Strategies for Constructing 3D Bioprinted Liver Organ Models

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Abstract. Liver diseases are a severe global health challenge. Liver transplantation, as the gold-standard therapy for end stage liver disease, still suffers the fundamental constraint of severe donor shortage. 3D bioprinting technology provides new opportunities to construct functional liver organ model. Metabolic zonation is the core organizational principle of liver function, which is driven by gradients of oxygen and signaling molecules formed during the blood flow from the portal tract to the central vein. The existing in vitro model can not recapitulate this spatial heterogeneity, which severely limits the accuracy of drug metabolism research and toxicity prediction. In this article, the capability boundaries of four mainstream bioprinting technologies, i.e. extrusion-based, DLP photocuring, coaxial printing and embedded sacrificial material printing in reconstructing metabolic zonation are systematically reviewed. On this basis, the multi-technology collaborative strategy is proposed which starts from the target metabolic zonation map and works backward to match the technologies, and the three-layer architecture framework span from microscale topology to macroscale perfusion is constructed. This strategy provides the theoretic reference for advancing 3D bioprinted liver models from morphological biomimicry towards functional biomimicry. The future research may further verify the real efficacy of this framework in reproducing zone-dependent metabolism in in vitro pharmacological screening, and to explore the standardized application pathway in high-throughput system.

Keywords: 3D bioprinting; liver; metabolic partitioning; liver lobules; organ model.

1. Introduction

Liver diseases represent a severe global public health problem: about 2 million dies every year, corresponding to 4% of the total global mortality [1]. Patients with end stage liver disease require liver transplantation. However, as the donor is severe shortage, only about 25% of those in the waiting list can be operated. Therefore, it is urgent to develop other therapeutic strategies and in vitro research models.

Drug development is very important for in vitro liver models. About 70% - 80% of the clinical drugs go through the hepatic metabolic transformation [2]. In traditional two dimensional culture, hepatocytes dedifferentiate quickly, and in the standard model, the hepatocytes are treated as a homogeneous population of which the spatial functional zonation is ignored, all of them directly affect the reliability of the toxicity prediction.

3D bioprinting shows specific advantages for the positioning accuracy, reproducibility and scale-up, by having the control of cells and materials layer by layer deposition [3]. With specific advantages of multicellular loading, microscale accuracy, one step tubular formation and geometric freedom, respectively, extrusion based, DLP photocuring, coaxial and embedded technology each provide the technical advantage to the construction of functional liver model.

However, whether the metabolic zonation characteristics of the liver can be recapitulated in printed tissue is of course the criterion for assessing the physiological relevance of the models, and this is the issue at the heart of the paper.

In this paper, by focusing on the core problem of metabolic zonation reconstruction, the capability boundaries of four mainstream bioprinting technologies are systematically reviewed, then, from the target metabolic zonation map, this paper proposes the multi-technology collaborative strategy to match the technologies from back, construct the three-layer architecture framework from microscale

topology to macroscale perfusion, and provide the theoretical reference for promoting the 3D bioprinted liver models from the morphological biomimicry toward functional biomimicry.

2. Functional biomimetic targets for liver bioprinting

2.1. Structure and Functional Organization of Hepatic Lobules

The hepatic lobule is the basic structural and functional unit of the liver. It presents a polyhedral prism morphology, of approximately 1-2 mm diameter, with the central vein at the center, and the radially arranged hepatic plates and liver sinusoids. The portal tract is at the periphery of the hepatic lobule, where the triad of the portal vein, of the hepatic artery and of the bile duct are located. The blood enters the sinusoids from the portal tract and flows toward the central vein, so that the oxygen partial pressure decreases from approximately 65 mmHg to 30 mmHg [4]. The nutrients, the hormones and the Wnt signaling molecules (such as Wnt2, Wnt9b) form concentration gradients along the path of the blood flow, which together shape the functional heterogeneity of the hepatocytes.

2.2. Molecular Mechanisms of Metabolic Zonation

The oxygen gradient-driven metabolic adaptation and the Wnt/ /catenin signaling pathway-mediated gene expression regulation are the core of the regulation of metabolic zonation [5]. The periportal zone (Zone 1) is in higher oxygen tension environment, in which gluconeogenic key enzymes (such as PEPCK), urea cycle enzymes (CPS-I) and bile acid synthesis enzyme CYP7A1 are in high expression and take synthetic and secretory function; the pericentral zone (Zone 3) is in hypoxic environment, in which metabolism is mostly based on glycolysis, and CYP450 enzyme systems (CYP1A2, CYP2E1, CYP3A4) are in high expression, and drug metabolism and detoxification are dominant. Ben-Moshe et al. found by single-molecule fluorescence in situ hybridization technology that about 50% of hepatocyte genes have significant zonal expression [6]. That shows the metabolic zonation is in the central position from the quantitative point of view.

2.3. Necessity of Metabolic Zonation Reconstruction

The main limitation of the standard in vitro culture is its lack of metabolic zonation. In the two-dimensional culture the hepatocytes are all exposed, which causes downregulation of the expression of CYP450 enzymes [7]; in the three-dimensional system the function is to some extent restored but there is no directional gradient drive [8].

The lack of metabolic zonation affects the accuracy of toxicity prediction. For example, the toxic intermediate NAPQI of acetaminophen is enriched in Zone 3 [9]; the models which can not recapitulate such a feature may not find the toxic drugs which is selective in zone. The functional liver model should satisfy three conditions: 1. recapitulate spatial topology, 2. have a perfusion system, 3. form the gradient microenvironment.

3. Capability Boundaries of Existing Printing Technologies in Zonation Reconstruction

As the existing studies show that different printing technologies each have their capability boundaries in reconstructing the closed loop of structure, gradient, and function. This section discusses the four mainstream technical approaches separately and then do horizontal comparison to analyze the core bottleneck that is facing now.

3.1. Extrusion-based bioprinting

In extrusion-based bioprinting, bioink is deposited layer by layer by using the pneumatic or mechanical pressure from a nozzle [10]. Extrusion-based bioprinting has relaxed the rheological requirement; it is compatible with different hydrogels, and the deposition of different bioinks could

be done synchronously, which is beneficial for the multicellular co-culture. For example, Kang et al. built the hepatic lobule arrays containing the hepatocytes, endothelial cells and the lumens [11], and Janani et al. formulated the bioink with dECM to print a tri-cellular system [12]. Both of the systems give rise to clinically relevant responses in the drug toxicity assessment.

The advantage of extrusion-based printing is high material compatibility, and it is suitable for the construction of millimeter scale tissue frameworks. However, the resolution of extrusion is not able to be less than 100 m, and the shear stress may affect the viability of fragility cells. In general, this kind of technology is not enough in gradient boundary clarity.

3.2. DLP Photopolymerization Bioprinting

DLP bioprinting cures photosensitive bioink layer by layer by means of digital micromirror devices projecting light patterns in a surface-forming manner. DLP uses a surface exposure strategy and has resolutions of 10- 50 μm, able to recapitulate fine channel arrays at the scale of liver sinusoids.

Mao et al. formulated bioink with liver dECM to print hepatic microtissues. The dECM conditions made the hepatocyte able to synthesize albumin and urea better. DLP can exactly control the geometric parameters of the microchannel. The fact that the oxygen gradient can induce zonal metabolism has been verified by Sato et al. [13,14]. The disadvantages of DLP are that it has a risk of phototoxicity, the range of materials is limited, and the cell loading density is not enough; It is not verified that the functionality can be kept in the long term.

3.3. Coaxial Bioprinting

Coaxial bioprinting takes the core-shell nozzle structure and it is able to form the hollow tubular constructs in one step. Generally speaking, this design has advantages for constructing a perfusable network in itself. Furthermore, the core-shell structure allows the microenvironment of different cellular compartments to be regulatable in independent.

Taymour et al. used the core-shell structures to perform the efficient co-culture of the hepatocytes and the fibroblasts [15], and Maji et al. printed a perfusable vascularized liver sinusoid model and showed higher hepatocyte viability and drug toxicity sensitivity under the perfusion condition [16].

Coaxial printing can indeed generate perfusion channels in one step; however, channel morphology is only regular cylinders, and it is hard to recapitulate the irregular branching structure of native liver sinusoids, and there is still a lack of functional vascularization.

3.4. Embedded Sacrificial Material Bioprinting

In embedded bioprinting the nozzle is immersed in a support bath with yield stress; the support bath is locally liquefied by the shear of the nozzle and is free to move. In principle, this method overcomes the strict restriction of the printing on the rheological properties, and one can in theory construct branching networks of arbitrary complexity.

Lv et al. built liver sinusoid-like vascular networks and a liver fibrosis model and showed that the matrix stiffness can lead to HSC activation [17]. Skylar-Scott et al. printed out the perfusable liver tissue structures and showed the potential of high density cell loading [18].

There remains a gap between its shape-creating advantages and function-creating chain. The geometric freedom of embedded printing holds important value for reconstructing the branching morphology of liver sinusoids, but the post-processing chain is prolonged and cumbersome, and whether gradient fields can be stably maintained lacks sufficient evidence.

3.5. Horizontal Comparison and Core Bottlenecks

Each technology has its capability boundaries. The four technologies correspond to different stages of zonation reconstruction: extrusion-based is suitable for macroscopic construction but does not have resolution; DLP offers high precision but is material-limited; coaxial is able to do one-step tubular formation but with regular geometry; embedded offers the greatest freedom but involves complex post-processing. Existing integration solutions mostly have the characteristics of technical

superposition and still do not have systematic technical division logic guided by target metabolic zonation maps [19]. The next chapter will fill the gap and propose a multi-technology collaborative strategy oriented toward zonation reconstruction.

4. Technology Collaborative Strategy for Zonation Reconstruction

4.1. Core Concept: Reverse Engineering Technology Integration from Zonation Targets

Almost all of the existing work takes the default way of first printing the structure then inducing zonation, while the way of introducing the gradients after the structure solidification is limited by the geometry. In this paper, what this paper is going to do is to change the design logic. It starts from the target metabolic zonation map and works backward to figure out the role of each technology in the chain of gradient construction.

The specific strategy is to clarify the desired zonation features and the spatial distribution of metabolic markers, plan the oxygen gradient decay curve and channel parameters, and at the end, match the printing technologies at different precision level in order that every technology will play his role at the more suitable position.

4.2. Three-Layer Technology Collaboration Framework

According to the above idea, in this paper put forward the technology collaborative conceptual scheme of three layers, namely the microscale topology layer, the macroscale channel layer, and the connecting transition layer.

The first layer, the microscale topology layer, is undertaken by DLP. It is the one where the hepatic plate and liver sinusoid micro-unit structures are recapitulated using ten-micron-scale resolution. Microchannel diameters are controlled at 50-100, and spacing at 100-200, using PEGDA-GelMA composite hydrogels.

The second layer, the macroscale channel layer, is undertaken by extrusion based or coaxial printing. The extrusion-based approach is suitable for laying large-diameter (200-500 m) perfusion trunks; the coaxial printing is suitable for generating medium-diameter (100-200 m) transition conduits. The layer-to-layer connection is done by embedded printing.

The third layer, the connecting transition layer, is undertaken by embedded printing, printing mesoscale branching networks to bridge the scale gap between DLP and coaxial printing. The channels after sacrificial material removal are endothelialized to confer selective permeability function to achieve a smooth transition of perfusion flow.

4.3. Design Principles: Gradual Scale Matching

The three-layer architecture follows the principle of gradient-driven scale matching: the DLP is deployed where the gradient boundary is precise, the extrusion-based/coaxial is deployed where the large-volume cell loading is needed, and the embedded is deployed where the flexible connection is required. The point is not if the technologies are more or less superior in inherent, but their adaption position on the zonation reconstruction chain. The three collaborate to form a complete gradient chain from macroscale to microscale, from supply to consumption. The three technologies are organized according to the spatial logic of gradient decay: the DLP layer is responsible for short-distance fine zonation, the macroscale channel layer is responsible for long-distance perfusion supply, and the connection layer is responsible for mesoscale transition and distribution. They hold promises to recapitulate the characteristic metabolic zonation map of hepatic lobules.

5. Challenges and Future Outlook

There are still several key challenges in the realization of multi-technology collaboration. The first is engineering integration: the three printing technologies have much different physical principles and process parameters and how to realize the collaborative operation in one device is a big challenge in

hardware design. Research should adopt modular printing platforms and use the way of replacing nozzles modules and variable design of build chamber to sequence the multiple process and also develop a unified slicing and path planning algorithm to coordinate the coordinate systems of different technologies.

Second is the biological challenge: keeping the metabolic heterogeneity dynamic during long-term culture is the core problem. Even if studies can establish the oxygen gradient successfully initially, cell proliferation, matrix remodeling and metabolic adaptation may gradually make the original gradient flat. One can at least partially solve this problem with continuous perfusion. However, the flow rate needs to be controlled in a proper way to avoid shear damage. Besides, it cannot fully recapitulate the mature microenvironment and the fenestration formation of endothelial cells in vitro condition, which also limits the efficiency of substance exchange.

Some technology fusion directions are worth considering. The organ-on-a-chip and microfluidic technology is introduced which can give the printed tissue the ability of perfusion control and real time monitoring, as it is expected that the perfusion flow rate, the oxygen concentration and the nutrient composition can be independent regulated for the printed tissue by those technologies. The AI assisted design can also be used to optimally set the topology of channel network and the perfusion parameters by machine learning to make the stability of zonal expression to be maximized. Yang et al. show that the 3D printed liver organoids can prolong the survival of the mice with the liver failure, which provides the preliminary proof of concept for the function effectiveness [20].

At the clinical translation level, standardization and reproducibility are the core requirements; at present, most of the research is still at the level of the laboratory, and there are large batch-to-batch variations. Full-process standard operating procedures should be established, from cell preparation, bioink formulation to the printing processes and the culture conditions. The clear regulation path is also very important. FDA and EMA have started to pay attention to the regulation of the 3D bioprinting products but have not issued the guidance targeted on the printed living tissues yet. When technology matures, ethical review and regulation approval will be the necessary links to connect the laboratory to the clinic.

6. Conclusion

This paper focused on the core problem of the reconstruction of metabolic zonation, systematically reviewed the boundary of the capability of the four kinds of bioprinting technology, which are the extrusion based, DLP photocuring, coaxial and embedded, in the resolution, the multicellular loading, the construction of vascularization and the maintenance of gradient, and showed that the single technology cannot complete the full reconstruction from the structure to the function independently. On this basis, a reverse design logic which start from the target metabolic zonation map is proposed, and the four technologies are deployed according to the principle of gradient-driven scale matching into the microscale topology layer, the macroscale channel layer, and the connecting transition layer, to form a systematic multi-technology collaborative framework. This strategy gives new theory reference for the advance of 3D bioprinted liver models from morphological biomimicry toward functional biomimicry; as the engineering integration, biological optimization and regulatory pathways gradually advance, the clinical translation prospects of the customized liver organ model are worthy of anticipation.

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