The Effectiveness of a Novel Cartridge-Based Bioreactor Design in Supporting Liver Cells

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There are a number of applications—ranging from temporary strategies for organ failure to pharmaceutical testing—that rely on effective bioreactor designs. The significance of these devices is that they provide an environment for maintaining cells in a way that allows them to perform key cellular and tissue functions. In the current study, a novel cartridge-based bioreactor was developed and evaluated. Its unique features include its capacity for cell support and the adaptable design of its cellular space. Specifically, it is able to accommodate functional and reasonably sized tissue ($>2.0 \times 10^8$ cells), and can be easily modified to support a range of anchorage-dependent cells. To evaluate its efficacy, it was applied to liver support in the current study. This involved evaluating the performance of rat primary hepatocytes within the unique cartridges in culture—sans bioreactor—and after being loaded within the novel bioreactor. Compared to collagen sandwich culture functional controls, hepatocytes within the unique cartridge design demonstrated significantly higher albumin production and urea secretion rates when cultured under dynamic flow conditions—reaching peak values of $170 \pm 22 \mu\text{g}/10^6$ cells/day and $195 \pm 18 \mu\text{g}/10^6$ cells/day, respectively. The bioreactor's effectiveness in supporting live and functioning primary hepatocytes is also presented. Cell viability at the end of 15 days of culture in the new bioreactor was $84 \pm 18\%$, suggesting that the new design is effective in maintaining primary hepatocytes for at least 2 weeks in culture. Liver-specific functions of urea secretion, albumin synthesis, and cytochrome P450 activity were also assessed. The results indicate that hepatocytes are able to achieve good functional performance when cultured within the novel bioreactor. This is especially true in the case of cytochrome P450 activity, where by day 15 of culture, hepatocytes within the bioreactor reached values that were 56.6% higher than achieved by the collagen sandwich functional control cultures. The success of the novel cartridge-based bioreactor in supporting hepatocytes with good viability and functional performance suggests that it is an effective design for supporting anchorage-dependent cells.

Introduction

BIOREACTORS, SUCH AS BIOARTIFICIAL LIVER devices, are extracorporeal devices used for a range of biomedical applications. One of their applications is a temporary bridge to transplantation, for patients suffering from organ failure disease or tissue loss, while pharmaceutical testing is yet another. The design goal of bioreactors is to effectively support a large mass of viable cells (e.g., hepatocytes) in a way that enables them to express high levels of differentiated functions. Although many bioreactor variants exist, typical considerations in the design include the cellular source, the culture environment established within the unit, the materials (and biomaterials) used within the design, the device's configuration, and the ability of the device to support its target cell population.

Liver bioreactor designs are typically classified into one of four categories: hollow fibers, flat plate with monolayer, suspension or encapsulation chambers, and perfused beds/scaffolds.¹ Of these, the capillary hollow-fiber-based systems have been most rapidly developed for clinical trials.^{2,3} Unfortunately, this configuration has the inherent physical limitations of constrained total mass diffusion distances, limited cellular mass capacity, and nonuniform cell distributions. While the designs of encapsulation and suspension chambers can provide uniform microenvironments and ease of scale-up,⁴⁻⁶ they can also offer poor cell stability (true for suspensions) and nutrient transport barriers (i.e., encapsulation systems). Moreover, the cells in such systems are typically exposed to higher shear forces. Contrastingly, perfused beds/scaffold designs offer an answer to the transport barrier limitations of encapsulation chambers,⁷⁻¹⁰ yet unfortunately

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encounter nonuniform perfusion profiles and clogging of membrane pores, and can expose the cells to shear forces.

The flat-plate design is one of the more extensively developed designs. The limitations of its low surface area-to-volume ratios and the difficulties of scaling up this configuration to the cell masses required for patient support are counterbalanced by the fact that this is a configuration that allows researchers to easily incorporate different tissue scaffolds and culture features.^{11–18} For example, Suzuki *et al.* used a polytetrafluorethylene (PTFE) membrane to support collagen sandwich-cultured hepatocytes.¹⁹ Tilles *et al.*¹⁴ and de Bartolo *et al.*^{20–22} have also demonstrated the benefits of using oxygen (O₂)-permeable membranes to improve O₂ delivery to the cellular spaces in flat-plate configurations. In general, tissue function is modulated by the communication of cells with extracellular matrices, soluble factors, and other cells.²³ In the specific case of the liver, the benefits of coculturing hepatocytes (the liver parenchymal cell) with support cells, in specific ratios, are known to positively impact hepatocyte functions. As such, technologies for exploring those interactions—such as microfabrication technology^{24–28}—have also been applied to the flat-plate design models.^{13,15,29} Unfortunately, tailored features such as these are not easily implemented in the other three bioreactor designs previously mentioned. Hence, development of a bioreactor with an adaptable cellular space would be beneficial. A recent paper by Poyck *et al.*³⁰ also highlighted the importance of a design that allows fractions of the cell space to be removed from the system (e.g., for analysis) without compromising the entire bioreactor. The adaptability of the novel bioreactor of this study offers solutions to each of these.

A variety of radial flow bioreactors, using various tissue culture scaffolds, have been developed to minimize the nutrient-cell transport barrier and maintained a uniform microenvironment for the cells.^{8,9,29,31–33} These radial flow bioreactor designs provide three-dimensional culture environments, and their results indicate potential in use as efficient *in vitro* systems. However, the designs did not succeed in minimizing the cells' exposure to shear stress. Our current bioreactor design seeks to overcome this limitation. Further, our bioreactor's tissue units can be easily removed from the bioreactor for storage or analysis. Finally, our unique design makes it easy to scale up or down the total cell volume supported by the bioreactor.

To test the effectiveness of the design in supporting hepatocytes, a series of characterization studies were conducted. This included the evaluation of the bioreactor's efficacy in supporting hepatocytes, through the completion of cell viability and function studies. Then to evaluate the effectiveness of the new bioreactor design in establishing normoxic conditions for its cells, hypoxia evaluation studies were conducted. The results indicate that hepatocytes within the bioreactor perform at a higher level than the cells of the functional controls.

Materials and Methods

Design of bioreactor device and its circulation system

Overall structure. The basic design of the bioreactor is schematically shown in Figure 1. In brief, the bioreactor has four quadrant chambers (shown in Fig. 1B), each of which has its own inlet (located at its base) and outlet (located at its

top). This feature has the benefit of allowing the bioreactor to be operated from ¼ to full (i.e., four quadrant) capacity. It also enables the transport path of the oxygenated nutrient medium flowing through a given quadrant to remain separate from that of the neighboring chambers. As shown in the Figure 1D, a perforated medium transport tube is at the corner of each quadrant, positioned adjacent to the geometric center of the bioreactor. As nutrient medium enters through the inlet at the base of a given quadrant, fluid is drawn upward and inward from the periphery to the perforated transport tube of that quadrant.

Rack-cartridge system of the bioreactor. The cellular spaces of the bioreactor are contained within individual cartridges, and each cartridge is secured within the chamber of the bioreactor by attaching it to a rack assembly (see Fig. 1C). Recall that one feature of the bioreactor is that it can be adapted to support a wide range of cells of various configurations. The cartridge design enables this by encompassing the cellular space (e.g., sandwich culture, micropatterned cell culture, and tissue slices) between permeable PTFE membranes (of 0.4 µm pore size and 30 µm thickness) secured within an autoclavable and biocompatible custom frame. The membrane-frame configuration of each tissue cartridge ensures that the cells it contains are protected from the damaging effects of shear stress by the nutrient medium flow within each quadrant chamber of the bioreactor. Custom clips were then used to attach each assembled cartridge to the rack (see Fig. 1B). A maximum of eight rectangular cartridges were attached to the circular rack of the device. The current prototype of the rack design (refer to Fig. 1C) enables each cartridge to hang vertically within the chamber at an angle of 10° from its neighbor, such that the largest distance between each neighboring cartridge is 0.45 inch. To further increase the cell capacity of the bioreactor, the rack-cartridge design is flexible enough to allow more cartridges to be installed within a given quadrant chamber. In the current version of the design, each individual cartridge has a surface area of 18.58 cm² available for cell support.

Cartridge frames made from either polycarbonate (PC) or stainless steel 316 (SS) have been used for the bioreactor. PC cartridge frames were machined using computer numerical control within the Center for Precision Metrology at UNC Charlotte. SS cartridge frames were manufactured using laser cutting. To prepare the PC cartridge frames for use, they were cleaned using sandpaper, immersed in 95% ethanol, and then sterilized by autoclaving at 121°C/15 psig using a 30-min cycle. Preparation of the SS cartridge frames for use required initial cleaning to remove all oxide particles and heat tint, followed by a 30-min soak in 20% nitric acid bath at 60°C (i.e., passivation). To remove the residual acid, the parts were then thoroughly rinsed in de-ionized water. Finally, the SS frames were sterilized via autoclaving at 121°C/15 psig using a 30-min cycle in preparation for tissue culture use. Before reusing either the SS or PC frames, fine sandpaper was used to clean the surfaces, followed by autoclaving at 121°C/15 psig using a 30-min cycle.

Before using the PTFE membrane of the cartridges, the membrane was also autoclaved using a 15-min liquid cycle at 121°C. Next, the membrane was attached to each frame using cyanoacrylate adhesive (Dymax, Torrington, CT). The assembled frame-membrane system was then stored within a

bio-safety cabinet for at least 24 h to allow the adhesive to fully cure.

Before using these membrane-frame assemblies for tissue culture, they were further sterilized by immersion in 95% ethanol until the membranes appeared transparent. The parts were then sterilized via immersion in 70% ethanol for a minimum of 1 h. The assembly was next washed three times in saline (0.9% NaCl), with a 20-min soak included in the second wash. The membrane-frame assembly was then dried in preparation for adding the tissue equivalent it would support.

To prepare the bioreactor for use, other critical parts (i.e., the chamber, connectors, silicone stoppers, various tubing, and also parts of the dynamic system) were sterilized before use by rinsing them first with 90% ethanol, followed by 70% ethanol, and then washed twice in 1× phosphate-buffered saline (PBS).

Circulation system. Figure 2 shows the circulation system of the bioreactor. As indicated, the circulation system includes a medium reservoir for removing gas bubbles from the medium; a gas exchanger made of gas-permeable Silastic tubing with a length of 5 m, ID×OD = 1.47×1.96 mm (Dow Corning, Midland, MI); a multifunction meter (Accumet XL60; Fisher

Scientific, Pittsburgh, PA) for documenting the level of dissolved O₂ within the medium and pH of the nutrient medium; a peristaltic pump coupled to a flow meter for directing the flow of the liquid nutrients; and an incubator for maintaining

FIG. 2. The schematic diagram of the circulation system of the bioreactor.

the bioreactor at 37°C with 5% CO₂/95% air. The priming volume of the circuit was 320 mL. The tissue culture medium was perfused at a rate of 55–60 mL/min starting on day 2 postisolation (a period of at least 24 h of static culture in the incubator is preferably provided to allow the hepatocytes to recover from the trauma of the enzymatic digestion of the liver). Medium was changed on days 6 and 12. The flow rate, pH, and pO₂ were checked regularly, and adjusted as needed.

Preparation of the bioreactor's cellular space

For each quadrant chamber of the bioreactor, the cartridges were attached to the rack and suspended vertically as shown in Figure 1C. This unique bioreactor design allows for easy disassembly of a single (or multiple) cartridges from the system. The membrane-frame design of each cartridge makes the bioreactor adaptable for use in supporting a wide range of tissue equivalents. As previously mentioned, the current investigation demonstrates its applicability to a cellular space consisting of hepatocytes sandwiched between two layers of type I collagen. Hepatocytes in the cartridges (and the controls) were seeded at a concentration of 2.1×10^5 cells/cm², such that each cartridge maintained 4 million cells. To ensure stabilization of the cells within the cartridge for 24 h before securing them within the bioreactor, the cartridge cultures were first incubated for 24 h within a 100 mm tissue culture dish at 37°C and 5% CO₂/95% air; then relocated under sterile conditions to the circular rack of the bioreactor. Because eight cartridges were loaded per quadrant chamber of the bioreactor, each quadrant chamber thus supported 32 million hepatocytes in the current study.

Hepatocyte isolation and culture medium. Rat hepatocytes were isolated from male Sprague-Dawley (Charles River Laboratories, Wilmington, MA) rats weighing 180–220 g, by collagenase perfusion using a method modified from Seglen.³⁴ In brief, the liver was perfused with collagenase solution (140 mg/dL) through the portal vein, and the digested liver was then filtered through a nylon mesh with a pore size of 105 µm (Small Parts, Miramar, FL). The hepatocytes were then separated from the nonparenchymal cell fractions by centrifugation (Thermo IEC Centra-CL3R; Thermo Scientific, Waltham, MA) at 50 g for 3 min. The viability of the hepatocytes, evaluated via trypan blue exclusion, was 88–95%. When the cell viability was below 85%, percoll centrifugation was performed. Hepatocytes were then resuspended in culture medium containing DMEM (Invitrogen (Gibco), Gaithersburg, MD) supplemented with 3.7 g of sodium bicarbonate, insulin (500 U/L), glucagon (7 µg/L), epidermal growth factor (20 µg/L), hydrocortisone (7.5 mg/L), penicillin

G (10,000 U/mL), streptomycin (10 mg/mL), amphotericin B (25 µg/mL), and 10% fetal bovine serum.³⁵

ECM preparation for the static culture in tissue culture plates. Static sandwich cultures in 35-mm-diameter tissue culture plates were used as the controls throughout the experiments (depicted in Fig. 3A). Collagen type I gel was first prepared by adding eight parts of 1.1 mg/mL PureCol collagen (Inamed, Fremont, CA) into one part of 10× DMEM, according to the instructions of the manufacturer. The pH of the solution was adjusted to 7.4 by adding 0.1 N NaOH or 0.01 N HCl. In the culture plates, 0.5 mL of collagen was coated in the tissue culture plates, 35 mm in diameter, and incubated at least for 1 h at 37°C and 5% CO₂/95% air to allow gelation. Then, 1 mL of 2.0×10^6 cells/mL hepatocytes was seeded in each culture plate to achieve a seeding density of 2.1×10^5 cells/cm². The medium was then changed after 1 h to remove unattached cells from the culture. After 24 h, 0.5 mL of collagen gel was added to each culture plate, and allowed to gel for 45 min at 37°C in a 5% CO₂, 95% air incubator. After this, culture medium was replaced daily.

ECM preparation for the cartridges. A modified sandwich culture was used for the cellular space, as shown in Figure 3B. As compared to the traditional sandwich culture (Fig. 3A), the membrane of the bottom unit of the cartridge was first coated with dried collagen film. The collagen film was prepared by diluting the stock Purcol collagen (3.1 mg/mL) 1:4 in 70% ethanol (one part collagen and three parts 70% ethanol) and mixed by vortex. Then, 1 mL of the diluted collagen was evenly coated on the membrane of the bottom unit. After incubating the collagen-coated membrane overnight at 37°C and 5% CO₂, 2 mL of hepatocytes (density: 2.0×10^6 cells/mL) was seeded. The final seeding density of hepatocytes for each cartridge was 2.1×10^5 cells/cm². The medium was changed after 1-h incubation to remove excess unattached hepatocytes. After 24 h of culture, 1 mL of collagen gel was added on top of cell layers and allowed to gel at 37°C in a 5% CO₂/95% air incubator for 45 min. Up to this point, custom supports were used to maintain an air interface at the membrane base of the bottom unit of the cartridge. After securing the top unit of the cartridge onto the bottom unit using custom clips, the completed cartridge was then placed in a 100-mm-diameter tissue culture plate, with 15 mL of nutrient medium.

Static and dynamic culture systems

The effectiveness of an individual cartridge in supporting cells was first evaluated using hepatocytes cultured within both static and dynamic systems, as schematically shown in

FIG. 3. Schematic of sandwich culture configuration used with tissue culture plates (A) and the bioreactor's cartridges (B). In the 35 mm tissue culture plates, cells access medium from their apical side only, whereas within the cartridges the cells can access medium from both sides.

Figure 4. In the static culture system (Fig. 4A), a single cartridge contained hepatocytes sandwiched between collagen type I gel (refer to Fig. 3B) was placed in a 100 mm tissue culture plate, and incubated at 37°C and 5% CO₂/95% air. To maintain the cell culture, 15 mL of cell culture medium was replaced every other day from day 2. In the dynamic culture system (Fig. 4B), same as the bioreactor, the medium circulation started on day 2 to allow the hepatocytes to recover from the trauma of the enzymatic digestion of the liver. On day 2, two cartridges identical to the hepatocyte supporting cartridges of the static system were placed in a modified 100 mm tissue culture plate, and retrofitted with an inlet and outlet to ensure medium flow. This dynamic system was also incubated at 37°C and 5% CO₂/95% air. A total of 50 mL of culture medium was circulated in the flow circuit (as shown in Fig. 4B) with a flow rate of 30 mL/min, and replaced every other day. Two cartridges were used for the dynamic system to avoid excessive dilution of the cells' metabolites within the medium. For both the static and dynamic culture systems, 1 mL of supernatant was sampled for cell function analysis from day 3 of the culture.

Evaluation of performance

Liver-specific functions. Urea and albumin secretion: The changes in urea concentration were quantitatively measured using Stanbio urea nitrogen (BUN) kit (Cat# 0580; Stanbio Laboratory, Boerne, TX), based on direct interaction of urea with diacetyl monoxime. The absorbance was measured at 540 nm with Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). Culture medium containing 5.0 mM ammonium chloride (NH₄Cl) was added to the cells to assess hepatocytes ability to metabolize ammonia. For individual cartridge evaluation, 5 mM NH₄Cl was added on day 7; for the bioreactor, fresh medium with 5 mM NH₄Cl was added on days 6 and 12. Further, albumin secretion was measured by a standard competitive enzyme-linked immunosorbent assay (ELISA) with the use of purified rat albumin and peroxidase-conjugated sheep anti-rat albumin antibody. Briefly, 100 µL of 50 µg/mL rat albumin (MP Biomedicals, Solon, OH) was added to 96-well plates and stored at 4°C for at least 24 h. The wells were washed with 0.05% Tween-20 in PBS, and nonspecific binding sites were blocked with Tween-20 at the same time. Next, 50 µL of sheep anti-rat albumin-peroxidase conjugate (MP Biomedicals) was added to each well and incubated for 24 h at 4°C. The wells were then washed with 0.05% Tween-20 for four

times and incubated for 15 min with *o*-phenylenediamine substrate (Sigma, St. Louis, MO). The reaction was stopped by 8 N sulfuric acid, and absorbance was measured at 450 nm with Synergy HT Multi-Detection Microplate Reader (BioTek Instruments). Both the urea and albumin results were calibrated to a standard curve, and concentration values were normalized by the nutrient medium volume, culture time, and number of seeded hepatocytes.

Ethoxyresorufin O-deethylase (EROD) assay: An important function of hepatocytes is to metabolize thousands of endogenous and exogenous compounds by a large group of heme-containing isoenzymes, that is, cytochrome P450 (CYP). They primarily locate in hepatocytes, within the membranes of the smooth endoplasmic reticulum. For rat primary hepatocytes, CYP1 enzymes present at a relatively higher level and are readily detectable as compared to other CYP families. EROD activity has been used as a catalytic monitor of CYP1 enzymes (chiefly CYP1A1).³⁶ Using ethoxyresorufin as the substrate, the rate of resorufin productivity is directly proportional to the EROD activity.

Sandwich cultures in 24-well plastic culture plates were used as negative and positive controls. CYP1A1/2 was induced by adding 2 µM 3-methylcholanthrene (3-MC; Sigma) to the medium for 48 h before the EROD assay. Three samples were used to perform each EROD assay. Each cartridge or the culture plate well was incubated in the Hank's-buffered salt solution containing 20 mM HEPES and 10 µM dicumarol (Sigma), which inhibits secondary metabolism of resorufin. After 10 min of incubation, assay buffer containing 5 µM ethoxyresorufin and 10 µM dicumarol was added. After 1-h incubation in 5% CO₂, 95% air at 37°C, the assay buffer was sampled at various time points (5, 15, 25 and 35 min). The cells in the cartridges and in the 24-well culture plates were washed twice with Hank's-buffered salt solution, fed with fresh medium, and returned to either the bioreactor or the incubator. Resorufin fluorescence (excitation at 530 nm and emission at 590 nm) was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments). To determine the net resorufin production, a resorufin standard curve (ranging from 2 to 200 pmol) was used to convert the fluorescence values obtained from the plate reader to picomoles of resorufin. Before serial dilution, the actual concentration of the super stock of resorufin, 200 µM in HEPES (pH 9), was checked on each assay date using a spectrophotometer (DU640; Beckman Coulter, Fullerton, CA). Rate of formation of resorufin, as calculated from the early linear increase in the fluorescence curve, was expressed as pmol/min. The

FIG. 4. Schematic of static culture (A) and dynamic culture (B) systems used to evaluate effectiveness of the bioreactor's cartridges in supporting cells.

results were normalized by the dilution of medium and number of seeded hepatocytes.

Evaluation of O₂ distribution within bioreactor

To evaluate the O₂ environment established within bioreactor, a single quadrant chamber of the bioreactor supporting eight cartridges was used for this study. Tissue culture medium containing 0.2 mM HypoxypromTM-1 (Pimonidazole Hydrochloride; Chemicon, Temecula, CA) was circulated through the chamber for 4 h. In this way, Hypoxyprom-1 is distributed to all cells in the cartridges, yet at 37°C it binds only to cells at O₂ tension of less than 10 mmHg. Meanwhile, three cartridges maintained statically within an incubator (5% CO₂, 21% O₂, 37°C) (Forma Scientific, Marietta, OH) were used as negative controls, whereas another three cartridges maintained in another incubator (5% CO₂, 1% O₂, 37°C) (Sanyo Biomedical, Bensenville, IL) were used as positive controls. After the 4-h incubation, samples were fixed in 4% paraformaldehyde (in 1× PBS) for 10 min at 4°C and stored in PBS until staining. The following immunohistochemical staining protocol was employed, where all steps took place at room temperature, and 1× PBS was used for each wash. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 10 min. Dako protein block (DakoCytomation, Glostrup, Denmark), used to block potential nonspecific binding sites in the cell/tissue, was applied for 15 min. Samples were then incubated with hypoxyprom-1 Mab1 conjugated with FITC (clone 3.11.3; Chemicon) at 1:50 for 40 min. As the negative controls, no primary antibody was added to the cells. A rocking platform (Cole Parmer, Vernon Hills, IL) was used with a speed of 30 rpm and 10° tilt angle, to ensure that all the hepatocytes were stained evenly. Anti-FITC Mab conjugated with HRP was used as the secondary antibody at 1:300 for 30 min. Labeling was viewed using liquid diaminobenzidine (Chemicon) for 5 min. Samples were then counterstained with Mayer's hematoxylin and kept in 1× PBS for image analysis on the same day.

Evaluation of cell viability

Cell viability was evaluated for hepatocytes within the static and dynamic systems, as compared with the control sandwich cultures on days 2, 7, and 11. In each case, an Olympus IX70 inverted microscope (Olympus America, Center Valley, PA) was used to document hepatocyte morphology immediately before initiating the viability assay. To evaluate the viability of the hepatocytes cultured within the cartridges of the bioreactor, the following procedure was followed. The medium was first removed, and then a small pair of scissors was used to cut off the membranes from the cartridge. The modified sandwich cultures were then carefully relocated from the cartridges to labeled 60 mm tissue culture plates and incubated for 30 min with 1 mL of a viability solution consisting of 2 μM calcein AM and 4 μM ethidium homodimer (# L-3224; Molecular Probe, Eugene,

OR) in medium. A rocking platform (Cole Parmer) was used with a speed of 30 rpm and 10° tilt angle to ensure that all the hepatocytes were stained evenly. Next, the cells were fixed with 10% buffered formalin solution in PBS. Viable and nonviable cells were examined using an Olympus IX70 Fluoroview confocal microscopy system with HeNe laser (FV500; Olympus America) using FITC and Texas red filter sets, respectively. Flouview v2.1.39 (Olympus America) and Metamorph Imaging system (Molecular Devices, Downingtown, PA) were then used to obtain and analyze the fluorescent images.

Data analysis

Plotted data are expressed as means ± standard deviation. The significance of differences was tested by *t*-test or one-way analysis of variance (ANOVA) followed by a Holm-Sidak test. *p*-Values less than 0.05 were considered significant.

Results

Evaluation of hepatocyte performance in individual cartridge

Each individual cartridge was first evaluated for the design's effectiveness in supporting hepatocyte viability, differentiation, and liver-specific functions under static and dynamic conditions. This helped verify whether the cartridge configuration provided a favorable environment for the hepatocytes. Culturing hepatocytes in a traditional sandwich configuration—between two layers of gelled extracellular matrix³⁵—within 35 mm tissue culture plates served as the functional control. Since cell-cell interactions strongly influence hepatocyte function in collagen gel,³⁷ seeding density was consistently kept as 2.1 × 10⁵ cells/cm² in both the controls and cartridges to ensure comparable results. This resulted in the seeding of 2.1 million cells on each P35 cell culture dish, and 4 million cells on each cartridge of the current study.

Hepatocyte morphology for the different culture conditions was documented daily, as shown in Figure 5. During the first 1 h of seeding, hepatocytes attached and started to spread on both the dried collagen film-coated cartridge membrane (refer to Fig. 3B) and the collagen gel surface (refer to Fig. 3A). However, the collagen film-coated cartridge membrane resulted in the cells spreading quickly with better interconnects between neighboring cells during the 24 h before the second layer of collagen was overlaid, as shown in Figure 5A, D, and G, during the culture. Similar to the control sandwich cultures (Fig. 5C), hepatocytes cultured within the cartridges developed and sustained polygonal morphology and exhibited distinct cell-cell borders for more than 11 days in both the static and dynamic systems (see Fig. 5F, I).

Next, cell viability was evaluated on days 2, 7, and 11 postisolation for hepatocytes cultured under the same three

FIG. 5. Hepatocyte morphologies on days 2, 7, and 11 cultured under three conditions: traditional sandwich culture in tissue culture plates, that is, the control (A–C); the modified sandwich culture within cartridges under static flow (D–F); and dynamic (G–I) culture conditions. Calibration bars indicate 50 μm.

Control – Sandwich
(A) Day2

Control – Sandwich
(B) Day7

Control – Sandwich
(C) Day11

Cartridge – Static System
(D) Day2

Cartridge – Static System
(E) Day7

Cartridge – Static System
(F) Day11

Cartridge – Dynamic System
(G) Day2

Cartridge – Dynamic System
(H) Day7

Cartridge – Dynamic System
(I) Day11

conditions. As shown in Figure 6, cell viability in the cartridges (both in static and dynamic) remained relatively stable during the 2-week culture period. No significant difference was observed between hepatocyte viability for the controls and cartridge.

The effectiveness of individual cartridges in supporting hepatocytes was evaluated by comparing liver-specific functions. Albumin production and urea secretion were measured for both the static and dynamic systems as compared with the control sandwich culture, throughout the 15-day culture period. Albumin is a highly soluble, single polypeptide protein with a molecular weight of 66,000, which is often measured as an indication of synthetic activity of hepatocytes. As shown in Figure 7, although hepatocytes had 12% higher albumin production rates when cultivated in the static cartridge system (peaking at $68 \pm 24 \mu\text{g}/10^6$ cells/day, $n=3$) compared to the control ($60 \pm 22 \mu\text{g}/10^6$ cells/day, $n=3$), this difference was not statistically significant. In contrast, in the dynamic system hepatocytes demonstrated significantly increased albumin production rates after day 5 compared to either the static system or the control ($p < 0.025$). The peak albumin secretion rate for the dynamic system reached an average value of $170 \pm 22 \mu\text{g}/10^6$ cells/day ($n=3$).

Urea secretion is an indicator of metabolic function of hepatocytes. To evaluate the urea secretion rate of hepatocytes cultured in three conditions, all cultures were spiked with NH_4Cl on day 7. As shown in Figure 8, the dynamic cartridge cultures yielded statistically higher urea secretion rate values compared to the other two systems. Specifically, this condition peaked at $195 \pm 18 \mu\text{g}/10^6$ cells/day on day 5 postisolation, and then progressively decreased in time until ammonia was re-introduced to the system on day 7. The urea secretion rate then continued to decrease for the dynamic cartridge system until it stabilized during the last 4 days of

FIG. 6. Hepatocyte viability results. Relatively stable cell viabilities were maintained for hepatocytes under all three culture conditions—control sandwich culture, and the modified sandwich configuration cultured in the cartridges under static and dynamic flow conditions. No significant differences were found between the three conditions.

FIG. 7. Albumin secretion rate of hepatocytes cultured within the bioreactor's cartridge under static and dynamic flow conditions, as compared to control sandwich cultures. The values are the mean $\pm$ SD, $n=3$. Statistically significant difference: * $p < 0.05$ and # $p < 0.025$.

the 2-week culture and averaged $124 \pm 22 \mu\text{g}/10^6$ cells/day. Interestingly, the urea secretion rates achieved by hepatocytes within the static cartridge and control systems were notably lower and more steady than that of the dynamic system, and weakly responsive to the day 7 addition of NH_4Cl . The static and control systems averaged 102 ± 5 and $97 \pm 7 \mu\text{g}/10^6$ cells/day, respectively, throughout the 2-week culture period. These results suggest that connecting the bioreactor's cartridge to a re-circulating flow significantly improves the ability of the cells it contains to secrete urea.

Performance of the bioreactor

The above results confirmed the efficacy of individual cartridge in supporting hepatocytes to maintain its morpho-

FIG. 8. Urea secretion rate of hepatocytes cultured within cartridges under static and dynamic flow conditions, as compared to control sandwich cultures. Five millimolar NH_4Cl was added on day 7 (labeled with $\downarrow$). The values are the mean $\pm$ SD, $n=3$. Statistically significant difference: * $p < 0.05$ and # $p < 0.025$.

FIG. 9. Hypoxia detection in different regions of a representative quadrant chamber of the bioreactor, compared to controls—where all cells have been treated with Hypoxyprobe-1 and hematoxylin. The positive control (A) demonstrates the characteristic brown labeling of hypoxic hepatocytes, which is not visible in the negative control (B), which shows the characteristic blue color of normoxic conditions. The micrographs of hepatocytes within the top (C) and bottom (D) regions of the chamber suggest that the cells are not exposed to oxygen tensions below 10 mmHg when cultured within the bioreactor.

logical development and cell survival. Further, the dynamic flow of nutrient medium enhances the cellular functional performance in the cartridges. The effectiveness of the bioreactor in supporting hepatocytes was evaluated by loading eight cartridges per chamber of the bioreactor, where each cartridge contained 4 million seeded hepatocytes. Since pH and O_2 levels are critical to cell survival and functional performance, the nutrient medium was sampled regularly to monitor these two parameters throughout the 2-week culture period of each experiment. Sampling indicated that the pH of the tissue culture medium was typically 7.4, yet variations between 6.9 and 7.5 were observed. The ideal physiological range is 7.2 to 7.4; hence, in cases when the values were deemed acidic, the pH was immediately corrected using NaOH. The following viability and functional assays' results suggest that the variations in pH were rare enough not to have a detrimental effect on cell viability.

O_2 diffusion could be facilitated by increasing the pO_2 using 95% O_2 plus 5% CO_2 . Nevertheless, to avoid hyperoxia encountered by hepatocytes, 21% O_2 /5% CO_2 and balanced with N_2 was used during the oxygenation of nutrient medium. The other way to augment the O_2 transfer rate is to increase the flow rate of the system. Because the unique membrane-frame design protected the encased cellular spaces from shear stress of flow, the flow rate could be set at a relatively high value, that is, 60 mL/min, without disturb-

ing the cells. The dissolved O_2 in the medium was measured at both inlet and outlet of the bioreactor, and ranged from 156 to 194 mmHg.

To verify whether hepatocytes within a given quadrant chamber of the bioreactor were exposed to hypoxic conditions, a hypoxic probe was used. Regions of hypoxia were assessed on day 2 postisolation (i.e., the first day of culture within the bioreactor). After 4 h of perfusion, hepatocytes located at the bottom region of the bioreactor (close to the inlet) were compared with the cells in the upper region (close to the chamber's outlet). As illustrated by the micrographs of Figure 9, hepatocytes of the positive control (incubated in severe hypoxic conditions of 1% O_2) homogeneously exhibit the characteristic brown stain of cells cultured in environments with pO_2 values less than 10 mmHg (Fig. 9A). In contrast, hepatocytes of the negative control (incubated at normoxic conditions of 21% O_2) only exhibited the characteristic blue staining of hematoxylin (Fig. 9B). For the hepatocytes supported by the bioreactor, the micrographs suggest that the unique flow pattern of the design is successful in ensuring that the majority of the cells in the bottom and top sections of the bioreactor (Fig. 9C and D, respectively) experience pO_2 values greater than 10 mmHg. The fact that a minority fraction of the cells in the top of the bioreactor exhibit the brown coloring displayed by the negative control (Fig. 9A) suggests that the level of O_2 available in the

FIG. 10. Micrographs demonstrating cell morphology of hepatocytes cultured in the bioreactor on days 2 (A), 6 (B), and 12 (C) postisolation. Calibration bars indicate 50 μ m.

medium has indeed been reduced by the time the flow reaches the top regions of the bioreactor.

Evaluation of whether this also translates to bioreactor's success in supporting viable and functioning cells has yet to be determined. Toward this goal, hepatocyte morphology was documented throughout the 15-day culture period. As shown in Figure 10, hepatocytes within the bioreactor maintained morphologies similar to that observed for hepatocytes in the individual cartridges of the dynamic culture conditions (recall Fig. 5G, I), which also show more cell-cell contact compared with sandwich controls. Further, in the bioreactor, distinct cell-cell borders were visible for a minimum of 11 days of the culture. After 15 days of perfusion, cartridges were removed from the bioreactor, and hepatocyte viability was evaluated. The average viability for hepatocytes in the bioreactor at this time point was $84 \pm 18\%$ ($n = 3$), compared with $79 \pm 17\%$ ($n = 3$) for the sandwich controls. This suggests that the bioreactor and its cartridge design are effective in prolonging cell viability.

Recall that hepatocyte support in the bioreactor began on day 2 postisolation. On days 6 and 12, the medium of the chamber and flow circuit was replaced with fresh medium

containing 5 mM NH_4Cl (Sigma), to assess the hepatocytes' ability to metabolize ammonia. The albumin production and urea secretion rates achieved by the hepatocytes of the bioreactor are presented in Figures 11 and 12. In both cases, the results, normalized by the dilution of the culture medium and number of cells, achieved values that were comparable to the range achieved by hepatocytes in individual cartridges. As shown in Figure 11, the rate of albumin production peaked at $127 \pm 10 \mu\text{g}/10^6$ cells/day on day 11, and reached its second highest level on day 15 (i.e., $110 \pm 16 \mu\text{g}/10^6$ cells/day). Regarding the time course of urea secretion during the 2 weeks of bioreactor perfusion (Fig. 12), the results illustrate that a progressive increase in urea synthesis was detected after addition of NH_4Cl to the tissue culture medium on days 6 and 12. The cell function values achieved 24 h after the perfusion of fresh medium (i.e., days 3, 7, and 13) can be used as a comparative baseline for deciphering the metabolic function of the hepatocytes maintained in the bioreactor. The fact that albumin production consistently increased (Fig. 11) while urea synthesis consistently decreased (Fig. 12)—compared to the baseline val-

FIG. 11. Albumin production rate of hepatocytes cultured in the bioreactor, where medium was changed on days 6 and 12 (labeled with $\star$). Days 3, 7, and 13 were used as the baseline control for comparison. The values are the mean $\pm$ SD, $n = 3$. Statistically significant difference: $*p < 0.05$ and $\#p < 0.025$.

FIG. 12. Urea secretion rate of hepatocytes cultured in the bioreactor. Nutrient medium was changed on days 6 and 12 (labeled with $\star$), and the cultures were spiked with NH_4Cl on days 6 and 12. Days 3, 7, and 13 were used as the baseline control for comparison. The values are the mean $\pm$ SD, $n = 3$. Statistically significant difference: $\#p < 0.025$.

FIG. 13. Ethoxyresorufin O-deethylase activity of hepatocytes before placement in the bioreactor (day 2 after isolation) and after (days 9 and 15). The values are the mean $\pm$ SD, $n = 3$. Statistically significant difference: * $p < 0.05$.

ues—illustrate that the hepatocytes maintained in the bioreactor were metabolically functioning throughout the timeline of the culture.

The inducibility of EROD was studied by initiating 3-MC exposure for 3 days beginning on days 6 and 12 of the bioreactor culture. Hepatocytes cultured in 24-well culture plates were used as the comparative control. In each case, resorufin production rates were normalized by the dilution of the incubation buffer and number of cells. Figure 13 illustrates the subsequent EROD activity evaluated on days 9 and 15. On day 3, before adding inducer, the cells in the plates and bioreactor exhibit very low EROD activity. After adding 3-MC, a maximum induction of EROD activity was obtained in both systems on day 9. Figure 13 reveals that the addition of inducer 3-MC had a 48.5% and 56.6% higher effect—on days 9 and 15, respectively—on the enzyme activity in cells of the bioreactor, as compared to the incubated sandwich cultures.

Discussion

A successful bioreactor design must establish a uniform environment that enables the cells it supports to perform key functions. In the current study, our novel bioreactor has been shown to support cells with high viability and superior function, as compared to its functional controls. Its success relies on lessons learned from the demonstrated advantages of radial flow bioreactor designs for liver support^{8,31–33}—that is, of providing a satisfactory distribution of nutrients and O₂ to enable supported hepatocytes to maintain highly differentiated cellular functions (e.g., ammonia removal, urea secretion, albumin synthesis, cytochrome P450 mRNA expression or enzyme activity, glucose consumption, and O₂ consumption rate). The bioreactor has the additional benefit of employing a cellular space (i.e., its cartridge design) that is unique in that it offers the flexibility to easily support any anchorage-dependant cell type. It can also be modified to incorporate modifications proven to further improve functional output (e.g., O₂ transport enhancements^{38–40} and micropatterned cocultures^{27,28}). The cartridge design also enables cells of the bioreactor to interact with the circulating

medium through both of the membrane surfaces of each individual cartridge. Multidirectional mass transport of this type is also achieved by the natural liver, *in vivo*. Finally, the design allows the four chambers configuration of the bioreactor. The use of chambers was partially inspired by a key feature of the natural liver—its lobular configuration. *In vivo* the liver lobule is the functional unit of the liver, and consists of cords of hepatic cells arranged radially around a central vein. It has two blood supplies. The largest amount of blood (about 75%) comes through the portal vein system, which carries deoxygenated blood to the liver from the gastrointestinal tract, including the intestines, the stomach, the spleen, and the pancreas draining into the portal vein and then into the liver. The other 25% is oxygenated blood provided to the liver via the hepatic artery, while deoxygenated blood leaves the liver via the hepatic central vein. This architecture ensures that the complex metabolic functions of the liver—such as regulation, synthesis, and secretion of glucose, proteins, bile, and lipids; storage of glucose, fat-soluble vitamins, and minerals; purification, transformation, and clearance of ammonia, bilirubin, hormones, drugs, and toxins—can be executed.⁴¹ In engineering our novel bioreactor, we focused on functionality rather than form. As such, the lobular configuration of the natural liver served to inspire the multichamber feature of the new bioreactor.

The hypoxia assay used to produce the results of Figure 9 is able to detect O₂ tensions below 10 mmHg. The fact that the majority of the cells in the top and bottom of the bioreactor (Fig. 9C, D, respectively) experienced the characteristic blue staining of the hematoxylin (e.g., the normoxic control) suggests that the supply and distribution of O₂ by the nutrient medium flow to the hepatocytes cultured in our new bioreactor is good. Yet, Figure 9C also illustrates that a fraction of the hepatocytes in the top regions of the bioreactor are exposed to O₂ tensions below 10 mmHg. Unfortunately, the Hypoxyprobe assay is not as effective in helping to discern if this intracellular pressure translates to hypoxic conditions for these cells since the literature indicates that pO₂ less than 10 mmHg are not necessarily hypoxic for hepatocytes maintained *in vitro*. More specifically, the O₂ tension reported for regular *in vitro* tissue cultures ranges between 2.3 and 5.3 mmHg, when the surrounding environment is maintained at 20% O₂.^{42–44} and has been shown to reach an intracellular pO₂ of only 11 mmHg after equilibrating the cell culture medium to 159 mmHg pO₂. Even, the physiological range of O₂ tension in the natural liver is known to span from 5 to 90 mmHg.⁴⁵ Together, these suggest that accurately deducing whether a cell, maintained *in vitro*, experiences hypoxia based on having an intracellular pO₂ below 10 mmHg value is system dependent. Fortunately, the results of Figure 9 together with the liver-specific function results suggest that, overall, the bioreactor is able to maintain a favorable microenvironment for the cells it contains. Yet, in future iterations of the design, making more O₂ available to the cells in the upper regions of the bioreactor's chambers would be one way to ensure that all supported cells experience pO₂ values above 10 mmHg.⁴⁶

The viability and function results of this study further clarified the potential of the bioreactor, since a well-established *in vitro* system—the traditional sandwich culture—was used as the functional comparative. The sandwich culture

configuration, originally developed by Dunn *et al.*,^{35,46} has been widely used as an *in vitro* culture configuration for the long-term maintenance of hepatocytes. Our results demonstrate that the cells maintained in the bioreactor's cartridges significantly outperform the sandwich controls. The cartridge design not only prolonged cell viability (refer to Fig. 6), but also maintained cellular morphologies with better cell-cell contacts (Figs. 5 and 10) and function than this comparative system. The results shown in Figures 7, 8, 11, 12, and 13 demonstrate that hepatocytes cultured in the cartridges and the bioreactor achieved notably higher performance (i.e., urea secretion and albumin production) levels than in the sandwich culture over the 2-week assessment period. Evaluation of the hepatocytes' ability to express cytochrome P450-dependent detoxification pathways also favors the bioreactor, since the inducibility of EROD activity in hepatocytes maintained within this bioreactor is ~50% higher than levels achieved for sandwich cultures.

The fundamental configuration that the bioreactor of this study is built upon is the stacked flat plate. In an earlier work, Uchino *et al.*¹⁷ presented a bioreactor containing hepatocyte monolayers where the albumin synthesis rate peaked at 29 $\mu\text{g}/\text{day}/10^6$ cells on day 5. de Bartolo *et al.*²² reached a slightly higher value of 34 $\mu\text{g}/\text{day}/10^6$ cells, achieved by incorporating internal membrane oxygenation into their flat-membrane bioreactor. In the current study, it is possible to surmise from Figure 11 that the average albumin synthesis rate peaked at 126 $\mu\text{g}/10^6$ cells/day, which was fourfold higher than those results. This indicates that the novel bioreactor introduced in this study is able to successfully maintain a microenvironment to support hepatocytes functional performance.

One of the major factors contributing to the functional performance of the new bioreactor could be the efficient nutrient exchange supplied by the dynamic medium flow established by the system. Since the nutrient medium serves as the blood equivalent in this study, its circulation of course delivers O_2 and vital nutrients to the bioreactor's cellular space; and carries away synthetic products and metabolic wastes (e.g., urea). The fact that our bioreactor relies on a unique cartridge design serves to protect the cells from shear stresses while simultaneously enabling them to effectively exchange with the circulating medium (recall Fig. 3). Comparing the flow rates utilized by other existing stacked flat-plate type of bioreactors, the cartridge design allows significantly higher flow rates to be used safely for hepatocytes support. For instance, Bartolo *et al.* used a flow rate of 9 mL/min for their full-scale flat-membrane bioreactor,²² whereas the rate used by Park *et al.* for their radial flow bioreactor that incorporated microchannel flat plates was 15 mL/min.²⁹ Interestingly, since the flat-plate bioreactor study of Taguchi and Matsushita also employed the shear stress benefits of the collagen sandwich culture configuration, they were able to safely employ a higher flow rate of 30 mL/min.⁴⁷ This rate is consistent with that applied in the current study for the cartridge dynamic flow studies. It is important to note that our current cartridge-based bioreactor design allows an overall medium flow rate of up to 60 mL/min to be maintained without adversely affecting the hepatocytes contained in the system.

Another benefit of the bioreactor's cartridge design is that it reduces the nutrient transport barrier (between the nutrient

source and the cells) to less than 80 μm , a dimension that includes the 30 μm thickness of the porous membrane. Another major impact factor could be the improved cell-cell contacts achieved by the hepatocytes maintained in the cartridges of the bioreactor (recall the morphological results of Figs. 5 and 11). The relevance here of course is that it is well established that cell-cell and cell-matrix contacts are essential for hepatocyte differentiation and metabolic functional performance.³⁸

The prototype of the new bioreactor of the current study was devised to demonstrate the efficacy of the unique design in supporting cultured cells. It should be mentioned that the seeding cell density used for the bioreactor in this study is 6 mL/million cells, which is relatively higher than the 1.4 mL/million cell density used by Park *et al.*²⁹ Loading each quadrant of the system at its full performance by further expanding the cellular space is one way to reduce that priming volume. This can be done by altering the rack shown in Figure 1 to enable more cartridges to be inserted. More specifically, the current design allows $\frac{1}{2}$ cartridges to be inserted between the full cartridges used in the current study, which is the focus of a future study. Another way to increase the number of cells that a given quadrant of the bioreactor can support is to take advantage of the adaptability of the cartridge design. Instead of supporting the monolayer design presented in the current study, a given cartridge can be adjusted to load multilayers of cell, cells entrapped within the tissue scaffold, or combinations consistent with the scale up and functional needs of a given application.

As previously mentioned, the flat-plate configuration was used in the current study for convenience and to maintain consistency with the collagen sandwich functional control. Yet, it is well known that internal membrane oxygenation,^{14,21,48} microfabrication, and coculturing hepatocytes with non-parenchymal cells^{13,29} have all been successfully used to improve hepatocyte performance. The flexibility of the new bioreactor is that its cartridge design allows the user to easily modify the cellular space to incorporate these techniques to further improve the functional performance reported in the current study.

Summary

In conclusion, this study presents a novel bioreactor in which cells are maintained in vertically suspended cartridges assembled within a unique rack design. To demonstrate the efficacy of the design, the prototype presented in the current work utilized cartridges loaded with hepatocytes sandwiched between collagen type I, and then secured between two PTFE membranes. The results illustrate that our novel bioreactor (patent pending) is a successful system for maintaining relatively large numbers (i.e., up to 128 million for the current laboratory prototype) of live and functioning cells. Hepatocyte performance within the cartridges and within the bioreactor was presented. The results indicate that our novel bioreactor was very effective in supporting liver hepatocytes, relative to the sandwich culture functional controls, especially in the case of cytochrome P450 activity. The strength of the bioreactor's design is that its cellular space can easily be adapted to incorporate a range of cell types and configurations, and its design lends itself easily to analysis and monitoring. As such, the bioreactor can be

applied to the support of a range of tissue and organ systems for biomedical applications.

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Disclosure Statement

No competing financial interests exist.

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