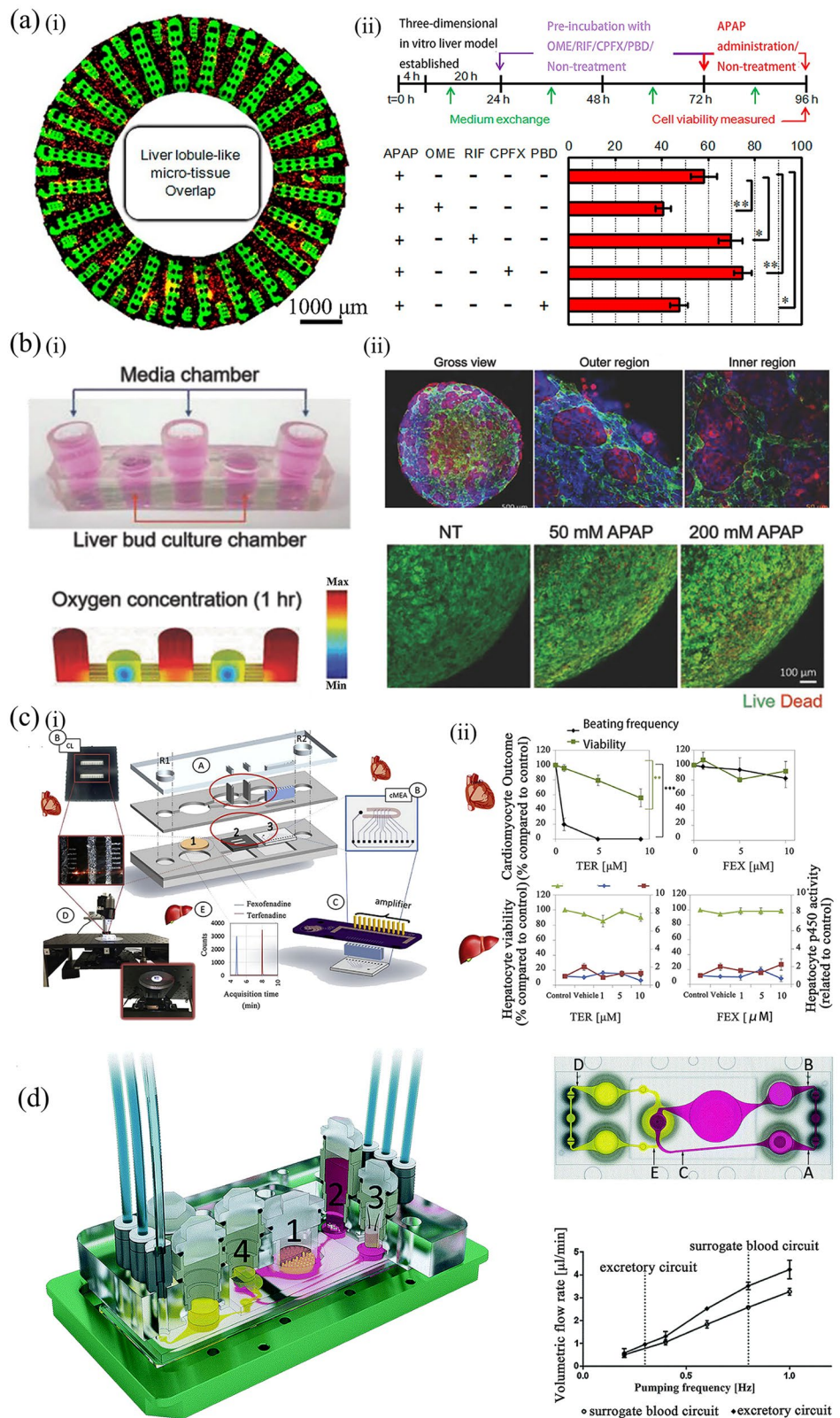
**Fig. 4** Microarrays and micropatterns for hepatotoxicity testing. **a** Schematic of micropillar/microwell chip components (i); scanned image of a combination of multiple drug-metabolizing enzymes expressed by THLE-2 cells. The chip was exposed to 200  $\mu$ M tamoxifen for 48 h (top) and normalized THLE-2 cell viability at different drug-metabolizing enzyme expression levels (bottom) (ii) (reproduced with permission [86], Copyright 2014, Springer Nature). **b**

Morphology and functional (albumin and urea secretion) characterization of MPCCs (reproduced with permission [90], Copyright 2016, ASPET). **c** Fabrication of gene-silenced MPCC to evaluate safety and efficacy of RNAi-based therapeutics (reproduced with permission [91], Copyright 2019, Elsevier). MPCC: micropatterned co-culture; RNAi: RNA interference

or dysfunction, an integrated liver chip was developed for drug screening and biophysical study of physiologically relevant microenvironments [100]. In this study, a dynamic fluid transport system was constructed by establishing an endothelial-like barrier that mimicked the continuous nutrient exchange and intercellular interaction between ventricular myocardium and hepatic sinusoid cells. Moreover,

using microfluidic channels and control of the fluid rate, the team imitated blood flow between the liver and heart. More importantly, the effect of different parameters, such as flow rate, was investigated via real-time detection of the morphology and metabolism of cultured cells. In another work, a heart-liver system was fabricated to better predict human drug metabolism (Fig. 5c) [101]. The heart-liver

**Fig. 5** Liver-on-a-chip for hepatotoxicity testing. **a** (i) Morphology of the 3D liver lobule-like microtissue, in which HepG2 cells and HAECs were, respectively, prestained green and red with cell tracker dyes. (ii) Time plan of a drug interaction detection experiment (reproduced with permission [98], Copyright 2016, ACS Publications). **b** (i) Top view of a microfluidic device with cultivation chambers interconnected via micron-sized channels (top) and simulated oxygen concentration after an hour in a device under flow conditions (bottom). (ii) Immunostaining of CD31 and albumin in 3D liver tissues encapsulated in the LEM hydrogel in the device and the device's application for APAP toxicity assessment (reproduced with permission [99], Copyright 2018, Wiley). **c** (i) Fabrication of a pumpless liver-heart microfluidic system. (ii) Dose response of terfenadine and its metabolite fexofenadine in human cardiomyocytes and hepatocytes (reproduced with permission [101], Copyright 2018, Elsevier). **d** 3D view of a microfluidic device comprising four organs and evaluation of fluid dynamics in this device (reproduced with permission [102], Copyright 2015, Royal Society of Chemistry). DDI: drug–drug interaction; LEM: liver extracellular matrix; APAP: acetaminophen



microfluidic system was integrated with a computational model to predict terfenadine and fexofenadine kinetics. This model recapitulated the fate of terfenadine, which improved the prediction of xenobiotic toxicity. To establish a drug absorption, distribution, metabolism, and excretion (ADME) map, and to perform a systemic toxicity test, the researchers proposed a four-organ chip (Fig. 5d) [102]. This four-organ chip system ensured a ratio of physiological fluid close to that of *in vivo* tissue. Intensive metabolic and genetic analysis indicated that the establishment of homeostasis in co-cultures for at least 28 days was independent of individual human cell lines or tissue/body background for each organ equivalent. The future direction of organ-on-a-chip research is likely to be aimed toward connecting these authentic functionalities to mimic a whole-body response for drug hepatotoxicity testing.

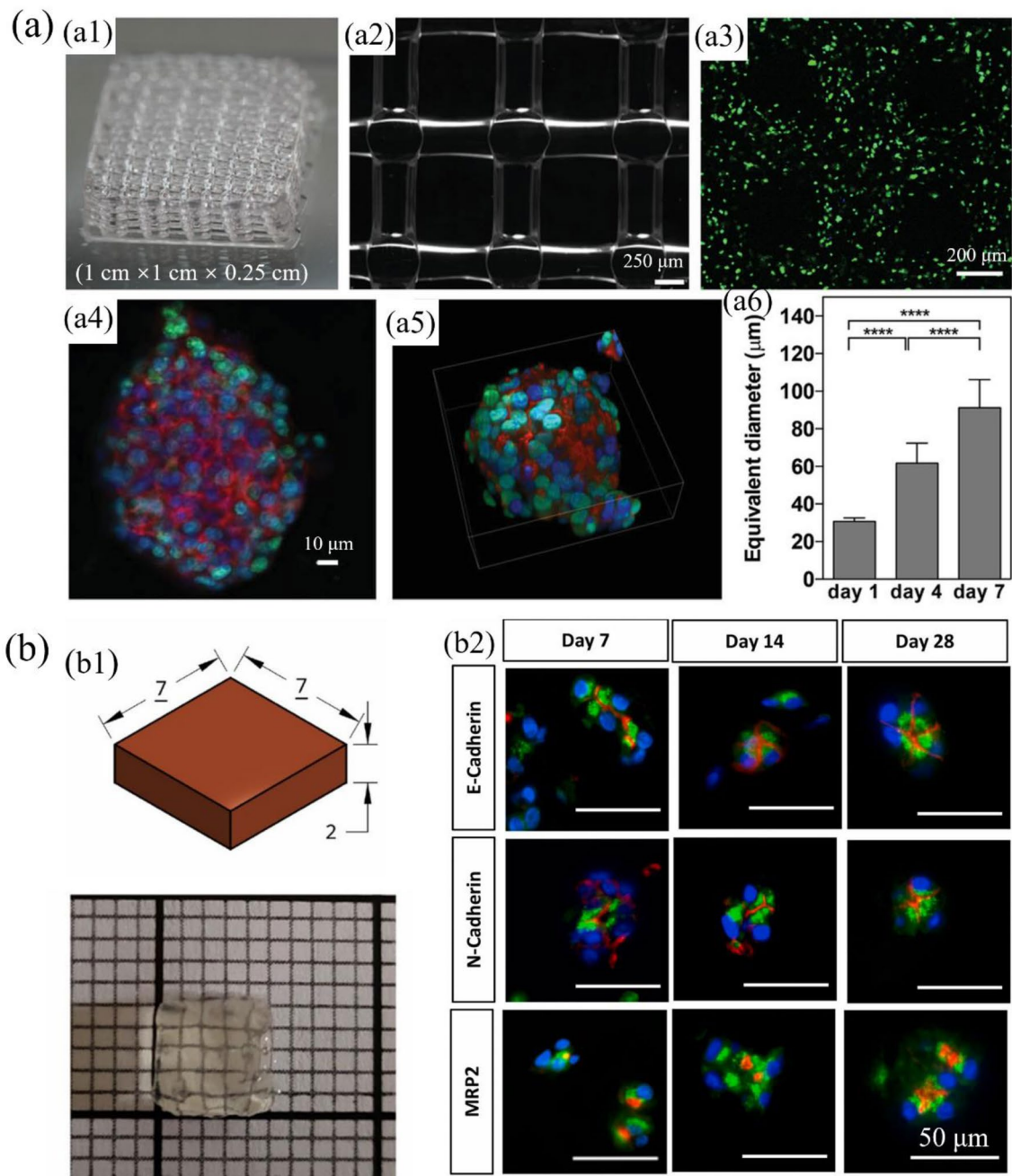
### 3D-printed livers

Recently, bioprinting approaches have facilitated automated and high-throughput fabrication of precisely controlled 3D liver architectures, which promotes accurate prediction of drug hepatotoxicity [103]. For example, Organovo's exVive3D liver is an already commercialized bioprinted human tissue model for drug hepatotoxicity assessment [104]. Hematoxylin and eosin staining have shown that exVive3D liver tissues have similar intercellular junctions, microvascular structures, and cellular compartments to the native liver. In addition, APAP damages exVive3D tissue, similar to clinical results. In another study, bioprinted Pluronic/alginate blends with HepG2/C3A cells were developed for investigating drug-induced hepatotoxicity (Fig. 6a) [105]. The Pluronic templating agent improved the diffusive properties of the hydrogel. In the 3D liver structure, cell viability and liver function improved compared to 2D cultures. Drug hepatotoxicity testing showed increased sensitivity to APAP compared to 2D adhesion controls. In addition, 3D extrusion bioprinting was used to fabricate a complex liver model [106]. The bioprinted structure showed 28-day cell viability and liver function. The study also provided a basis for multicellular models, which combine hepatic parenchymal cells with stellate cells and endothelial cells to study fibrosis development. Recently, a novel liver cancer model constructed using patient-derived hepatocytes was established via 3D bioprinting [107] (Fig. 6b). In long-term culture, the 3D model retained the genetic alterations and expression profile of the original tumor. In addition, the tumorigenic potential and histological features of the 3D model were preserved after long-term culture, meaning that it can be used for evaluating the efficacy of multiple candidate drugs for hepatocellular carcinoma patients.

3D bioprinting combined with microfluidics has become a very hot topic, since it improves models' bionics and complexity [108–111]. For instance, a hepatic bioreactor culture platform integrated with a bioprinter and biomarker analysis module was developed for predicting drug hepatotoxicity (Fig. 7a) [108]. This engineered bioreactor was interfaced with a bioprinter to promote the fabrication of 3D hepatic spheroids encapsulated within a photocrosslinkable GelMA hydrogel. Hepatocyte markers were assessed via detection of the secretion of alpha-1 antitrypsin, albumin, transferrin, and ceruloplasmin; and immunofluorescence staining of hepatocyte markers (i.e., cytokeratin 18, MRP2 bile canalicular protein, and tight junction protein ZO-1) demonstrated that the engineered liver constructs remained functional during 30 days of culture. Additionally, treatment with 15 mM APAP was found to induce toxicity in liver constructs, which was similar to published animal and other *in vitro* model studies, and provided a practical proof-of-use for this liver-on-a-chip platform for toxicity assessment. Another study showed that 3D-cell-printed livers-on-a-chip have a liver microenvironment and biliary system (Fig. 7b) [109]. In this model, multiple cell types encapsulated in liver decellularized bioink were deposited on a chip via 3D cell-printing. In addition, cells were connected through the fluidic channels to mimic vascular and biliary systems. Compared to a chip without a biliary component, liver specific functions were better maintained, and APAP toxicity were more effectively detected in the chip with a biliary fluidic channel. Another key study combined the advantages of 3D printing, co-culture, and microfluidics to develop a 3D co-culture microfluidic model (3DPF) for drug screening research [110]. In this work, 3D printing and subsequent culture were employed to acquire uniform-sized hepatoma cell clusters, and a kind of thermo-sensitive hydrogel maintained the clusters' location and morphology. More importantly, the 3DPF model showed a similar pharmacodynamic character of metuzumab to animal experiments, demonstrating its potential to assess the hepatotoxicity of antibody-based drugs.

### Conclusions and perspectives

In this review, we discussed both the components (i.e., cells and materials) and construction techniques of *in vitro* liver models to evaluate hepatotoxicity for drug development and precise medication. We suggest that livers-on-a-chip are a particularly attractive tool for drug hepatotoxicity assessment due to their ability to simulate *in vivo* drug hepatotoxicity induction *in vitro*. Integration of 3D printing significantly increases the automation and precise regulation of *in vitro* hepatotoxicity assessment models owing to its personalized, rapid, and efficient properties. For example, using decellularized LEM

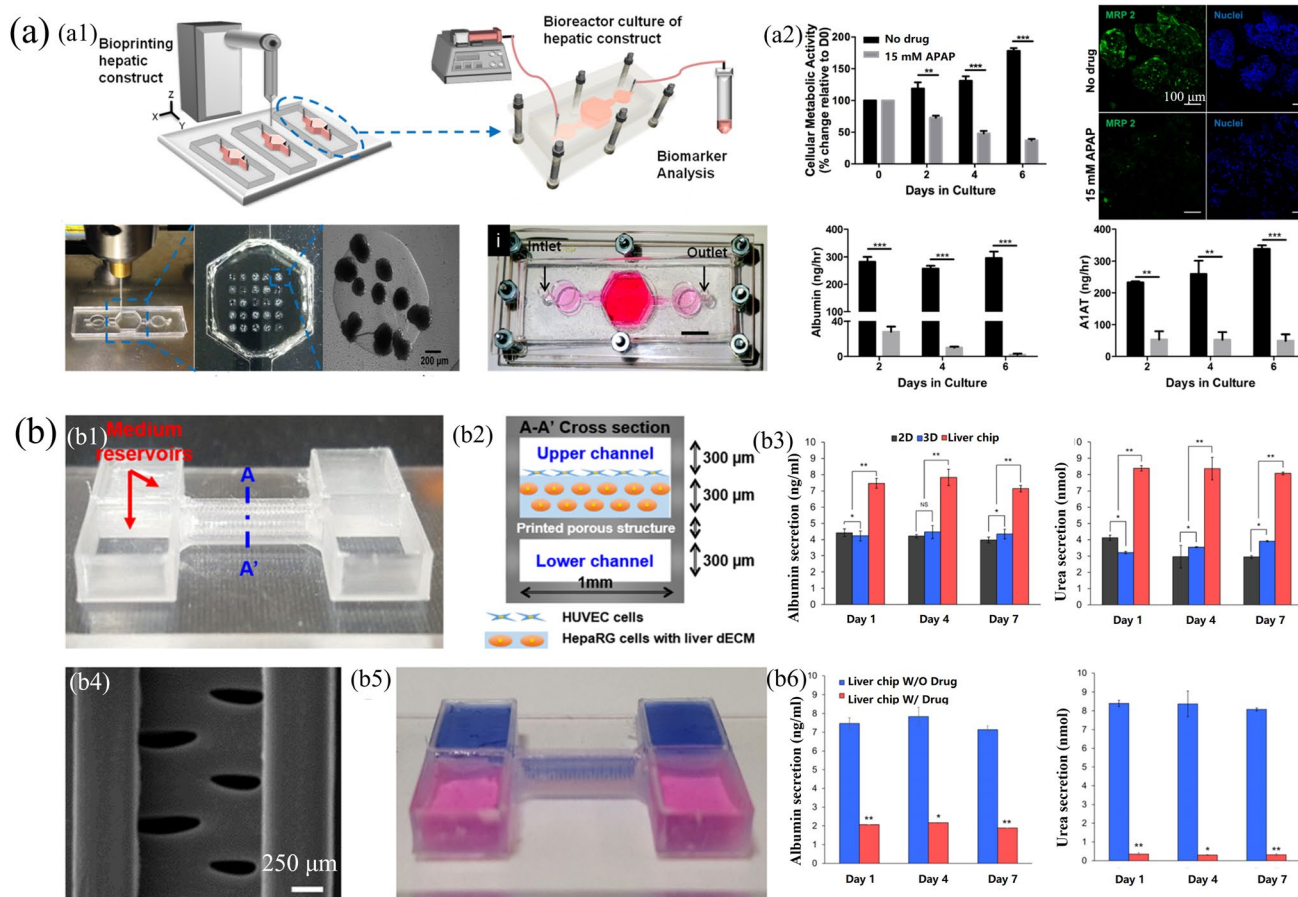


**Fig. 6** Application of 3D printing technology to fabricate a hepatotoxicity testing model. **a** (a1, a2) Microscopic characterization of 3D printed scaffold; (a3) confocal image of HepG2 cells in scaffold at day 0; (a4, a5) confocal images of HepG2 cells in hydrogel scaffolds at day 7; (a6) equivalent diameter of cell clusters in 3D-printed scaffolds (reproduced with permission [105], Copyright 2020, Wiley). **b**

(b1) CAD design (mm) and image of a 3D-printed 5% GelMA scaffold; (b2) immunostaining for E-cadherin, N-cadherin and MRP2 in the 3D-printed scaffolds (reproduced with permission [106], Copyright 2020, Elsevier). CAD: computer-aided design; GelMA: gelatin methacryloyl

bioink material in 3D printing provides a biomimetic element in building a hepatotoxicity detection platform, which offers a more complex internal microenvironment. Microarrays and micropatterns are generally integrated with natural materials

for precise hepatotoxicity testing due to their high-throughput properties. Therefore, multidisciplinary efforts have been dedicated to the development of in vitro liver models for drug hepatotoxicity detection.



**Fig. 7** Application of 3D-printing-based microfluidic hepatotoxicity testing. **a** (a1) Schematic of the hepatic culture platform integrated with a bioprinter module in which a GelMA hydrogel-based hepatic construct was printed in the device; (a2) evaluating 15 mM APAP-induced hepatotoxicity using the liver-on-a-chip platform (reproduced with permission [108], Copyright 2016, IOP SCIENCE). **b** (b1) Image of 3D-printed liver-on-a-chip; (b2) cross-sectional view

of upper and lower channels; (b3) SEM images of the printed porous structure; (b4) image of dual fluidic upper and lower channels; (b5) albumin secretion and urea secretion in 2D culture, 3D culture, and 3D liver-on-a-chip; (b6) evaluation of drug-induced hepatotoxicity with a 3D-printed liver-on-a-chip (reproduced with permission [109], Copyright 2019, IOP SCIENCE). GelMA: gelatin methacryloyl; APAP: acetaminophen; SEM: scanning electron microscopy

However, researchers still seek to establish more precise in vitro liver hepatotoxicity assessment models for better evaluation of drug candidates in clinical diagnosis and drug development [112, 113]. First of all, it is difficult to ensure that the cells used to fabricate a liver model can maintain complete physiological function in a long-term in vitro culture environment. Although iPSC-derived hepatocytes exhibit outstanding advantages in drug toxicity testing, fabricating stably induced hepatocytes is still a difficult point. Secondly, a liver model constructed by in vitro distribution of materials and cells cannot exactly match in vivo features due to the complex structural features and functions of liver tissue. Thirdly, in vitro liver models such as livers-on-a-chip lack interconnectedness with other organs and thus cannot accurately simulate the characteristics of drug metabolism in the body. Therefore, we would encourage continuous efforts for clinical application of in vitro liver hepatotoxicity assessment models.

To conclude, organ-on-a-chip and 3D printing technologies have attracted extensive public attention because of their ability to detect drug hepatotoxicity, allowing personalized healthcare and rapid drug development (as well as reduced economic costs). Meanwhile, organ-on-a-chip combined with 3D printing can accurately simulate the in vivo fluid environment, reduce batch-to-batch variation in liver-on-a-chip models, and achieve cost-effective drug hepatotoxicity detection. More importantly, multi-organ-on-a-chip systems accurately reflect inter-organ effects, have drug metabolism that mimics in vivo conditions, and accurately display hepatotoxic effects. Moreover, a combination of the signal acquisition component and an in vitro hepatotoxicity detection chip is able to realize personalized precise diagnosis and treatment, and improve the accuracy and efficiency of hepatotoxicity detection. We thus envision that hepatotoxicity models for assessment of drug hepatotoxicity will be integrated with organ-on-a-chip

technology, 3D bioprinting, and signal acquisition components for next-generation precise medical devices.

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## Declarations

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** All institutional and national guidelines for the care and use of laboratory animals were followed.

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