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A robust high-throughput sandwich cell-based drug screening platform

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ABSTRACT

Hepatotoxicity evaluation of pharmaceutical lead compounds in early stages of drug development has drawn increasing attention. Sandwiched hepatocytes exhibiting stable functions in culture represent a standard model for hepatotoxicity testing of drugs. We have developed a robust and high-throughput hepatotoxicity testing platform based on the sandwiched hepatocytes for drug screening. The platform involves a galactosylated microfabricated membrane sandwich to support cellular function through uniform and efficient mass transfer while protecting cells from excessive shear. Perfusion bioreactor further enhances mass transfer and cellular functions over long period; and hepatoctyes are readily transferred to 96-well plate for high-throughput robotic liquid handling. The bioreactor design and perfusion flow rate are optimized by computational fluid dynamics simulation and experimentally. The cultured hepatocytes preserved 3D cell morphology, urea production and cytochrome p450 activity of the mature hepatocytes for 14 days. When the perfusion-cultured sandwich is transferred to 96-well plate for drug testing, the hepatocytes exhibited improved drug sensitivity and low variability in hepatotoxicity responses amongst cells transferred from different dates of perfusion culture. The platform enables robust high-throughput screening of drug candidates.

1. Introduction

In vitro hepatocyte culture models are gaining increasing attention from the pharmaceutical industry for early stage drug toxicity screening [1]. Despite the advent of many *in vitro* liver models [2,3], adaptation of these models into industry-scale drug testing application still faces many challenges. Beside re-establishing hepatocyte *in vivo* functions, the ideal *in vitro* liver model for industry-scale drug screening demands high reproducibility with minimal variations in screening results, high-throughput and automated processing capability [4]. Among the various *in vitro* models, such as perfusion liver slices [5], microsomes [6], cell lines [7] etc, isolated primary hepatocytes are the preferred model as they strike a balance between throughput and basic cellular architecture and functions [8]; but they lose differentiated functions rapidly under conventional culture configuration [9]. Isolated hepatocytes can be sandwiched between two layers of extracellular matrices (e.g. collagen) [10,11] to reestablish the differentiated hepatocyte functions such as urea and albumin secretion [11], biotransformation enzyme functions [12], polarity and transporter

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activities [13]; making it the model recommended by the US Food and Drug Administration for *in vitro* drug metabolism and toxicity studies [14]. Still, collagen sandwich culture suffers from batch to batch collagen variations [15], variable drug absorptions [16] and poor mass transfer across collagen gel especially in static culture over extended culture period [17]. Each of these factors contributes to variable drug toxicity measurements. Adapting collagen sandwich culture into perfusion system for drug testing is difficult because collagen gel overlaying the hepatocytes can be washed off by perfusion flow, resulting in increased drug access variability and possibly in the shear stress experienced by the hepatocytes.

Perfusion culture can maintain and improve cell viability and metabolic activities of cultured hepatocytes [18] due to improved oxygen or nutrient delivery and waste removal [19]. Many hepatocyte perfusion culture systems developed [2,20,21] are low throughput [22] as they are not compatible with the industry-scale multi-well plates for high-throughput drug screening. Multi-well plate perfusion culture systems that conform to standard multi-well culture plate dimension developed previously [22,23] rely upon online drug administration in fluidic flow that compromises on controls of shear stress, cellular functions and throughput. Here we report a high-throughput and robust drug-induced hepatotoxicity screening platform (RoboTox) using perfusion sandwich-cultured hepatocytes.

2. Materials and methods

2.1. Materials

100 μm thick biaxially oriented polyethylene terephthalate (PET) films were purchased from Goodfellow Inc. (Cambridge, UK). The galactose ligand, 1-O-(6′-aminohexyl)-D-galactopyranoside (AHG, M.W. 279) was synthesized as described previously [24,25] and verified by NMR spectrum. All other chemicals were purchased from Sigma-Aldrich (Singapore) unless otherwise stated. Low stress porous Si_3N_4 membrane was designed, fabricated, surface functionalized and characterized as described previously [26,27].

2.2. Primary rat hepatocyte isolation and culture

Hepatocytes were harvested from male Wistar rats weighing 250–300 g by a two-step *in situ* collagenase perfusion method [28]. Animals were handled according to the Institutional Animal Care and Use Committee (IACUC) protocol approved by the IACUC of the National University of Singapore. Only hepatocyte with viability of >85% as determined by Trypan Blue exclusion assay was used. Freshly isolated hepatocytes were seeded onto collagen-coated or galactose-immobilized PET films at 1×10^5 cells/cm^2 in a 96-well plate. The cells were cultured in William's E medium supplemented with 10 mM NaHCO3, 1 mg/ml BSA, 10 ng/ml EGF, 0.5 μg/ml insulin, 5 nM dexamethasone, 50 ng/ml linoleic acid, 100 units/ml pencillin and 100 μg/ml streptomycin; and incubated with 37 °C, 5% CO_2, 95% humidity for 24 h. Collagen sandwich was assembled by placing a collagen-coated polycarbonate membrane (Isopore™, Milipore, USA) on top of the hepatocytes seeded on collagen-coated PET film. Si_3N_4 sandwich culture was assembled by placing galactose-immobilized porous Si_3N_4 membrane (20 μm pore size and 20 μm inter-pore distance, corresponding to 20% porosity) on top of the cells on galactose-immobilized PET films.

2.3. RoboTox platform

Hepatocytes were first seeded on galactose-immobilized PET film for 24 h, and then overlaid with galactose-immobilized porous Si_3N_4 membrane (Fig. 1A). Hepatocytes were cultured in this Si_3N_4 sandwich culture configuration for another 24 h before transferring into the RoboTox bioreactor. The bioreactor comprises of three subunits: an upper lock plate, a middle sieve plate and a lower perfusion bioreactor. It was machined to dimension from a polycarbonate block and sealed with o-rings and screws (Fig. 1B). The lower perfusion bioreactor was fabricated according to the dimension of a typical 96-well plate, except the wells are interconnected in series by a 3 mm diameter fluid channel. The wells of the middle sieve plate are slightly smaller than the wells of lower perfusion bioreactor so that they can fit into the bioreactor and conventional 96-well plate. The middle sieve plate holds the Si_3N_4 sandwich and facilitates easy transfer of the cells between the perfusion bioreactor and standard 96-well plate. The upper lock plate provides uniform pressure to seal the entire assembly. To improve the delivery of oxygen which is important for hepatocyte culture [29], circular openings corresponding to the bioreactor wells were drilled into the upper lock plate. An oxygen-permeable membrane (Breathe-Easy®, Diversified Biotech, USA) was placed between the sieve plate and the upper lock plate to preserve the closed circuit while allow adequate oxygen diffusion at the same time. The bioreactor was connected to a recirculating closed perfusion loop so that it is isolated from the external environment (Fig. 1C). The perfusion loop consists of RoboTox bioreactor, medium reservoir, peristaltic pump (Ismatec SA, Switzerland), three-direction valves (Upchurch Scientific, USA), stopping valves (Upchurch Scientific, USA), connectors (Upchurch Scientific, USA) and oxygen-permeable silicone tubing (Ismatec SA, Switzerland), and placed in a 37 °C incubator with 5% CO_2 and 95% humidity. Online sampling and monitoring was achieved via three-direction valve. For high-throughput drug testing, the middle sieve plate holding the perfusion-cultured Si_3N_4 sandwiches were removed from the bioreactor and placed into the conventional 96-well plate for multiplex drug testing using robotic liquid handler (Fig. 1D).

2.4. Fluid flow modeling

Finite Element Analysis (FEA) software, COMSOL Multiphysics 3.5a (COMSOL Inc., Burlington, MA) was used for the fluid dynamic simulations. A 3D model which represented the fluid body containing oxygen and hepatocytes was constructed. Customized meshing was used and resulted in 744578 tetrahedral elements. The model consisted of one subdomain in where the steady state incompressible Navier-Stokes application mode and the steady state convection and diffusion application mode were applied to simulate for the fluid motion and the oxygen mass transfer. The characteristic parameters used to define the models are summarized in Table 1. The governing equations for the subdomain are

$$\rho(u\cdot\nabla)u = -\nabla\rho + \eta\nabla^2 u$$

$$\nabla\cdot(-D\nabla c) = -u\cdot\nabla c$$

where $u[m^2\cdot s^{-1}]$ denotes the fluid velocity field and ∇ is the standard del (*nabla)* operator.

A fully developed flow condition was applied at the inlet boundary with oxygen partial pressure set at the atmospheric pressure. Outlet boundary conditions were zero pressure outflow and convective-only oxygen transport. The fluid parts of the bioreactor which are exposed to air were modeled as boundaries with the slip wall condition having atmospheric oxygen partial pressure. The no-slip wall boundary conditions were used along the wall of the model.

The hepatocytes in the bioreactor were assumed to be uniformly distributed on the bases of the wells. They were represented as boundaries with oxygen out-flux condition. The magnitude of the flux is in accordance to the Oxygen Uptake Rate (OUR) of the hepatocytes as described in the equation

$$\mathrm{OUR} = V_m \times \frac{\frac{c}{\alpha}}{\frac{c}{\alpha} + K_m}$$

where c is the local oxygen concentration. This equation follows the Michaelis–Menten kinetics and has been widely used as a mathematical model to represents Oxygen Uptake Rate of many cells including hepatocytes [30].

2.5. Mass transfer efficiency measurement

Mass transfer efficiency was measured by limited diffusion of cell labeling agent CellTracker™ Green (Invitrogen, USA). Hepatocytes were first thoroughly labeled with 20 μM CellTracker™ Orange (Invitrogen, USA) in culture media. The media was replaced with 2 μM of CellTracker™ Green under static and perfusion condition for 2 h. The cells were rinsed with phosphate buffered saline (PBS) and fixed with 3.7% paraformaldehyde. Z-stack images from three independent experiments were taken with confocal microscope (Fluoview 300, Olympus, Japan). Quantification of the labeled cells was performed by Matlab (R2009a). After removing noise using a low-pass filter, z-stacks of binary masks were created by thresholding the red channel images. Zero values in the mask represent background; while red signals represent the space occupied by cells. The total cell area for one z-stack was defined to be the total number of all positive pixels in the corresponding masks. Mass transfer was represented by total intensity in the green channel. Total intensity was defined as the sum of all pixel intensities in the positive areas of the corresponding green channel. The mass transfer efficiency was calculated by total intensity/total cell area.

2.6. Cell viability measurement

Cell viabilities of primary rat hepatocytes cultured in RoboTox system for 6 days at the flow rates of 0.1, 0.06, 0.03, 0.015 ml/min were assessed by live and dead staining of the cells with Calcein AM (Molecular Probes, USA) and propidium iodide (PI). Cells were washed with PBS and incubated with 5 mM of Calcein AM and 25 mg/ml of PI in culture medium at 37 °C for 30 min. Cells were then washed with PBS, placed in fluorescent mounting medium (Dako, Denmark) and viewed by confocal microscopy (Fluoview 300, Olympus, Japan). Cell viability was also measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS Assay, Promega, USA).