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Three-dimensional fabrication of thick and densely populated soft constructs with complex and actively perfused channel network

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ABSTRACT

One of the fundamental steps needed to design functional tissues and, ultimately organs is the ability to fabricate thick and densely populated tissue constructs with controlled vasculature and microenvironment. To date, bioprinting methods have been employed to manufacture tissue constructs with open vasculature in a square-lattice geometry, where the majority lacks the ability to be directly perfused. Moreover, it appears to be difficult to fabricate vascular tissue constructs targeting the stiffness of soft tissues such as the liver. Here we present a method for the fabrication of thick (e.g. 1 cm) and densely populated (e.g. 10 million cells·mL^{-1}) tissue constructs with a three-dimensional (3D) four arm branch network and stiffness in the range of soft tissues (1–10 kPa), which can be directly perfused on a fluidic platform for long time periods (>14 days). Specifically, we co-print a 3D four-arm branch using water-soluble Poly(vinyl alcohol) (PVA) as main material and Poly(lactic acid) (PLA) as the support structure. The PLA support structure was selectively removed, and the water soluble PVA structure was used for creating a 3D vascular network within a customized extracellular matrix (ECM) targeting the stiffness of the liver and with encapsulated hepatocellular carcinoma (HepG2) cells. These constructs were directly perfused with medium inducing the proliferation of HepG2 cells and the formation of spheroids. The highest spheroid density was obtained with perfusion, but overall the tissue construct displayed two distinct zones, one of rapid proliferation and one with almost no cell division and high cell death. The created model, therefore, simulate gradients in tissues of necrotic regions in tumors. This versatile method could represent a fundamental step in the fabrication of large functional and complex tissues and finally organs.

Statement of Significance

Vascularization within hydrogels with mechanical properties in the range of soft tissues remains a challenge. To date, bioprinting have been employed to manufacture tissue constructs with open vasculature in a square-lattice geometry that are most of the time not perfused. This study shows the creation of densely populated tissue constructs with a 3D four arm branch network and stiffness in the range of soft tissues, which can be directly perfused. The cells encapsulated within the construct showed proliferation as a function of the vasculature distance, and the control of the micro-environment induced the encapsulated cells to aggregate in spheroids in specific positions. This method could be used for modeling tumors and for fabricating more complex and densely populated tissue constructs with translational potential.

1. Introduction

In the last decade, significant effort has been made to fabricate functional three-dimensional (3D) tissue engineering constructs to simulate a portion of a human tissue [1–6]. Such constructs could open up for a new world of applications in drug delivery [7,8], 3D cell culture [9–11], disease model platforms [12] and tissue regeneration [13–16].

The current challenge in fabricating functional 3D constructs relies on the difficulties in creating relevant perfusable vasculatures, necessary to overcome the regarded diffusion limit of oxygen

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and nutrients (i.e., 200 μm) [17], within an ECM simulating soft tissue stiffness (e.g. liver) [18,19]. This is mainly due to the fact that it is hard to maintain the physical integrity of the ECM and the channels within [20]. In addition, to keep densely populated tissue constructs alive (cell density in the range of 10–500 million cells·mL^{-1} [21]), an open vasculature without active perfusion would probably not be sufficient [22]. Therefore, to guarantee the final success of the fabricated construct it is necessary to developed a strategy to actively perfuse these tissues constructs [23].

Direct 3D printing has gained an important role in the fabrication of thick, vascularized, and complex 3D constructs [14,15,18,22,24–32], since it allows layer-by-layer printing of a variety of complex shapes. The drawback is that it is not possible to print structures with 3D degree of freedom without having a support structure underneath. This has mainly led to the fabrication of simple self-sustained square lattices which do not represent the complexity of real tissues [15,21,25,27,30,33]. Kolesky et al. [33] reported a technique where they printed two different inks at the same time followed by casting an ECM into the printed structure. The first ink used was Pluronic F127, which was the sacrificial material responsible for defining the vascular channels. The second ink was a mixture of cells and suitable ECM that was made by a blend of gelatin and fibrinogen, which was direct printed in the positions along the vascular channels. Then, another mixture of gelatin and fibrinogen cross-linked by a dual-enzymatic strategy by using thrombin and transglutaminase (TG) was cast around the direct printed structure. The thrombin was used to rapidly polymerize fibrinogen, and TG, a slow-acting Ca^{2+}-dependent enzymatic cross-linker, acted on the gelatin to give mechanical stability on the final printed construct.

An interesting approach to obtain free form 3D is to have a support structure bath where the direct printed hydrogels are supported by a slurry of gelatin beads [18]. Hence two component printing as described above is not needed. However, this method requires precise thermal control and custom modified printers. The technique is able to create hollow channels with cross section lumens of 1–3 mm in size. It is unclear how soft hydrogels can be printed using this method.

Another strategy that has been employed to fabricate 3D constructs is the 3D sacrificial molding technique [21,28,33–45]. Bioprinters have been used to print gelatin [44,45], agarose [46], Pluronic F127 [43] as sacrificial material to create vascular networks. Miller et al. [21] reported a method for fabricating 3D vascular constructs, in which a modified Fused Deposition Modelling (FDM) printer has been used to 3D print carbohydrate glass structures serving as sacrificial template. Then, a poly(ethylene glycol) (PEG) based hydrogel with cells encapsulated was cast around the sacrificial template. Subsequently, the sacrificial material was removed from the PEG hydrogel leaving behind a network of open microchannels. FDM has been also used to print sacrificial templates in PVA [39,40,42]. However, in some cases the 3D printed structures were used to create porous elastomeric scaffolds [39,40] for 2D cell culture. In another case, although custom modified machines allowed the realization of more complex PVA structures, the ECM used was extremely stiff with no cells encapsulated in it [42]. Finally, molds have been used to create sacrificial templates using sodium alginate [37] and PVA [38]. Although direct printing and 3D molding strategies have shown advantages in fabricating tissue engineered constructs with perfusable vasculature there are some aspects that needs to be addressed. The majority of the proposed vascular networks were composed by single [44,46] or multiple microchannels in a single plane [21,28,37,38,42,43,47], or in a square lattices-like structure [15,26,27,30,33,48]. It appears to be difficult to manufacture thick tissue constructs able to integrates (1) soft tissues stiffness (2) relevant cell density (3) complex vascular networks and (4) perfusion. We believe these four points are critical for the final success of an engineered construct and this is what are addressing in this paper.

Here, we describe a method for the fabrication of a thick and densely populate tissue construct with full 3D perfusable network and stiffness in the range of soft tissues. The obtained 3D tissue constructs are relatively thick (1cm), connected and perfused with a custom fluidic platform for extended time periods (>14 days). Long term cell culture demonstrated strong dependence of nutrient and oxygen feed for cell growth.

2. Materials and methods

2.1. Hydrogel preparation

Gelatin cross-linked with transglutaminase (Activa-TI, Ajinomoto North America Inc., Illinois, US, (TG)) was used in this study. Briefly, 7.5% (w/v) gelatin (48723 Fluka) was mixed with cell culture medium (Roswell Park Memorial Institute, RPMI 1640) supplemented with 10% fetal bovine serum (FBS; Gibco Labs), and penicillin/streptomycin at 60 °C and stirred until fully solubilized. The degree of cross-linking of the resulting gelatin/TG hydrogel was controlled by varying the amount of TG with respect to the mass of the gelatin. The appropriate mass of TG (Activa-TI, Ajinomoto, Inc., activity of approximately 100 U·g^{-1} of TG according to the manufacturer) was dissolved in 15 mL of PBS (Sigma Aldrich) to give concentrations of 2.5, 5, 10, and 20 units of TG per g of gelatin. The final concentrations of the gelatin/TG hydrogel used in this study were 5% (w/v) gelatin, with 2.5, 5, 10, and 20 units of TG per g of gelatin.

2.2. Mechanical properties

Mechanical tests were performed on cylindrical shaped samples (n = 6). Briefly, 1 mL of the 5% (w/v) gelatin/TG pre-polymer solutions (ratio of TG (2.5, 5, 10 and 20 units of TG per g of gelatin) was cast in a cylindrical PMMA mold (10 mm in diameter, 10 mm in thickness). Samples were incubated at 37 °C for 24 h allowing gelatin cross-linking. The cylindrical samples were tested at 37 °C, and compressed to 10% of the initial length using 0.1% strain rate using the Solid Analyzer instrument (0.1 μN force resolution, 0.5 mN minimum force, 1 nm displacement resolution) (RSA II, Rheometrics, Inc.). The compressive elastic modulus of each sample was calculated from the stress-strain curve (first linear zone, 2% strain).

2.3. Design and 3D printing of the 3D sacrificial structures

The 3D sacrificial structures were designed and converted into STL files using Solid Works CAD software (computer aided design, Dassault Systemes SolidWorks Corporation, US). All STL files were processed by KISSlicer (www.kisslicer.com) software and sliced into 100-μm-thick layers to generate G-code instructions for the 3D printer. G-code instruction sets were sent to the printer using Repetier-host (https://www.repetier.com), an open-source 3D printer host program. Then commercially available PVA and PLA filaments (Makerbot Industries, Brooklyn, NY, USA) were printed using the Felix 3 (Felix Printers, IJsselstein, Netherlands) printer with a nozzle of 250 μm.

2.4. Dissolution of support material in the 3D sacrificial structures

The 3D sacrificial structures were printed with PLA as support material. Then, the structures were immersed in 200 mL of chloroform (Sigma Aldrich) under continuous stirring until all the solid PLA support was dissolved. After the total dissolution of the PLA,

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