An insight into in vitro construction of implantable liver: from “bottom up” integrated with “top down” aspects

Yuan PANG, Stephanie Liana Utami SUTOKO, Yohei HORIMOTO, Masahiro ANZAI
Toshiki NIINO and Yasuyuki SAKAI

Abstract

In this paper, we focus on integrated approaches to liver tissue equivalent that can be finally used as substitute for those damaged original liver organ. To achieve the large implantable liver, a tissue construct of 500 cm$^3$ in volume with in vivo comparable per-volume-based functionalities is necessary. This could hardly be realized by current technologies due to insufficient mass transfer problems in in vitro engineered tissues. We established a novel engineering methodology by integration of both bottom-up and top-down tissue engineering technologies, which provided an efficient way of arranging engineered liver tissue with improved mass transfer simultaneously from micro- and macro-scales, meanwhile addressing to a clinically relevant size with well recovered per-volume-based functions.

1. Introduction

Liver is one of the largest, complex and most important vital organs playing the central role in biotransformation, drug metabolism and toxicity. It fulfills the multiple and finely tuned the critical functions for homeostasis of the human body. The failure of this organ results in functional replacement presenting one of the most difficult challenges in substitutive medicine. Orthotopic liver transplantation (OLP) has been proven the only effective clinical treatment for patients with end-stage liver disease, and additionally temporary liver support system is to some extent efficient. However, these treatments are purposed either to bridge patients to liver transplantation or to allow the native liver to recover from injury, which still face the difficulties of donor shortage. A potential method to completely solve this problem is to engineer implantable liver tissue equivalents that can ultimately substitute for the original liver organ, where the techniques of tissue engineering (TE) provide a promising access to the final goal.

In the last two decades, the technology of tissue engineering has been applied for addressing a liver construct to provide an alternative option for ADME/Tox applications, liver disease treatment and transplantation. The technologies range from hepatocytes suspension infusion, hepatocyte transplantation with various ECMs, hepatocytes culturing in biodegradable polymer scaffolds of 3D macroporous structure to cell sheet technology. However, most of these methodologies are still small-scale research, which more specified in liver diseases treatment or partly recovering liver functions while waiting for in vivo regeneration. To construct an in vitro model for implantation and reliably predicting the in vivo drug responses, the core issue is to realize adequate cell supply and stability of liver-specific functions closely matching those measured in vivo.
Recently, some novel approaches have been proposed by engineers aiming at completely building larger implantable liver tissue equivalents. These approaches can be classified as: (1) *In vivo* approach of “decellularization” - constructing the liver substitute based on hepatocyte perfusion into a decellularized liver matrix. Immunohistochemical staining of recellularized tissue elements from micro scale. The most famous and pioneering research is the microelectromechanical systems (MEMS)-related technology by the group of Vacanti with a success in creation of liver lobule-like unit having 2D microvascular networks of the same dimension as liver sinusoid. McGuigan and Sefton further developed bottom up approach to the so called “modular assembly” method by immobilization of liver cells in a hydrogel element whose surface being covered with endothelial cells, and finally the cell-loaded modules were packed in a small volume of a space for perfusion culture; (3) *In vitro* approaches from top down aspect—fabricating a scaffold where cells are seeded from macro scale. The earliest example of this approach is the lotus-shaped biodegradable 3D scaffold of 10 mm × 10 mm height by the 3D printing process using poly-L-lactic acid and salt particles. We improved this method by flow channel design based on the oxygen diffusion/consumption around the channel, with one inlet and outlet to uniformly supply the entire scaffold with culture medium or blood perfusion.

Although above mentioned approaches have contributed remarkable steps to the progress of liver tissue engineering, to date, a 500 cm³-scale liver tissue equivalent with a comparably high cell density (about 1.4 × 10⁸ cells/cm³-tissue *in vivo*) and functionality for implantation has not yet been successfully achieved. Each of the approaches faces their own heel of achilles leading the unaccomplished large liver tissue construction: the decellularized liver matrix goes well in small animal but faces uncertainty in scaling up and also the donor shortage issue; it is very much difficult and laborious to have much larger tissues by stacking 2D plates from MEMS with smooth branching and joining; when apply the “modular assembly” idea to much smaller modules of cellular aggregates which are around 100 to 200 μm in diameter, perfusion failed in 48 h due to insufficient oxygen and nutrient supply between densely packed aggregates; although 3D scaffold design and fabrication undoubtedly function as a very promising approach to large liver tissue construction from macro scale, the resolution

<table>
<thead>
<tr>
<th>Table 1. Achievements in engineering of implantable liver tissue equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell amount</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td><strong>Hepatocytes suspension infusion</strong></td>
</tr>
<tr>
<td><strong>Hepatocyte transplantation with ECMs</strong></td>
</tr>
<tr>
<td><strong>Macroporous scaffold</strong></td>
</tr>
<tr>
<td><strong>Cell sheet</strong></td>
</tr>
<tr>
<td><strong>Decellularization</strong></td>
</tr>
<tr>
<td><strong>MEMES</strong></td>
</tr>
<tr>
<td><strong>Modular assembly</strong></td>
</tr>
<tr>
<td><strong>Perfusion of HUVECs-coated hepatocyte aggregates</strong></td>
</tr>
<tr>
<td><strong>3D scaffold fabrication</strong></td>
</tr>
</tbody>
</table>
of 3D fabrication process is still in several hundreds μm, which didn’t reach the demand of oxygen consumption by the cells according to a simple equation describing decrease in oxygen concentration around a single flow channel based on oxygen diffusion\(^{28}\). This resulted in an overall cell density of \(2 \times 10^7\) cells/cm\(^2\)-scaffold which cannot be compared to real liver cell density.

To sustain the homeostasis of human body, it is reported that at least 20-30% of hepatic functions should be retained\(^{29,30}\). This also indicates the necessary volume for implantable liver tissue is around 20-30% of real liver organ size. To successfully obtain thick tissue with complex structure as liver in vitro, the two major issues need to be fulfilled regarding to limitations of existing technologies; (1) Organization of the medium-delivering systems (vascular-like structure) in the engineered tissue for sufficient mass transfer; (2) Realizing adequate cell supply and stability of liver-specific functions closely matching those measured in vivo (Fig. 1). Herein, we proposed a new methodology of “integration of bottom-up and top-down technologies” towards implantable liver tissue construction (Fig. 2). We worked to resolve the insufficient mass transfer simultaneously from micro- and macro-scale, while addressing a clinically significant mass.

### 2. Improved cellular aggregate assembly concept from “bottom up” aspect

First, cellular aggregate assembly was improved by applying “spacing tissue elements” concept, namely creating some spacing between the packed aggregates for securing better mass transfer by immobilizing them with biodegradable single poly-L-lactic acid (PLLA) fibers in a small perfusion bioreactor. To achieve this, a novel culture method whereby aggregates were constructed using a polydimethylsiloxane (PDMS)-based honeycomb microwell arrays (Fig. 3a). Primary rat hepatocyte aggregates around 100 μm in diameter coated with human umbilical vein endothelial cells (HUVECs) were spontaneously and quickly formed after 12 h of incubation, thanks to the continuous supply of oxygen by diffusion through the PDMS microwell device. Then, recovered endothelialized rat hepatocyte aggregates were mixed with PLLA fibers in suspension, and packed into a PDMS-based bioreactor (Fig. 3a). 7 days of perfusion culture was successfully achieved with more than 73.8% cells retained in the bioreactor. As expected, the fibers acted as spacers between aggregates, which was evidenced from the enhanced albumin production and more spherical morphology (Fig. 3b) compared with fibre-free packing. The results showed the advantages of using PDMS-based microwells to form heterotypic aggregates and also demonstrates the feasibility of spacing tissue elements for improving oxygen and nutrient supply to engineered tissues from micro-scale.

---

**Figure 1.** Roadmap of development of liver tissue equivalents of clinical significant size for implantation

**Figure 2.** Concept of the integrated methodology from “bottom up” and “top down” aspects

**Figure 3.** (a) Cellular aggregates formation in PDMS microwells and culture in bioreactor. (b) Cellular morphology after 7 days of perfusion culture by H&E staining. Scale bar: 100 μm
3. Poly-ε-caprolactone(PCL) micro-modules as tissue elements towards scaling up

During the perfusion culture, we found the cellular aggregate disintegration gradually happen. The broken aggregate parts may result in floating debris that hinders the perfusion to some areas. Thus, to maintain the homogeneous flow with sufficient mass transfer, stable cellular modules with enough mechanical strength are necessary. To circumvent this obstacle of cellular aggregate disintegration, we designed a module with a hollow structure, and fabricated it using poly-ε-caprolactone (PCL). The module was designed to be a cylinder with a hollow central canal 500 μm in diameter and an outer diameter varying from 1100 to 1300 μm (Fig. 4a). Hep G2 cells, which were considered as proliferative progenitors, were found to be well immobilized on the PCL module after collagen coating (Fig. 4b). Given that 200 μm is the limitation of oxygen diffusion in vivo 101, PCL hollow modules were able to sufficiently secure good mass transfer to the cultured cells. Hep G2 cells were found to maintain higher cell viability along with improved hepatic function (suggested by increased cellular albumin production and glucose consumption) when cultured in the module with the inner canal. As possessing the advantages of cell affinity, maintaining hepatic cell functions and stable in culture medium, PCL hollow modules with loaded cells are expected to replace the pure cellular aggregates as tissue elements for scaled-up modular constructs in the future.

![Figure 4](image-url) (a) Fabricated PCL modules. (b) PCL module with Hep G2 cells laden. Cells were labeled with PKH 67, and image was taken by confocal microscopy.

4. Design and fabrication of a three-dimensional scaffold from “top down” aspect

To further produce a large liver tissue equivalent by scaling up the "spacing cellular aggregates" along with improved macro-circulation of medium for better oxygen and nutrient supply, a 3D scaffold was designed with flow channels homogeneously delivering medium to the whole construct. The scaffold was designed based on the real liver structure, which comprised 43 subunits assembled on three layers (Fig. 5). 12 units are posited on the 1st and the 3rd layer, and 19 units on the 2nd layer, producing a construct with symmetrical outer shape. Then, the construct of 43 units was arranged again in the same way of pattern to finally achieve a structure 500 cm³ in volume. Subunits for cell culture were designed with a hexagonal section, in order to mimic the liver lobule structure, and were about 0.27 cm³ in volume. Interconnected flow-channels were designed to deliver culture medium independently to each of the subunits. The diameter of each flow channel was calculated based on Murray’s law 102 and Hagen-Poiseuille’s equation 103 to ensure the same pressure drop in the flow to each subunit. The scaffold was fabricated using nylon-12 due to its high mechanical properties via selective laser sintering (SLS) technique in Niino lab. 104

The efficacy of the present 3D scaffold was evaluated by perfusion culture of spaced aggregates. Endothelialized hepatic aggregates were obtained by co-culture of Hep G2 and TMNK-1 cells, which are the immortalized human liver sinusoidal endothelial cells, using PDMS microwells 200 μm in diameter. Formed aggregates were collected and mixed with a PLLA fiber suspension and inoculated to the scaffold. After 7-day perfusion culture of the spacing aggregates in the scaffold, cellular hepatic functions were analyzed in terms of albumin production and glucose consumption, and cell viability was assessed by H&E staining of cultured cells in the subunits. The results of increased albumin production, glucose consumption and high cell viability (Fig. 6) demonstrated that such 3D scaffold consisting of subunits for cell culture and independent flow channels for medium delivery was essential for proper cell growth and function.

![Figure 5](image-url) Sketch up data of scalable units inside the scaffold

![Figure 6](image-url) H&E staining of thick cell layer on the bottom of the subunits in the 3D scaffold after 7 days of perfusion culture. Scale bar: 100 μm
5. Conclusive remark and future perspectives

In seeking for engineering a large liver tissue equivalent of a clinically significant mass (500 cm$^3$) with the cell density and functionalities being equal to those of the real tissue, we proposed a possible roadmap showing the first proof-of-concept stage with efficient methodologies arranging simultaneously from "bottom-up" and "top-down" aspects (Fig. 1). Related approaches were summarized based on present technologies, and we cleared up the remaining issues of insufficient mass transfer that limited the in vitro scaling up of liver tissues. We then introduced our attempts to maintain micro- and macro-circulation of medium for better oxygen and nutrient transport, that is, investigation on the potential improvements to packed cellular elements (dimensions, mechanical strength, etc), the packing method itself (with PLLA fibers as spacers), and the scaling up of modular assembly of cellular elements (using a finely designed 3D scaffold).

As explained above before, the established integrated approach provides the engineered liver tissue with improved mass transfer from bottom-up aspect, meanwhile the scalable feature of the scaffold design guarantee the possibility of approaching to a clinically relevant size with well revered per-volume-based functionalities from top-down aspect. The developed methodologies are not only promising in liver tissue engineering, but also have the potential to apply to other organs.

Acknowledgement

This work was supported by Grant-in-aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology in Japan; and also by Chinese Science Council (CSC).

References