



Computer-Aided Design, Modeling, and Freeform Fabrication of 3D Tissue Constructs for Drug Metabolism Studies

Robert Chang¹, Jae Nam² and Wei Sun^{3*}

¹Drexel University, rcc34@drexel.edu

²Drexel University, jhn23@drexel.edu

³Drexel University, sunwei@drexel.edu

ABSTRACT

A novel targeted application of tissue engineering is the development of an in vitro 3D tissue model for drug screening and toxicology. This paper discusses the modeling, design, and freeform fabrication of 3D cell-embedded tissue constructs for creating a pharmacokinetic model. This is achieved using a combinatorial setup involving a CAD-driven automated syringe-based, layered direct cell writing process in conjunction with soft lithographic micro-patterning techniques. This enables the rational design of a microscale in vitro device housing a bioprinted 3D tissue construct (or micro-organ) that biomimics the cell's physiological microenvironment for enhanced functionality. This paper specifically addresses issues related to the development and implementation of a unique direct cell writing process for biofabrication of 3D cell-encapsulated hydrogel-based tissue constructs with defined patterns, the direct integration onto a microfluidic device, and the perfusion of the 3D tissue constructs for pharmacokinetic study. Micron-sized features enables the achievement of large hydrodynamic shear forces on our tissue constructs while preserving predictable laminar flow regimes. It has been demonstrated in literature that these shear stresses serve as mechanical stimuli which cells mechanotransduce to influence drug response. The motivation for the design and modeling of the bioprinted flow pattern is to predict, tune, and optimize the metabolic drug response of 3D bio-printed liver tissue to hydrodynamic perturbations under varying experimental flow conditions and structural flow patterns.

Keywords: microfluidics, cell printing, tissue engineering, solid freeform fabrication, hydrogels.

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1. INTRODUCTION

The recent development and confluence of two enabling technologies and research domains has greatly facilitated the engineering of tissues for in vitro applications: solid freeform fabrication of cell-embedded tissue constructs within 3D cellular microenvironments and microfabrication of microfluidic flow platforms to precisely control nutrient transport and fluid shear stress. While regeneration medicine is rapidly evolving in its attempts to engage innate physiological processes to reconstitute tissue and organs for implantation, the application of tissue engineering principles in designing in vitro physiological models to study disease pathogenesis, as robust sensors for rapid identification of biological and chemical agents, and platforms for pharmacokinetic study are also extremely promising [1]. One possible near-future application of in vitro physiological models is in the area of pharmaceutical drug screening and new drug discovery and development. In vitro cell culture models with engineered liver tissue have already shown great potential in

* Corresponding author: Wei Sun, sunwei@drexel.edu

predicting drug toxicity and metabolism in the pharmaceutical industry as an alternative to animal testing [2]. The present work specifically explores engineering underpinnings for the modeling, design, development, and the characterization of an *in vitro* 3D microfluidic microanalytical microorgan device for simulation of the physiological human response to drug administrations and toxic chemical exposure. The applied solid freeform fabrication (SFF) technology is a viable direct cell writing process for layer-by-layer extrusion of 3D cell-encapsulated hydrogel-based tissue constructs. By fabricating a 3D *in vitro* tissue analog consisting of an array of channels with tissue-embedded chambers, one can selectively biomimic different mammalian tissues for a multitude of applications for clinical pharmaceutical screening of drug efficacy and toxicity. The research conducted is aimed at the achievement of high-throughput reproducible fabrication of bioprinted tissue constructs and 3D organ chambers, maintenance of structural integrity and direct integration with the microfluidic platform, and enhancement of cell viability and display of cellular-level differentiation and tissue-level function, specifically metabolic function for drug screening applications.

2. COMPUTER-AIDED FREEFORM FABRICATION FOR DIRECT CELL WRITING

A proprietary multi-nozzle direct cell writing system has been developed for computer-aided freeform fabrication of 3D cell-embedded tissue constructs [3-6]. This direct cell writing process is designed to operate at bio-friendly conditions of room temperature and low pressure conditions to deposit multiple cell types and bioactive factors such as proteins in controlled amounts with precise spatial arrangements to form pre-designed, cell-embedded tissue constructs with easily adaptable patterned architectures. Compared to other cell dispensing systems limited to a single nozzle for cell printing, this multi-nozzle direct cell writing system enables the simultaneous or sequential deposition of cells, growth factors, and scaffolding biomaterials to form heterogeneous or functionally gradient tissue constructs to guide the highly orchestrated events of cell growth, differentiation, and organization. The direct cell writing system configuration is shown in Fig. 1a.

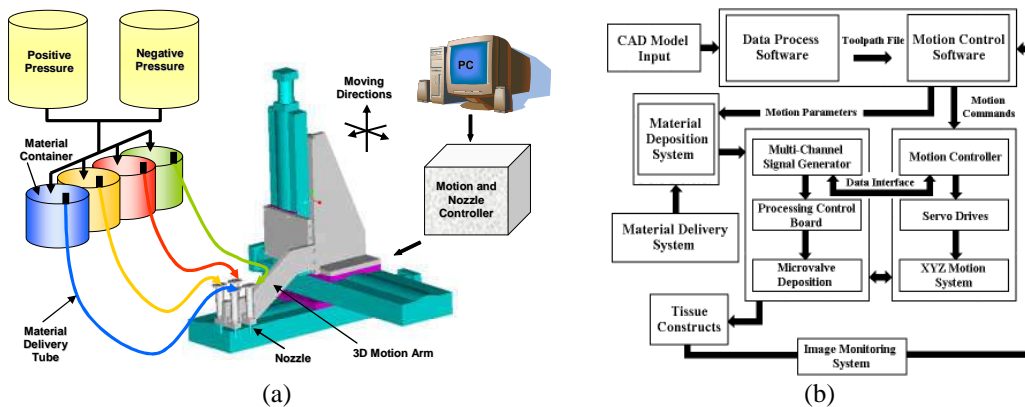


Fig. 1: System configuration for direct cell writing (DCW) biofabrication process. (a) Direct cell writing system and (b) Process information pipeline.

An information pipeline of the direct cell writing system for freeform fabrication of tissue constructs is also presented in Fig. 1b. As shown in the figure, the data processing software processes the designed CAD model of the tissue construct and converts it into a layered two-dimensional process tool path. The motion control system is driven by a layered manufacturing technique. The material delivery system supplies various nozzles with the appropriate biopolymer or biological factor. The system implements multiple nozzles with different types and sizes, thus enabling the deposition of specified hydrogels with different viscosities for constructing 3D tissue constructs.

In the development of the direct cell writing system, several micro-nozzle systems have been investigated to evaluate their performances and feasibility to deposit biopolymer solutions for tissue engineered constructs. The specific biopolymer deposition process performed in this study implements a pneumatic microvalve which is a typical mechanical valve that opens and closes the valve via an applied air pressure regulated by a controller (EFD Inc., East Providence, RI).

3. APPLICATION OF DIRECT CELL WRITING MICRO-ORGAN FOR DRUG METABOLISM STUDY

The direct cell writing process is integrated with a microfluidic device to fabricate 3D tissue constructs, as opposed to producing 2D cell monolayers (Fig. 2) for subsequent drug metabolism study. Biological studies have revealed the unstable cellular phenotype and reduced tissue-specific gene expression with conventional monolayer in vitro culture techniques [7-9]. A 3D tissue model will, in contrast, induce and foster improved retention of cell-specific function.

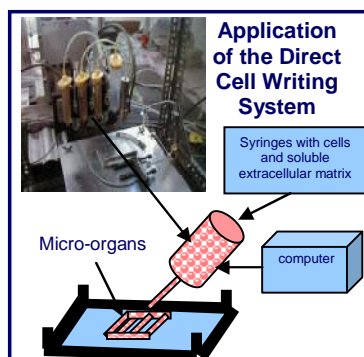


Fig. 2: Application of direct cell writing micro-organ.

Furthermore, direct cell writing of encapsulated cells offers tighter control over the spatial distribution of cells, allowing one to feasibly incorporate high cell density or co-culture multiple cell types within a 3D construct. This can create tissue structures that more closely resemble the in vivo physiology. Furthermore, optimization of process parameters (e.g. nozzle pressure, motion arm velocity, nozzle tip size etc.) and material parameters (e.g. biopolymer viscosity, crosslinking agent concentrations, etc.) have been done to achieve high-fidelity 3D structures and seamless integration onto a microfluidic tissue micro-organ chambers [3-6].

The microfluidic device consists of two components fabricated using standard soft-lithographic microfabrication methods: 1) a PDMS substrate containing a chamber to house the bioprinted tissue construct and 2) a glass cover layer with inlet and outlet ports for dynamic flow studies. The glass layer consists of an etched bifurcating micro-channel pattern that drain into and out of the tissue chamber. The PDMS component contains a central 10mm x 10mm x 750 μ m deep tissue chamber which constrains the dimensions of the bioprinted tissue construct during design considerations.

The schematic of the experimental apparatus setup of pharmacokinetic study is shown in Fig. 3a. By designing and biofabricating various microscale 3D physiological tissue engineered constructs within tissue chambers on a microfluidic chip, one can selectively model an interconnected network of differentiated mammalian tissues for a number of applications, among them pharmaceutical screening for both drug toxicity and efficacy. A fully perfused 3D micro-organ is shown in Fig. 3b.

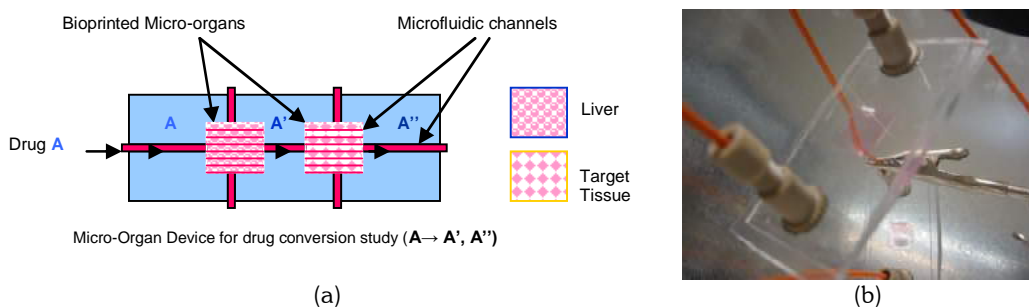


Fig. 3: (a) Schematic diagram of flowpath of drug through micro-organ device for pharmacokinetic study; (b) A fully perfused tissue chamber.

To demonstrate effective drug metabolism in the liver chamber, a non-fluorescent prodrug is fed into the system through the inlet port, metabolized by the liver chamber, and then produces an effluent fluorescent metabolite for analysis collected at the outlet port. Results of such analysis can then be used to understand the relative pharmacokinetic efficiency as well as relevancy of the tissue chamber design for human application. The drug flow study protocol includes a reactant EFC (7-ethoxy-4-trifluoromethyl coumarin) (Molecular Probes, Inc., Carlsbad, CA) reactant for syringe pump infusion. The micro-organ device is then connected to the pump for simultaneous infusion at the inlet port and withdrawal at the outlet at a flow rate of 0.25 uL/hr. The drug substrate EFC is then metabolized by the bioprinted tissue construct into the drug product HFC (7-hydroxy-4-trifluoromethyl coumarin) (Molecular Probes, Inc., Carlsbad, CA). The perfused micro-organ is placed within an incubator along with static bioprinted control constructs. The effluent is collected within the withdrawal syringe and assayed with a cytofluorometer for HFC drug metabolic conversion with a drug residence time consistent with the static controls.

4. IN VIVO LIVER PHYSIOLOGY AS RATIONAL FOR FLOW PATTERN DESIGN

Since the liver is the most important site of drug metabolism, a pre-requisite for a pharmacologically relevant predictive screen requires that a model system be developed that biomimics essential aspects of the in vivo biotransformation pathways of the human liver. The liver organ possesses a sophisticated engineering design with a large, highly structured reactor bed, an intricate flow manifold, and a separation system that efficiently delivers metabolic products to the bloodstream. The complex architecture of the liver is closely intertwined with its response to xenobiotic compounds such as drug compounds. The liver receives blood flow from the hepatic artery and the portal vein, which carries ingested drugs from the small intestine to be metabolized by liver hepatocytes. A schematic diagram of the hepatocyte microenvironment in liver tissue is shown in Fig. 4 with its associated feature dimensions. The in vivo architecture provides a low pressure blood flow travels from branches of the hepatic arteries and hepatic portal vein through the liver sinusoidal capillary bed and drains out via the central vein. The hepatocytes reside in thin cords and form extensive cell-cell contacts. The human liver consists of approximately 10⁹ parallel sinusoid units.

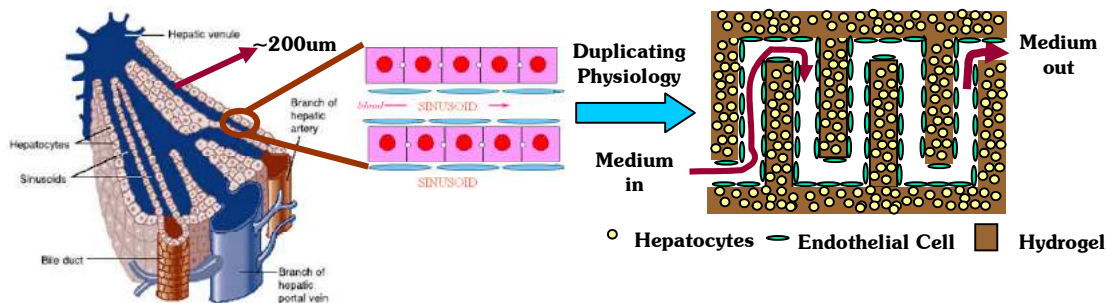


Fig. 4: Schematic diagram of the hepatocyte microenvironment in liver tissue [10].

The sinusoid space is bordered by a sheet of highly fenestrated endothelial cells which lack a basement membrane. This barrier is therefore highly permeable, and enables the hepatocytes to be bathed in blood plasma. Based on this microarchitecture as our guiding principle for design and fabrication of the tissue constructs, we would like to biomimic the liver microenvironment by creating flow pattern geometries of a similar shape and feature dimension order of the sinusoidal capillaries for the in vivo liver.

Micron-sized features enables us to achieve large hydrodynamic shear forces on our liver tissue constructs while preserving predictable laminar flow regimes. These shear stresses serve as mechanical stimuli which liver cells mechanotransduce to influence drug response. Transmission of fluid shear stresses influences liver cell biology (i.e. drug metabolism) whereby steady shear stress serves as mechanical stimulus for hormone-receptor mediated signal transduction across cell membranes for intracellular metabolism [11]. The physiological limit is approximately 2 dyne/cm² for shear stresses in body tissues such as liver [12-14]. Furthermore, high shear stress (>5 dyne/cm²) can compromise hepatic function while lower shear stresses (0.33 dyne/cm²) manages to preserve stable hepatocyte function [15].

4.1 Computational Fluid Dynamic (CFD) Modeling to Predict Wall Shear Stress in Micro-device

In vivo studies aimed at understanding cellular responses to shear stresses have the inherent problem that they cannot quantitatively define the exact features of the hemodynamic environment. Moreover, it is very difficult to attribute the resultant response is due to shear stress or some other feature associated with the hemodynamic environment. Cell culture studies and techniques for exposing cells to a controlled shear environment in vitro have been increasingly used to elucidate cellular responses to shear stress and flow. The use of the parallel plate flow chamber allows one to have a controlled and well-defined flow environment based on the chamber geometry (fixed) and the flow rate through the chamber (variable). In addition, individual cells can be visualized in real time using video microscopy. Assuming parallel plate geometry and Newtonian fluid behavior, the wall shear stress on the cell monolayer in the flow chamber is calculated as follows with a Poiseuille flow model:

$$\tau_w = \frac{6Q\mu}{bh^2} \quad (1)$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, b is the channel width, and τ_w is the wall shear stress. The flow chambers are designed such that the entrance length is very small compared to the effective length of the chamber [11]. Therefore, entry effects can be neglected, and the flow is fully developed and parabolic over nearly the entire length of the flow chamber.

Shear stresses and pressure drops can also be calculated using the analytical solution for rectangular channel flow to evaluate the accuracy of the simple Poiseuille model [16]. The analytical expression below represents the linear velocity, u , in terms of the spatial coordinates (x in the flow direction, y the wall-wise direction, and z the span-wise direction):

$$u = -\frac{\Delta p}{2\mu l} y(y-h) + \sum_{m=1}^{\infty} \sin\left(\frac{m\pi y}{h}\right) \left(A_m \cosh \frac{m\pi z}{h} + B_m \sinh \frac{m\pi z}{h} \right) \quad (2)$$

$$A_m = \frac{h^2 \Delta p}{\mu m^3 \pi^3 l} (\cos m\pi - 1),$$

$$\text{where } B_m = \frac{A_m (\cos m\eta\pi - 1)}{\sin m\eta\pi},$$

$$\eta = \frac{w}{h}$$

The wall shear stress is the derivative of u with respect to position y evaluated at $y = 0$:

$$\tau_w = -\mu \frac{du}{dy} \Big|_{y=0} = \mu \frac{\Delta p}{2\mu l} \left(\frac{1}{2} y - h \right) - \mu \sum_{m=1}^{\infty} \left(\frac{m\pi}{h} \right) \cos\left(\frac{m\pi y}{h}\right) \left(A_m \cosh \frac{m\pi z}{h} + B_m \sinh \frac{m\pi z}{h} \right) \quad (3)$$

The pressure drop is determined from the flow rate Q by integrating the velocity expression with respect to y and z :

$$\frac{\Delta p}{l} = Q / \left[\left[\frac{1}{24\mu} hw(h^2 + w^2) - \frac{8}{\pi^5 \mu} \sum_{n=1}^{\infty} \frac{1}{(2n-1)^5} \right] \times \left[h^4 \tanh\left(\frac{2n-1}{2h} \pi w\right) + w^4 \tanh\left(\frac{2n-1}{2h} \pi h\right) \right] \right] \quad (4)$$

Computational fluid dynamic (CFD) software (ANSYS, Inc.) was used to predict wall shear stress of various shapes and dimensions of 3D flow patterns under a constant flow rate. The design criteria was that the shear stress on the walls of the bioprinted construct must mimic liver physiological conditions (i.e. 4.7 dyne/cm²) [2]. Further, the constant flow rate ($Q=0.25\mu\text{L}/\text{min}$) was selected based on experimental testing of static controls which previously demonstrated that a sufficient fluid residence time (i.e. 4 hrs) was necessary to ensure proper diffusion of drug into construct for measurable metabolic drug interaction and response. The design constraints for the candidate flow patterns included the 10mm x 10mm x 750 μm deep tissue chamber dimensions and a constant volume to ensure constant cell numbers.

Based on liver physiology, various sinusoidal flow pattern designs were constructed with cross-sectional widths of 0.50mm, 0.35mm, and 0.25mm satisfied the design criteria for wall shear stress with maximum shear stresses of 4.9 dyne/cm², 10.8 dyne/cm², and 17.9 dyne/cm², respectively as shown in Fig. 5. Since the aspect ratio of the flow microchannels was greater than 10:1, the predicted shear stresses in the numerical model compared well with that predicted by the Poiseuille model.

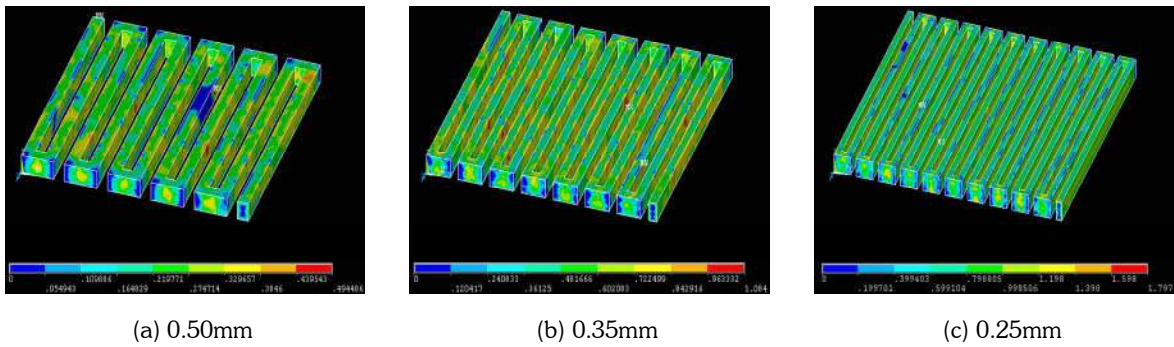


Fig. 5: Shear stress profiles for tissue constructs of varying channel widths.

4.2 Mass Transfer Modeling of Drug Flow through Tissue Chamber

In this study, substrate concentrations and shear stresses at the may be computed from a 3D numerical flow-model incorporating mass transport. To model this flow system, the governing conservation equations for continuity, momentum and substrate transport are expressed in the form of the Cartesian tensor notation as follows:

$$\nabla \cdot \vec{U} = 0 \tag{5}$$

$$\rho(\nabla \cdot \vec{U}) = -\nabla p + \mu \nabla^2 \vec{U} \tag{6}$$

$$\vec{U} \cdot \nabla C = D \nabla^2 C \tag{7}$$

where U is the velocity, p is the pressure, ρ is the density, μ is the viscosity, C is the concentration, and D is the drug flow diffusivity.

The flow boundary conditions are as follows: at the inlet, the axial velocity is uniform and specified; at the outlet, the axial velocity is fully developed; at the wall, the no-slip condition is applied. There, the drug concentration boundary conditions are denoted as follows:

$$C = C_{in} \text{ at } x = 0 \text{ and } \frac{dC}{dx} = 0 \text{ at } x = l \tag{8}$$

$$\frac{dC}{dx} = 0 \text{ at } z = 0 \text{ and } \frac{dC}{dz} = 0 \text{ at } z = \frac{w}{2} \quad (9)$$

$$D \frac{\partial C(x, 0, z)}{\partial y} = \frac{V_m \cdot \gamma \cdot C(x, 0, z)}{K_m + C(x, 0, z)} \quad (10)$$

$$\text{at } y = 0 \text{ and } \frac{\partial C}{\partial y} = 0 \text{ at } y = h$$

where l is the channel length; C_{in} is the inlet concentration, which is uniform and specified; V_m is the maximal substrate uptake rate per cell; K_m is the Michaelis-Menten constant or substrate concentration at which the V_m is half-maximal; and γ is the cell density.

5. STATIC CELL CULTURE RESULTS TO DETERMINE DRUG RESIDENCE TIME AND CELL DENSITY

Static cell culture experiments were carried out to determine drug residence time and cell density to obtain a detectable drug response from liver cells encapsulated in alginate hydrogels. To prepare the pre-polymer solution of alginate, medium viscosity sodium alginate powder (Sigma, St. Louis, MO) was dissolved in deionized water as a 3.0% (w/v) solution. The ionic cross-linking solution was prepared by dissolving 5.0% (w/v) calcium chloride (Sigma, St. Louis, MO) in deionized water. The HepG2 liver cell line (ATCC, MA) was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, ATCC), supplemented with 10% (w/v) fetal bovine serum and maintained in the incubator at 5% CO₂ and 37°C. The cells were gently mixed in 1 mL of viscous alginate solution with Pasteur pipet to ensure uniform cell distribution with hemocytometer readings to measure the cell counts. Each sample was reacted with 120µM EFC and measured with cytofluorometer for drug metabolization with the results shown in Fig. 6.

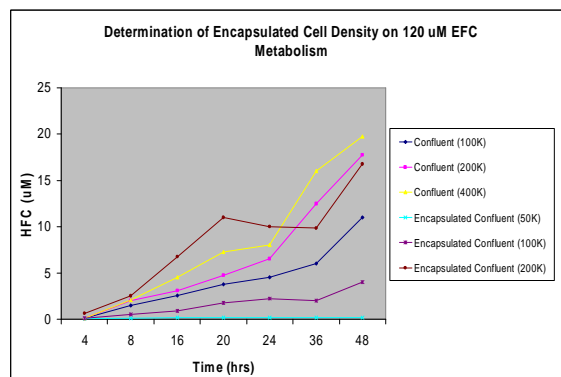


Fig. 6: Determination of adequate encapsulated cell density for detectable drug response.

From this cell study, it is determined that a drug residence time of at least 4 hours and a encapsulated cell density of 200,000 cells/mL alginate is necessary to obtain an appreciable amount of drug metabolite response. The volumetric flowrate Q for the numerical simulations is determined by the volumetric amount of bioprinted tissue construct divided by the drug residence time. This biological data therefore provides useful benchmarks for future modeling, design, and experimentation of dynamic drug flow studies.

6. CONCLUSIONS AND SUMMARY

Appropriate complex tissue models can serve as promising testbeds to more accurately reflect human physiological responses. Herein a methodology is formulated to rationally design, model, fabricate, and characterize a microfluidic device serving as an in vitro physiological liver tissue model. Such miniaturization allows for parallelization and automatization for high-throughput pharmacokinetic drug analysis. Furthermore, since cells receive messages from multiple sources within its microenvironment to initiate a cascade of intracellular responses that ultimately determine its phenotypic outcome, a physiological relevant 3D tissue model is configured based on in vivo liver architecture and

physiology. This is achieved by implementing a CAD-driven direct cell writing process for bioprinting cell-embedded tissue constructs within a 3D microenvironment. Static cell culture experiments were first carried out to determine the sufficient drug residence time and cell density to obtain a detectable drug response from liver cells encapsulated in alginate hydrogels. The experimentally determined flow rate is then incorporated into the computational fluid dynamic studies to satisfy the shear stress design criteria from literature. This will permit the accurate prediction and characterization of the hydrodynamic and transport properties of the in vitro micro-device under conditions used experimentally in future dynamic drug flow studies. Ongoing and future work includes a combined diffusion-convective numerical model and experimental modeling of varying flow conditions and dimensional parameters to tune and optimize the biological drug response of 3D bio-printed liver tissue to hydrodynamic perturbations with relation to fluid dynamical parameters and mass transport.

7. ACKNOWLEDGEMENTS

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