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3D bioprinting strategies for recapitulation of hepatic structure and function in bioengineered liver: A state-of-the-art review

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Abstract

3D bioprinting has recently emerged as a successful biofabrication strategy for replicating the complex *in vivo* hepatic milieu. Significant research advances in this field have allowed for the fabrication of biomimetic hepatic tissues with potential applications in the healthcare (regeneration, transplantation, drug discovery) and diagnostic sectors (*in vitro* disease models). This article initially delves into describing the hepatic tissue architecture and function, followed by a rational exposition of how 3D bioprinting potentiates the better development of functional liver tissue compared to traditional tissue engineering approaches and 3D cell culture platforms. This review then highlights the recent breakthroughs and reliable strategies for replicating liver structure and function through bioprinting approaches. In this context, we have systematically described the current landscape of hepatic bioprinting, initially focusing on the cell sources used, followed by the biomaterials and strategies implemented to prolong their *in vitro* viability. Proceeding forward, we have critically highlighted essential aspects of hepatic bioprinting, such as developing tissue-specific bioinks, strategies to induce vascularization within bioprinted liver constructs, and replication of native liver tissue heterogeneity through spatial distribution of multiple cell types in predetermined patterns. In our concluding remarks, we discuss the existing bottlenecks that prevail in this field and provide our viewpoint regarding possible future directions to overcome them.

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Introduction

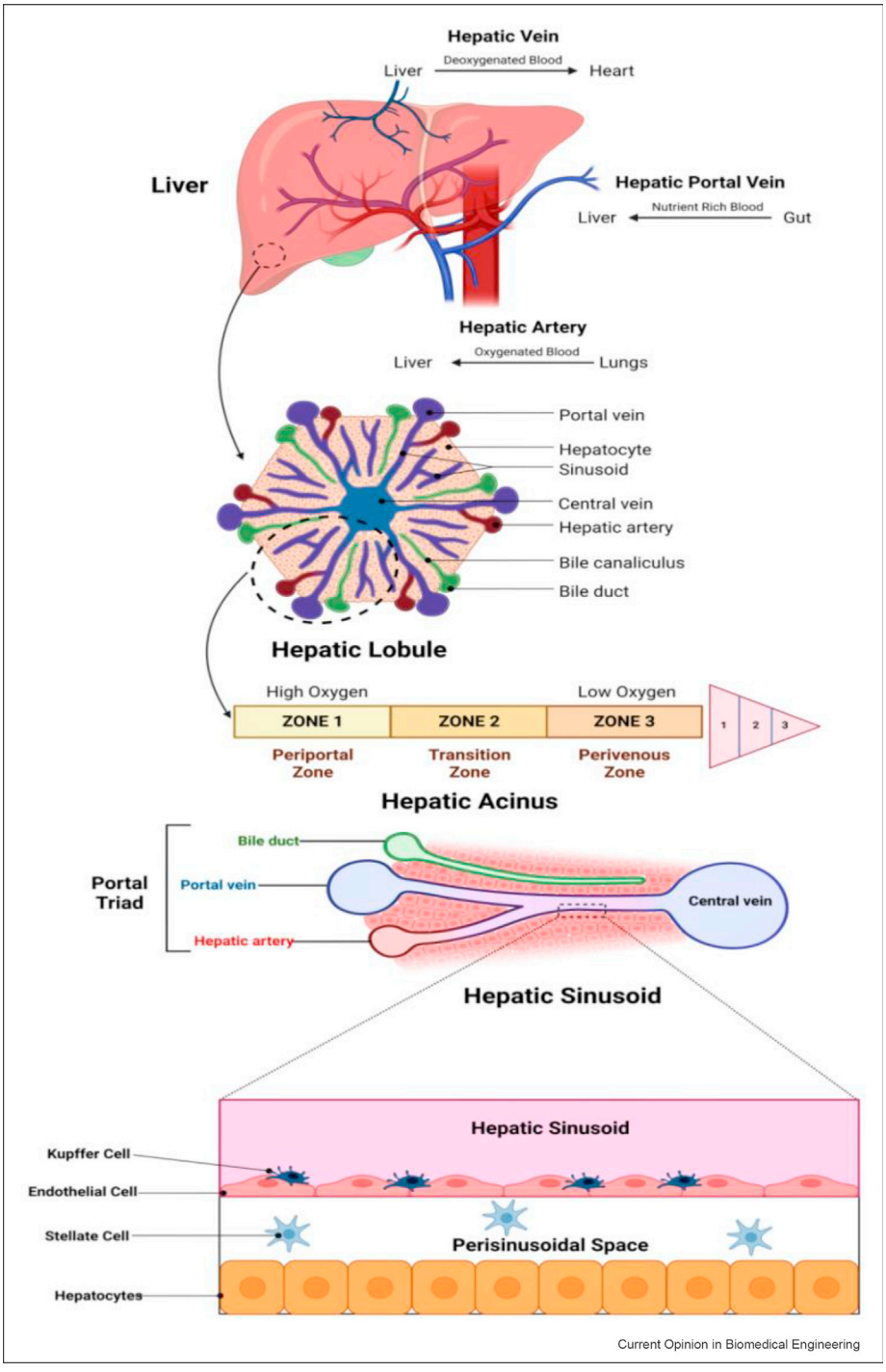
The liver, the largest internal organ in our body, is located in the upper right abdomen. Our liver is associated with multiple life-supporting functions, including glycogen storage, metabolism of biomacromolecules, synthesis of clotting factors, and xenobiotic detoxification, in addition to performing an array of functions that are both endocrine (i.e. hepatokine production and hormone metabolism) and exocrine (i.e. bile production) in nature [1]. The liver's distinctive architectural features and precise interactions between different resident cells and the extracellular matrix (ECM) are critical for its appropriate functioning. Despite being widely acknowledged to possess a high regenerative ability, the liver is frequently exposed to numerous chemicals and biological damage, which inevitably harms the tissue [2,3]. Common risk factors for liver damage include obesity, alcohol abuse, type 2 diabetes, and prolonged drug exposure (drug-induced hepatotoxicity). Additionally, various diseases such as hepatitis B, hepatitis C, cirrhosis, and liver cancer considerably affect liver tissue. Although transplantation is a viable treatment option for patients with clinical deficiencies of a functional liver, the acute scarcity of organs and a poor degree of engraftment serve as major bottlenecks [2]. Furthermore, newer treatment strategies, such as bioartificial liver support systems and cellular therapy-based approaches, are either short-lasting or have poor therapeutic outcomes [4]. In this context, liver bioengineering has recently gained prominence as an alternative for fabricating artificial analogs for assisting repair and regeneration. Such approaches are critically dependent on the selection of appropriate biomaterials and cell sources, as well as the incorporation of appropriate microenvironmental cues and the optimization of appropriate culture conditions. Because of the liver's complexity, including its structural and functional characteristics, replicating it is a challenging task. At this juncture, implementing 3D printing technology to fabricate tissue scaffolding and cell-laden constructs with intricate geometries has emerged as a substantial breakthrough toward generating functional tissues. This advent of 3D bioprinting brings forth exciting opportunities in the field of tissue engineering through the precise spatiotemporal distribution of a bioink in a layer-by-layer approach. In the current review, we briefly describe the structure of the liver, summarize the recent developments and existing bottlenecks in liver bioengineering, and propose a rational perspective on

bioprinting approaches for replicating this structure and function.

Anatomy of the liver tissue

A comprehensive understanding of the liver microstructure (Figure 1) is crucial to successfully developing methodologies for bioprinting the liver. The hexagonal lobular organization of the liver is a critical feature in hepatic microarchitecture, wherein the hepatic lobules (structural units of the liver) organize themselves into irregular polygons demarcated by connective tissue. Additionally, they are composed of plates of resident

Figure 1



Cellular microarchitecture and the basic structural units of the hepatic tissue. A brief outline of the components of the hepatic lobule, hepatic acinus, and hepatic sinusoid.

hepatocytes that radiate outwards from the central vein to the portal triads located at the periphery [5]. The hepatic sinusoids are channels that connect the hepatic plates and carry blood from the portal tracts toward the hepatic venule. These sinusoids are lined by endothelial cells and Kupffer cells (KCs) [6]. Essentially, the endothelial lining is plate-like and fenestrated, facilitating easy blood exchange between the sinusoidal lumen and hepatocytes via the perisinusoidal space. In contrast, KCs are resident macrophages demonstrating phagocytic function [7]. Furthermore, quiescent fibroblasts known as stellate cells generally exist in the perisinusoidal area, which, upon inflammatory activation, commence collagen synthesis [8]. The hepatic lobule is a heterogeneous unit divided into three concentric, diamond-shaped zones of hepatocytes, known as the acinus [9]. These zones of the hepatic acinus are based on the distinct metabolic functions arising from the gradient of oxygen, nutrients, metabolites, and signaling factors that develop as blood circulates in one direction from the peripheral area toward the central vein. At the same time, bile travels in a reverse direction [7]. Hepatocytes in the periportal zone are the first to receive oxygen and nutrient-rich blood due to their proximity to the portal regions. They are metabolically active, thereby engaging in gluconeogenesis, cholesterol biosynthesis, ureagenesis, and bile production. On the other hand, hepatocytes in the perivenous zone receive oxygen and nutrient-deficient blood and are mainly associated with xenobiotic transformation. Hepatocytes from the transitional zone containing mostly oval cells have high regenerative potential [10]. Importantly, the intricate protein network of the liver ECM forms a fibrous scaffold, providing an interface for cell adhesion, space for cell proliferation and migration, and serving as a reservoir for signaling molecules [11]. Hence, the liver is a complex organ, with its distinct microenvironment playing an essential role in its maintenance. Bioprinting strategies for recreating this niche must combine tissue developmental biology, cell composition, cell source, cell positioning, and biomaterials to resemble the native microenvironment of the liver in terms of structure, composition, and local stiffness.

Developmental biology of the liver

A better understanding of the developmental biology of the liver and the molecular interplay between its cells and the surrounding microenvironment will result in significant breakthroughs in liver bioengineering strategies [12]. The current understanding is that the Wnt/β-catenin and fibroblast growth factor (FGF) signaling pathways regulate hepatobiliary development and cell fate during embryogenesis by patterning the endoderm into fore-gut, mid-gut, and hind-gut during gastrulation and early somite stages [13]. The creation of the hepatic diverticulum marks the onset of embryonic liver development. The liver and intrahepatic biliary tree are formed by the anterior portion of the hepatic diverticulum, whereas the posterior portion forms the gall bladder and extrahepatic bile ducts. The liver bud then accelerates its expansion as it is vascularized and colonized by hematopoietic cells. The maturation of functioning hepatocytes is then followed by creation of a biliary network connecting to the extrahepatic bile ducts [13]. Overall, multiple stages of selection and differentiation occur throughout the course of liver development. FGFs, bone marrow morphogenetic proteins (BMPs), and Wnt produced by the mesoderm serve as active mediators assisting the development of hepatoblasts from liver parenchymal cells [14]. Hepatoblasts, which are fetal bipotential liver progenitor cells (LPCs), become abundant in the fetal liver during the mid-gestation period [15]. The activation of the Notch and transforming growth factor (TGF)-β signaling pathways in the fetal liver commits hepatoblasts along the portal vein toward cholangiocytes. During late gestation and the neonatal phase, juvenile cholangiocytes function as LPCs. These LPCs can differentiate into albumin-positive hepatocytes and mature into cholangiocytes through the cumulative effect of hepatocyte growth factor (HGF), Wnt, bone marrow morphogenetic protein (BMP), and FGF signals [16].

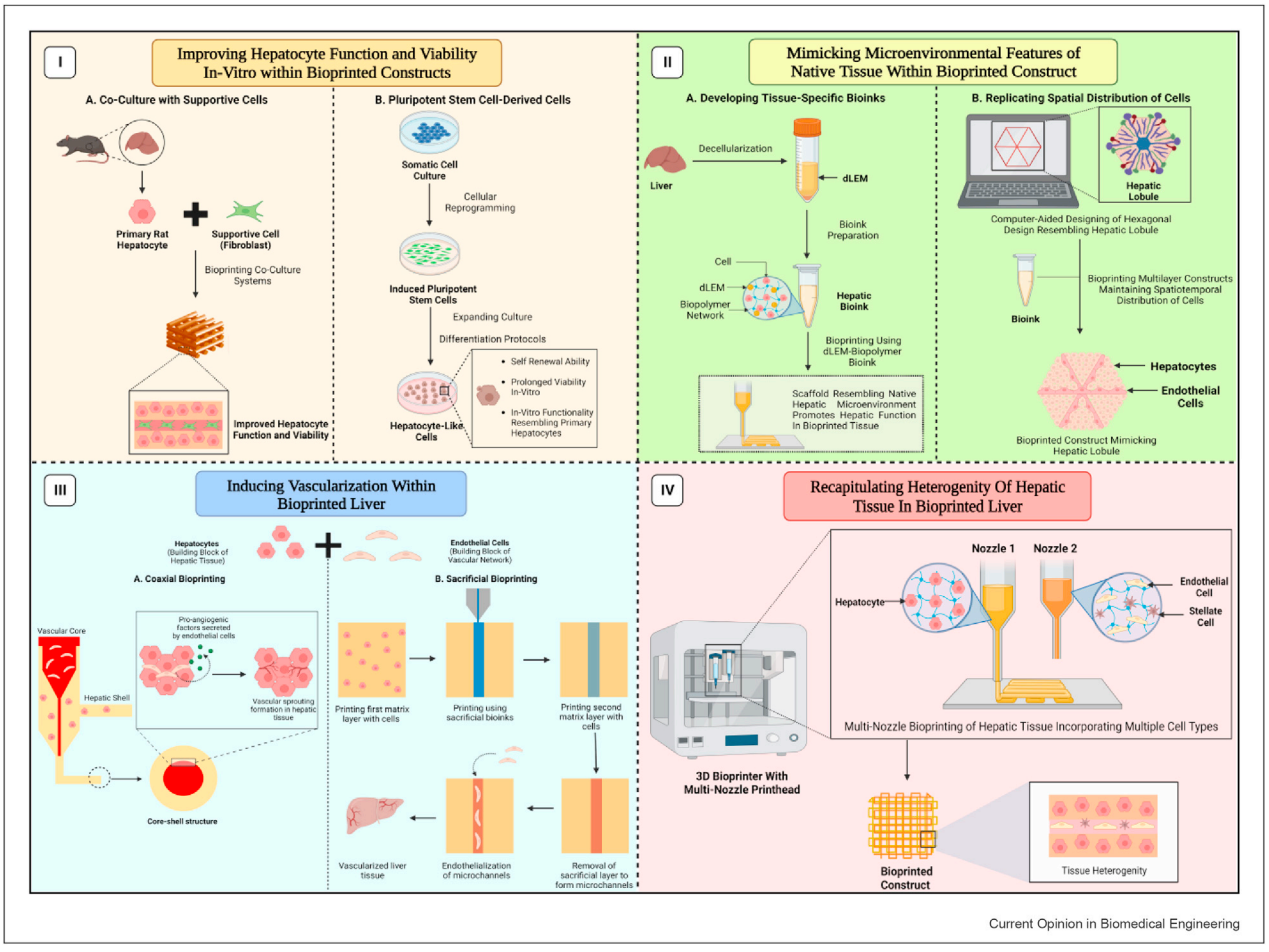
Current landscape of bioengineering the liver using 3D bioprinting

The primary approach to liver bioengineering is establishing a scaffolding platform that can provide necessary biochemical, physiochemical, biomechanical, and microarchitectural properties that replicate developmental biology aspects of native liver tissue. These characteristics determine cell long-term survival *in vitro* and *in vivo*, physiological and functional phenotypes, interaction with the host system during implantation, toxicological aspects, immunological responses, etc. Multiple viewpoints exist on which 3D bioprinting strategies could partially or wholly recapitulate the structure and function of the liver (Figure 2). In the sections ahead, we justify the suitability of bioprinting hepatic tissue and present the current landscape on this topic, highlighting the essential aspects and noteworthy breakthroughs surrounding bioprinting technology for bioengineering the liver.

Need for 3D bioprinting strategies for the biofabricating liver tissue

A significant roadblock faced during bioengineering hepatic tissue is the inherent physiological deterioration of hepatic cells *in vitro*. During conventional culture conditions, such as monolayer cultures, films, electrospun nanofibers, and cell sheets, hepatocytes are exposed to a 2D-matrix environment, which causes loss of cellular phenotype over a short period of time [17]. In such conditions, the cells exist in a flattened-extended

Figure 2



Schematically summarizing state-of-the-art strategies for bioengineering liver tissue utilizing 3D bioprinting technology. I - The first point of concern is prolonging the *in vitro* culture of hepatic cells, which tend to lose their viability and functionality. Emerging bioprinting studies have indicated the use of supportive cells (e.g. fibroblasts) in bioprinted scaffolds to help improve the *in vitro* survivability of hepatocytes. Another strategy has been focused on developing bioinks using alternative cell sources such as iPSCs, which exhibit almost all the properties of native PHs. II - Secondly, mimicking the native *in vivo* microenvironment of the hepatic tissue has been advocated as a promising strategy to develop liver tissue with improved functionality. Tissue-specific bioink development utilizing dLEM is the most commonly reported proof-of-concept, wherein dLEM-bioinks have been known to improve the functionality of encapsulated cells. At the same time, more sophisticated CAD designs replicating the hexagonal lobule-like structure of the liver have been researched with the idea of positioning cells as they are found *in vivo*. III - Thirdly, achieving vascularization in bioprinted liver tissue is a mandate since it governs nutrient perfusion within the neo-tissue and is directly linked to its long-term viability engraftment at implanted site. In this regard, newer studies have considered emerging bioprinting approaches like coaxial and sacrificial bioprinting, which serve as proof-of-concept studies. IV - Lastly, one of the more recent bioprinting approaches is multi-nozzle bioprinting, which, in principle, aims to recapitulate the tissue heterogeneity of native liver through micro-patterning of multiple cell types in specific regions by using multiple nozzles in a single printhead or multiple printheads, each having one nozzle.

morphology characterized by a high proliferation index and high cytoskeletal protein expression levels. A considerable decrease in liver-specific gene expression and other functional aspects often follows, necessitating the need for 3D geometrical platforms to positively influence seeded cells' viability, proliferation, and functionality. Overall, hepatic grafts fabricated using conventionally utilized tissue engineering approaches are associated with lower cell retention, poor engraftment, poor durability of the graft, and complications, including portal hypertension. With the realization of the importance of 3D culture conditions for hepatic development, researchers have previously sought to develop 3D printed scaffolds for seeding hepatic cells and providing them with a suitable environment for engineering *in vitro* liver tissue equivalents [14]. However, such methods frequently fail to mimic the intricacy of native liver tissue and are ineffective in distributing several cell types in desirable patterns [19]. The rapid development of conventional 3D-printing techniques for fabricating 3D structures that emulate complicated geometries and structures at the tissue level enabled the

genesis of bioprinting technology. Bioprinting enables rapid fabrication of tissues with great precision and control over size, as well as variations to the shape, porosity, stiffness, mechanical strength, and biodegradability of constructs. Bioprinting allows the manipulation of cell-cell interactions through the precise spatiotemporal positioning of cells. Hence, it can circumvent issues inherent in conventional 2D and 3D models, such as low engraftment efficiency and poor functioning of cells arising from a lack of appropriate cellular crosstalk [20]. Furthermore, bioinks may be combined with various paracrine components, including growth factors, whose release could be further controlled spatiotemporally to facilitate effective neo-tissue formation. Therefore, bioprinting technology has progressively been advocated as a method to mimic the complexity and architecture of native liver tissue. Overall, the advent of 3D bioprinting has shown promise in expediting the goal of the fabrication of fully functional liver tissues, potentially making it a forthcoming reality.

Hepatic cells

The choice of cells represents an indispensable component of the bioink since they are the building blocks associated with neo-tissue formation. Towards this end, multiple studies have reported the development of bioprinted liver tissues using hepatic cell lines (e.g. HepG2, Huh-7) [18,21]. Despite their robustness and ease of culture up to infinite passages, cell lines eventually tend to undergo genotypic and phenotypic changes during culture and, hence, may not represent primary cells adequately due to altered metabolic functions [22]. Therefore, researchers have sought to harvest primary hepatocytes (PHs) and use them for bioink preparation. PHs are often regarded as the gold standard cell source for hepatic bioprinting because of their high metabolic activities. However, PHs are inherently quite difficult to maintain. Following removal from their native microenvironment, PHs begin to undergo oxidative stress, resulting in a sharp decline in their functionality and becoming "fibroblast-like," characterized by the loss of their cuboidal morphology, the development of actin stress fibers, and eventually undergoing apoptosis [23]. Moreover, during standard 2D culture, PHs are known to de-differentiate within a few days and subsequently show declining hepatocyte-specific gene expression and functions. Efforts to promote their viability *in vitro* have relied on mimicking the native microenvironment using co-culture systems, wherein their interactions with nonparenchymal cells, such as fibroblasts and endothelial cells, improved their survival and functionality due to cell-to-cell interactions and the release of soluble factors by the supporting cells [24]. Taymour et al. reported the fabrication of functional liver tissue by implementing a coaxial bioprinting approach involving a co-culture system, wherein core-shell structures accommodating fibroblasts and hepatocytes in the 'core' and 'shell' regions, respectively, allowed for the long-term functionality of the hepatocytes [25]. Therefore, the microenvironment of hepatocytes could be designed to mimic their microenvironmental conditions through specific tailoring of the bioink composition and coaxial printing of two separate bioinks in the core-shell form to allow spatial arrangement of cells resembling the hepatic lobule.

An alternative aspect investigated involves the use of human-induced pluripotent stem cells (hiPSCs) possessing the ability to self-renew, being grown *in vitro* up to high cell numbers, and being differentiated into hepatocyte-like cells (HLCs) [26,27]. Most hepatocyte-like cells generated from hiPSCs have an immature phenotype and show limited metabolic capabilities compared to primary hepatocytes. Nonetheless, hiPSCs have the potential to be a promising cell source for the development of functioning hepatocytes that can reach full maturity with further efforts. In line with these efforts, He et al. successfully bioprinted hiPSC-derived HLCs to develop a hepatic model for *in vitro* toxicological studies [28]. The HLCs within the bioprinted scaffolds demonstrated excellent growth and spheroid formation, possibly attributed to interconnected microchannels facilitating nutrient diffusion. Additionally, the cellular activity of bioprinted HLCs was well maintained, resulting in higher mRNA expression levels of liver function-related proteins. This study provides significant impetus for further research involving the generation of HLCs as a suitable cell source encompassing the characteristics of native PHs for bioprinting applications.

Bioinks for bioengineering liver

Hepatocytes are essentially adherent cells; hence, the ECM is necessary for their survival and functionality [29]. As a result, different biopolymers have been explored as potential bioinks for successful bioprinting with hepatic cells. The ultimate goal here is to simulate the *in vivo* hepatic microenvironment as closely as possible to provide instructive cues for cell attachment, proliferation, differentiation, and neo-tissue formation. Hydrogels, which are hydrated, crosslinked polymer networks possessing optimizable stiffness, are best suited to stimulate the native stiffness and topology of the hepatic ECM [30]. Since protein- and polysaccharide-based ECM make up the majority of the liver, hydrogel bioinks constituting such biopolymers have a significant promoting effect on protecting and supporting the cell growth of liver tissue *in vitro*. In other words, such biopolymers are more conducive to restoring the cellular microenvironment *in vivo* of the liver cells. Due to the liver's intricate structural and physiological properties, using single-component bioinks to bioprint 3D *in vitro* liver structures remains challenging. Multicomponent bioinks have become an appealing strategy for bioprinting

liver tissues with better mechanical characteristics of the printed constructs and cellular functioning [31]. Consequently, multiple biopolymers have been used as bioinks for hepatic bioengineering and have been elaborately reviewed recently [29]. Polysaccharide-based bioinks, despite their well-reported biocompatibility, possess certain limitations. For instance, although exhibiting cytocompatible gelling mediated by Ca^{2+} ions, alginate is bioinert and lacks cell attachment motifs [32]. On the other hand, chitosan possesses excellent cytocompatibility and antibacterial and antioxidant properties, which can protect cell proliferation, viability, and functionality [33]. Moreover, modified chitosan derivatives such as galactosylated chitosan possessing galactose moieties have reportedly been utilized for providing anchorage sites to hepatocytes [34]. Nevertheless, chitosan is insoluble in water at physiological pH and precipitates at pH values beyond 6.2 [35]. Contrastingly, protein-based bioinks such as collagen and its hydrolyzed counterpart, gelatin, are known to possess Arginyl-glycyl-aspartic acid (RGD)-motifs necessary for cytoadherence but exhibit weak mechanical properties necessitating coblending with other polymers. A recent study involving culturing hepatoblast-like cells on the gelatin scaffolds reported their differentiation into hepatocyte-like cells (HLCs) following transplantation into partial-hepatectomized mice. Additionally, the expression of liver-specific genes and the cell proliferation marker, Ki67, indicated appropriate proliferation and functioning of cells within these scaffolds [36]. Silk fibroin bioinks, despite lacking putative cell attachment motifs, have been reported to upregulate the Wnt/β-catenin and FGF signaling pathways, which are known to regulate hepatobiliary development [18,37].

Recently, incorporating decellularized ECM in bioinks can provide the encapsulated cells with a more appropriate microenvironment similar to their native one. Studies have revealed that encapsulating hepatocytes in decellularized liver ECM (dLEM) hydrogels significantly improves their viability and functionality [38]. However, the primary limitation of employing these scaffolds is that the dLEM scaffolds do not have the optimal mechanical properties necessary to sustain the long-term survivability and functionality of hepatocytes. This problem can be solved by co-blending dLEM with other polymers and introducing crosslinking between the dLEM-polymer polymer backbone, resulting in a stable structure with improved biological activity. An important consideration here would be to control the degree of crosslinking, which directly affects the hydrogel stiffness [39]. The stiffness of the scaffold inevitably affects the metabolism and functionality of encapsulated cells. For instance, scaffolds with stiffness close to that of native liver tissue promoted cell attachment, migration, and hence functionality in comparison to stiffer scaffolds [40]. Photo-crosslinkable liver-specific bioinks encapsulating human-induced hepatocytes (hiHeps) were developed by Mao et al. by combining GelMA with dLEM for fabricating hepatic microtissue using a digital light processing (DLP)-based 3D bioprinting approach [41]. The results corroborated the hypothesis that dLEM addition in polymeric solutions improves the tissue-specific properties of the resulting bioink. To avoid printing dLEM-based bioinks at low temperature ranges, which is not favorable for extended print duration with cells, Khati et al. developed a novel dLEM-based bioink that enabled printing at physiological temperature ranges [42]. The results provide strong evidence to substantiate that dECM-based bioinks provide intrinsic biochemical cues to cells, thereby promoting cell attachment and their downstream activities within bioprinted constructs.

3D bioprinting approaches for fabricating liver tissue

Bioprinting technologies are mainly classified into three approaches: inkjet-based bioprinting, extrusion-based bioprinting, and laser-assisted bioprinting, with more sophisticated approaches usually based on either of the ones mentioned above (Table 1) [43]. Extrusion-based bioprinting is the most versatile approach compared to other forms of bioprinting. Consequently, there has been an active exploration towards bringing forth more sophisticated and state-of-the-art extrusion-based approaches such as multi-nozzle bioprinting, coaxial bioprinting [25], and sacrificial bioprinting [44] for bioprinting liver. In terms of microenvironmental features of the liver, the hepatic lobule forms the liver's basic structural and functional unit. Replicating this spatial distribution between hepatic and vascular cells within the liver is essential for developing platforms to recapitulate its structure and function *in vitro*. To engineer similar multi-scale heterogeneous tissues, Kang et al. fabricated hepatic lobule-like structures containing hepatocytes (HepG2), endothelial cells, and a lumen using a preset bioprinting technique [45]. Each cell type with spatial cell patterning in bioink has been demonstrated to expedite cellular organization, maintain structural integrity, and increasecellular activities. The work by Leva et al. involved the laser-induced forward transfer (LIFT) technique to laser print the Huh7 on a porous collagen-glycosaminoglycan (GAG) matrix [46]. The findings demonstrated the advantages of using laser bioprinting to precisely position and immobilize hepatocyte cells into porous collagen scaffolds for innovative, custom-made implants for regenerative medicine applications.

The ultimate goal envisaged for fabricating an artificial liver includes incorporating all the structural and functional components of hepatic tissue, with particular attention on mimicking the microenvironment and spatial distribution of cell types in specific compartments predetermined during printing design. Notably, there has been active research involving bioprinting co-culture systems towards improving cell function and

Table 1

Summary of the major 3D bioprinting approaches utilized for bioprinting liver tissue with their advantages and disadvantages.

Type of 3D bioprinting		Description	Advantages	Disadvantages	References
Inkjet based bioprinting		A noncontact printing technology based on traditional inkjet printing in which successive droplets of dilute bioinks are deposited onto a substrate utilizing thermal energy, piezoelectric impulses, or microvalve forces.	1. It is very fast and has a high printing resolution of approximately 50 μm. 2. Low costs involved.	1. Requirement of low viscosity bioinks (<10 cP) which result in constructs exhibiting weak mechanical integrity. 2. Lack of precision in droplet placement and nonuniformity of droplet size. 3. High cell density causes clogging of nozzle.	[65]
Extrusion based bioprinting	Multi-nozzle bioprinting	It is a printing approach that relies on a single printhead or multiple printheads comprising multiple printing nozzles, each capable of extruding a different polymeric bioink and/or cells simultaneously using mechanical or pneumatic forces.	1. It can be used to print using a wide range of viscosities. 2. It is possible to print using multiple cell types with varying densities. 3. It offers the possibility of micropatterning cells in specific positions to fabricate heterogeneous tissues like the liver.	1. It is challenging to enhance printing speed and resolution. 2. It elicits detrimental effects on cell viability since cells are subjected to shear during extrusion.	[49]
	Coaxial extrusion bioprinting	An extrusion-based bioprinting approach featuring a concentrically stacked nozzle is usually capable of dispensing two bioinks as a single filament arranged concentrically in a core-shell layout.	1. It enables the printing of coculture systems with well-oriented compartments ideal for developing vascularized tissues resembling liver sinusoids. 2. Relatively short printing duration. 3. Low setup costs making it cost effective.	1. It is challenging to mimic anatomical bifurcate structures. 2. It is difficult to stack hierarchical constructs.	[25]
	Sacrificial extrusion bioprinting	It is a highly developed method based on extrusion bioprinting that uses supporting structures generated using sacrificial bioinks, which eventually get dissolved to create tissue blocks with empty channels that imitate the vascular network.	1. The hollow channels generated by the sacrificial inks enable the transport of oxygen and nutrients to cells, improving cell viability. 2. It is effective in creating micro-channels for developing organ-on-a-chip models.	1. Limited availability of sacrificial materials is available (e.g. PVA, gelatin, alginate, pluronic F127). 2. Lower printing accuracy. 3. Difficult to print complex, delicate structures such as biliary trees.	[44]
Laser assisted bioprinting		A nozzle-free printing technology in which the bioink is suspended at the bottom of a thin ribbon and is transferred onto a receiving substrate when vaporized by a laser pulse.	1. It is possible to print using high viscosity bioink and high cell densities without causing nozzle clogging. 2. High degree of precision and resolution of approximately 10 μm.	1. The process of ribbon preparation is time consuming. 2. The post printing viability of cells is low. 3. Higher costs involved.	[65]
Stereolithography		A layer-by-layer fabrication technology in which a moving laser beam is focused on the unbound surface of a liquid photopolymer resin, i.e. the bioink, in order to trigger polymerization and change the liquid into a polymerized solid.	1. It offers a high degree of fabrication accuracy, with a resolution ranging from 40 to 150 μm. 2. Low printing durations. 3. It ensures high post printing cell viability (>90%).	1. Limited choice of biomaterials since only photocurable materials can be used as bioinks. 2. Lengthy post processing time.	[66]

vascularization. While such studies have reported recapitulation of certain aspects of liver function and vascularization, there is immense scope for research to accommodate other cell types in the liver. Earlier studies have tried to replicate the heterogeneous layered architecture of native liver tissues through coculture 3D liver models comprising multiple cell types sequentially seeded on hollow fiber membranes to promote organotypic interactions [47]. The coculture system, comprising hepatocytes with sinusoidal endothelial and hepatic stellate cells, successfully preserved the structural architecture of hepatic tissue and improved the liver-specific functions compared to hepatocyte monoculture. Other studies have also demonstrated the favorable effect of heterotypic interactions on liver-specific processes, emphasizing the importance of nonparenchymal cells in the maintenance of hepatocyte phenotype and function [48]. With its ability to precisely micro-pattern cells and biomaterials, bioprinting technology can serve as a more robust and efficient tool for fabricating biomimetic *in vitro* liver models with increasing structural complexity [19]. Bioprinting offers the scope to print complex heterogeneous tissues comprising multiple cell types, such as the liver, by using multinozzle printhead systems. Such systems can fabricate fully functional tissues through the deposition of multiple bioinks encapsulating different cell types, which is achieved by the programmed switching of the printing nozzle. This would inevitably allow the user to have better control over governing the cell distribution and cellular crosstalk towards more accurate mimicking of native tissue environments in both healthy and diseased conditions. A steppingstone in this direction has been put forward by researchers at Organovo, a US-based company, who, using their proprietary NovoGen Bioprinter® platform, successfully developed heterogeneous hepatic tissues. The developed hepatic tissue patches termed ExVive™ reportedly allowed the controlled incorporation of primary human hepatocytes, and endothelial cells, hepatic stellate cells, Kupffer cells in an automated and precise fashion [49].

A pertinent limitation encountered during scaffold-based tissue model systems is the immobilization of encapsulated cells within polymeric biomaterials, which limits liver-specific cell—cell interactions and cell orientation and slows down cell maturation. Scaffold-free bioinks have been identified as an exciting development in tissue fabrication given that they are essentially cell aggregates (e.g. spheroids, organoids, cell sheets) that are entirely biological and biocompatible, can provide high cell density and facilitate ECM deposition with better cell-cell interaction. Compared to scaffold-based bioinks, the time required for neo-tissue formation is considerably reduced due to the high initial cell seeding density, unhindered cell proliferation, and migration [50]. Implementing such approaches expedites neo-tissue formation and integration into its surroundings because there are no other biomaterials to degrade and adjust to the environment. It also avoids the rejection and tissue failure issues common with biopolymer scaffolds [51]. There are numerous methods for fabricating cell aggregates (magnetic levitation, hanging drop, hydrogel microwells, spinner flasks, etc.); the hydrogel microwell method is widely accepted for producing freely floating spheroids on antiadherent U-bottom 96 wells, which can be directly bioprinted [52]. A relatively newer approach known as the Kenzan method was adopted by Kizawa et al. wherein the establishment of a scaffold-free bioprinted model of hepatic tissue was reported using coculture spheroids derived from cryopreserved hepatocytes and mouse fibroblasts [53]. Using a Regenova® printer, the spheroids were positioned at precise locations on skewers, which subsequently fused to form larger and more complicated structures resembling miniature liver tissue. The formation of hepatic microarchitectural features such as bile duct and blood capillary-like sinusoid-like structures within the bioprinted tissue demonstrated that the bioprinted liver tissue was unique given that it exerted diverse liver metabolic functions for several weeks.

Culturing technique (static vs dynamic)

A frequently ignored and decisive factor that often controls the fate of bioprinted cells is postprinting maturation. Since oxygen is less soluble in an aqueous medium than glucose, it is typically the limiting nutrient in tissue-engineered structures. The scenario is exacerbated in high-hepatocyte density cultures and under static conditions. Moreover, it is worth noting that when hepatocytes are cocultured with other nonparenchymal cells, the heterotypic interactions have a synergistic effect on hepatocyte metabolism and proliferation activity, which increases oxygen demand [47]. In such a scenario, dynamic cultures can allow perfusion through bioengineered tissues, thereby improving the oxygen uptake by cells and enabling their long-term viability and functionality. Hence, even though most studies report the maintenance of bioprinted constructs under static conditions, it must be pointed out that the dynamic culturing of bioprinted liver constructs is not given its due importance. During static culturing, constructs are incubated within a biological incubator with periodic media changes and growth factor supplementation to modulate essential cell signaling pathways. It is worth mentioning that the expression of many drug-metabolizing enzymes' is considerably downregulated in static 3D bioprinted hepatic cultures. Static cultures diminish cell signaling, critical in maintaining stable liver-specific activities, and induce nonsteady state conditions due to decreasing substrate concentrations and waste product accumulation [54]. Although static culture techniques are easy to employ, they lack the complexity of *in vivo* liver tissue. Dynamic cell culture

refers to cell culture *in vitro* in the presence of mechanical stress. The capacity to control the fluid flow, such as medium, over cells while they flourish is of considerable emerging interest. Dynamic culture conditions promote mass transfer and mechanotransduction, resulting in more functioning cells [55]. Furthermore, bioreactors directly utilize mechanical forces to establish physiologic conditions and accelerate differentiation towards a specific lineage in dynamic differentiation processes. Dynamic 3D culture models are becoming increasingly crucial in liver bioengineering research because they better replicate perfusion conditions in native tissue than static culture systems. Compared to static culture models, such systems have been proven to sustain liver cell activities for extended durations and provide exceptional sensitivity to enzyme induction [56]. For example, Ahmed et al. reported that coculturing hepatocytes, endothelial cells, and stellate cells under dynamic hollow fiber membrane culture conditions was associated with prolonged albumin and urea secretion for 28 days, in addition to maintaining high levels of diazepam bio-transformation activity [47]. The membrane bioreactor fostered a physiologically realistic milieu for cells by ensuring that nutrients and metabolites perfuse through the porous wall while removing catabolic and particular cellular products from the extracapillary area.

Induction of vascularization

Perfusion through the liver vasculature is necessary for maintaining cell viability and functionality. Hence, induction of neovascularization is a prerequisite for *in vitro*-generated liver tissues. 3D bioprinting allows for the fabrication of liver tissue with precise microvasculature, which may develop in a predefined pattern that not only preserves structure but also encourages cell-to-cell contact [57]. Microvascular induction methods target the creation of vascular channels by taking advantage of cells' proclivity to expand towards an increasing nutrition gradient. This technology overcomes the restrictions of printing resolution and allows for the development of physiologically relevant microvascular networks. Studies have demonstrated that adding endothelial cells to the culture medium causes the production of microvessels and, eventually, angiogenic sprouts in the bioprinted construct due to the controlled endogenous release of proangiogenic factors [58]. Moreover, coculturing hepatocytes and endothelial cells in biomimetic ratios creates paracrine signaling, supporting vasculature formation involving a self-assembly process of endothelial and supporting cells into functional capillary-like structures. While human-umbilical vein endothelial cells (HUVECs) are commonly used, liver sinusoidal endothelial cells (LSECs) better recapitulate the fenestrated phenotype critical for substance exchange. However, isolating primary LSECs is tedious, and purity is difficult to control. A recent study reported the development of a hepatic sinusoid-like model consisting of an angiogenesis-supporting collagen-fibrin-gelatin (CFG) core compartment containing HUVECs and fibroblasts, in addition to a plasma-algMC shell compartment containing hepatocytes, using a coaxial bioprinting approach [59]. In the presence of HepG2 in the shell, HUVECs encapsulated within the CFG core and human fibroblasts as supporting cells developed a prevascular network. Although hepatocytes and HUVECs were reported to compete for the supportive fibroblasts, the overall cellular interactions in the triple culture model demonstrated an increase in net albumin production due to increased cell viability arising from developing vascular networks within the core-shell constructs.

In producing vascular networks, sacrificial printing methods for forming a hollow channel-like structure through heating and dissolving temperature-sensitive materials strongly have been proposed. Such a bioprinting approach entails the use of sacrificial materials as interim templates for vessel development. Sacrificial materials can be printed alongside the cells and then removed, leaving hollow channels that can be infiltrated with endothelial cells to form blood arteries. In this regard, Liu et al. developed soft, vascularized liver tissues via dissolving Pluronic F127 and incubating HUVECs to create channels and vascular beds [60]. The printed constructs, also containing HepG2 and mesenchymal stromal cells (MSCs), could effectively be perfused through branched endothelial vasculature to support well-formed 3D microcapillary networks, mimicking mature and functioning liver tissue in terms of liver-specific protein synthesis. A more recent study by Fan et al. involved a dot extrusion printing technology where sacrificial printing of HUVEC-laden gelatin microbeads was carried out on a C3A-laden GelMA microbead to form millimeter-scale hexagonal hepatic lobule-like constructs with vascular networks [61]. Despite all the progress made, replicating the complicated architecture and hierarchical structure of the liver's vasculature is still complicated. Bioprinting techniques must correctly reproduce the various vessel sizes, branching patterns, and linkages in the liver. However, cell-laden constructs comprising tailored microchannels have been observed to improve cell survival and differentiation capacity when compared to constructs without microchannels. One of the major issues that must be addressed is the small size of capillaries for printing. An elaborate preset design is required for the coupling of microchannels via angiogenesis. Future research is strongly recommended to develop more sophisticated designs to promote vascular channel formation within hepatocyte cords, as in native hepatic lobules, instead of forming limited vascular branching within bioprinted constructs. Overall, bioprinting is more conducive to fabricating liver tissues

with intricate vascular networks that can form according to a preset pattern that preserves structure well and promotes cell-to-cell interactions.

Challenges and future directions

Despite substantial advances in liver tissue engineering, hepatic tissue remains difficult to replicate due to its structural and physiological heterogeneity. The mechanobiological properties of a bioprinted construct, particularly its matrix stiffness, influence the balance of forces involved in the cell-matrix interaction. Regarding hepatic matrix stiffness, the matrix is soft in the proximity of hepatocytes but stiffer in the periportal region, wherein cholangiocyte differentiation and bile duct morphogenesis occur. Aside from the specific arrangement of distinct cells found in hepatic tissue, this difference in local stiffness is exceptionally complicated to reproduce. Developing biomaterials that can be tailored to mimic the stiffness of the healthy liver or the stiffness of various diseased states for *in vitro* investigation is a significant problem in bioprinting technologies. Bioprinting has tremendous potential to address this challenge owing to its versatility, customizability, reproducibility, and microscale controllability in positioning diverse cell types in predetermined patterns [19]. By implementing multi-nozzle bioprinting approaches involving multicomponent bioinks with varying crosslinking ratios, it is possible to bioprint hepatic constructs with spatially varying mechanical stiffness as in their native counterparts. The capacity of 3D bioprinting approaches to produce intricate structures is a promising development in liver tissue engineering. Nonetheless, guaranteeing printed tissues' long-term functional and structural stability is critical to their success. High cell densities in bioprinted structures are required for appropriate tissue function.

It is also critical to ensure that the cells are uniformly dispersed within the biomaterial scaffold to ensure proper cellular crosstalk with one another throughout the construct. This can be difficult since cells would remodel, migrate within the hydrogel bioink, and reconfigure during culture. Hence, protecting the function of bioprinted tissues by minimizing distortion and preserving its original structure is critical. Another impending challenge yet to be addressed in the field of liver engineering is to successfully develop a hepatobiliary network within bioengineered liver tissue by populating the bile duct with functioning cells (e.g. cholangiocytes). The resolution of 3D bioprinting is, at present, inadequate for replicating complicated hepatic microenvironments since the printed hydrogel architecture is too large for controlling cells, and the scaffold's randomly dispersed cells cannot preserve the delicate anisotropy. Nevertheless, studies have shown that dECM hydrogels, in particular, when populated with immortalized mouse cholangiocytes, were associated with intrahepatic bile duct formation [62]. Moreover, subtle changes in oxygen and nutrient concentration gradients in different liver zones are challenging to replicate while incubating bioprinted constructs in a culture medium [63]. In light of addressing these challenges, efforts have been made to integrate bioprinting approaches with other emerging technologies, such as organ-on-a-chip technology based on microfluidics and organoid engineering, for the creation of micro-organs with zonal heterogeneity, ideal 3D cellular arrangements, tissue-specific functions, and even circulatory motions in microfluidic devices [64]. Another plausible approach that should be explored is to spatio-temporally localize cells at different densities within bioprinted constructs to tailor the oxygen gradient within them. Higher cell densities correlate with lower oxygen gradients, and vice versa. Here also the concern lies to maintain the oxygen gradient within an optimal range, which would not pose any detrimental effects on the cells.

This review presents different directions of research that have been carried out recently with the common goal of developing long-term, fully functional, and vascularized hepatic tissue analogs replicating the native tissue heterogeneity. It is prudent to conduct future research toward integrating these approaches coherently. This would be the determining factor in making the successful fabrication of an artificial liver a reality. Overall, 3D bioprinted *in vitro* liver architectures provide promising findings for drug screening, disease models, and liver regeneration and transplantation applications. However, clinical translation and commercialization of such bioprinted liver tissue are dependent on how well the 3D bioprinted model mimics the activities of a native liver. This necessitates substantial optimization during the bioprinting process. 3D bioprinting can potentially develop macroscopic and microscopic parts of an organ, making it a one-of-a-kind technology for precisely fabricating vascular and ductular systems in the liver. Consequently, it is expected that the development of *in vitro* liver structures using 3D bioprinting will exponentially rise in the future due to advanced current research in biomaterials and a better fundamental understanding of liver development and physiology.

CRediT authorship contribution statement

Arka Sanyal: Conceptualization, Data Curation, Writing - original draft,Visualization. **Sourabh Ghosh**: Conceptualization, Supervision, Writing - review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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