Published in final edited form as:

Acta Biomater. 2018 June ; 73: 217–227. doi:10.1016/j.actbio.2018.02.009.

Nanofibrous PLGA Electrospun Scaffolds Modified with Type I Collagen Influence Hepatocyte Function and Support Viability *In Vitro*

Jessica H. Brown^{1,2}, Prativa Das³, Michael D. DiVito^{1,2}, David Ivancic^{1,2}, Lay Poh Tan^{3,a}, and Jason A. Wertheim^{1,2,4,5,6,7,a}

¹Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611

²Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611

³Interdisciplinary Graduate School, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

⁴Chemistry of Life Processes Institute, Northwestern University, Evanston, Illinois, 60208

⁵Department of Surgery, Jesse Brown VA Medical Center, Chicago, Illinois, 60612

⁶Simpson Querrey Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, Illinois, 60611

⁷Department of Biomedical Engineering, Northwestern University, Evanston, Illinois, 60208

Abstract

A major challenge of maintaining primary hepatocytes *in vitro* is progressive loss of hepatocyte-specific functions, such as protein synthesis and cytochrome P450 (CYP450) catalytic activity. We developed a three-dimensional (3D) nanofibrous scaffold made from poly(L-lactide-co-glycolide) (PLGA) polymer using a newly optimized wet electrospinning technique that resulted in a highly porous structure that accommodated inclusion of primary human hepatocytes. Extracellular matrix (ECM) proteins (type I collagen or fibronectin) at varying concentrations were chemically linked to electrospun PLGA using amine coupling to develop an *in vitro* culture system containing the minimal essential ECM components of the liver micro-environment that preserve hepatocyte function *in vitro*. Cell-laden nanofiber scaffolds were tested *in vitro* to maintain hepatocyte function over a two-week period. Incorporation of type I collagen onto PLGA scaffolds (PLGA- C_{high} : 100 μ g/mL) led to 10-fold greater albumin secretion, 4-fold higher urea synthesis, and

^aCorresponding authors: Lay Poh Tan, Ph.D., Interdisciplinary Graduate School, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore, School of Materials Science & Engineering, College of Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore, lptan@ntu.edu.sg. Jason A. Wertheim, M.D., Ph.D., Department of Surgery, Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611, jason.wertheim@northwestern.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

elevated transcription of hepatocyte-specific CYP450 genes (CYP3A4, 3.5-fold increase and CYP2C9, 3-fold increase) in primary human hepatocytes compared to the same cells grown within unmodified PLGA scaffolds over two weeks. These indices, measured using collagen-bonded scaffolds, were also higher than scaffolds coupled to fibronectin or an ECM control sandwich culture composed of type I collagen and Matrigel. Induction of CYP2C9 activity was also higher in these same type I collagen PLGA scaffolds compared to other ECM-modified or unmodified PLGA constructs and was equivalent to the ECM control at 7 days. Together, we demonstrate a minimalist ECM-based 3D synthetic scaffold that accommodates primary human hepatocyte inclusion into the matrix, maintains long-term *in vitro* survival and stimulates function, which can be attributed to coupling of type I collagen.

Graphical Abstract

Keywords

electrospun nanofibers; tissue engineering; primary hepatocyte culture; extracellular matrix (ECM) modification; 3D scaffold

1. Introduction

While hepatocytes comprise ~80% of the liver parenchyma and drive its major functions such as protein synthesis, glycogen storage, metabolic regulation and detoxification, the acellular fraction of the liver micro-environment, its extracellular matrix (ECM), plays an essential role in regulating the *in vivo* maturation and function of primary hepatocytes. The liver ECM is comprised of macromolecules such as collagens and fibronectins that, along with heterotypic cell-cell binding and soluble or matrix-associated biochemical cues, maintain the phenotype and physiological potency of hepatocytes, characteristics that are oftentimes lost when hepatocytes are removed from their natural *in vivo* liver micro-environment.[1] As a result, hepatocytes undergo dedifferentiation when cultured *ex vivo* in which liver-specific activities progressively deteriorate.[1, 2] This loss of function poses a significant obstacle to the use of primary hepatocytes for *in vivo* pharmacology testing or development of cell-based therapies such as liver assist devices or liver tissue engineering applications.[3]

One of the earliest examples of *in vitro* ECM-based cell culture systems for hepatocytes is the ‘gold standard’ ECM sandwich culture in which hepatocytes are placed between layers of ECM components (such as collagen or Matrigel). This configuration improves hepatocyte function compared to growth of cells in an uncovered monolayer. However, hepatocyte function still diminishes over time, perhaps due to a lack of a 3D layering structure. The

non-uniform composition of Matrigel and its production by transformed, cancerous cell lines limits its use in the culture of potentially therapeutic cells.[4–7] This study is motivated by the need to develop scaffolds with essential ECM components that maintain primary human hepatocytes *in vitro*, but are developed from minimal and defined biological components using molecular synthesis. An additional important scaffold characteristic is the need for a highly porous 3D structure that supports inclusion of primary human hepatocytes that together with ECM components recreate a minimalistic micro-environment for hepatocyte culture.

Electrospinning is a well-known technique in which nanofibers are produced that resemble the size and structure of native ECM.[8, 9] Advantages of this fabrication method include: 1) versatility in its application, in that almost all soluble polymers can be processed into nanofibers, 2) ease of use, due to the simplicity of the process, and 3) cost-effectiveness, which only requires readily available materials and a basic set-up.[10] Electrospinning is a synthetic technique to produce matrices for the bioengineering of tissue substitutes and has been used for various medical applications such as cardiac[11], neural[12], bone[13], and skin[14]. One critical drawback of conventional electrospinning is the high density and tight packing of adjacent fibers that collect on a metal collector, leading to high fiber density that impedes cell infiltration into the scaffold.[15] Conversely, the use of a wet electrospinning technique, also known as a liquid-collecting technique, allows for the production of nanofibers that are less dense and more porous.[16] During wet electrospinning the nanofibers are collected in a liquid bath that prevents dense packing as nanofibers accumulate.

The wet electrospinning technique results in a highly porous bundle of nanofibers that resemble a 3D spongiform scaffold.[17] In this study we utilized wet electrospinning because the morphology of scaffolds developed using this technique is reminiscent of a liver matrix with high porosity. We further modified electrospun PLGA with an ECM component, type I collagen or fibronectin, to provide adhesive support for primary human hepatocytes and to potentially enable biochemical stimulation via intracellular signaling originating from integrin-mediated binding. Type I collagen and fibronectin were chosen as the ECM components to use in this system because type I collagen is the major component of the liver ECM and fibronectin is a critical ligand for integrin-mediated cell attachment.[18] The objective is to evaluate these minimalistic, biosynthetic hybrid materials (PLGA with ECM constituents) as new 3D nanofibrous scaffolds to preserve, and possibly enhance, primary hepatocyte function *in vitro* that may later be used as a platform to screen new medications or preserve hepatocyte function for use in human cell therapy. In comparison to unmodified PLGA scaffolds and those grafted with fibronectin, we show that type I collagen leads to increased hepatic gene expression and simulation of functional parameters characteristic of primary hepatocytes.

2. Materials and methods

2.1. Materials

PLGA (80:20 L-lactide:glycolide) was purchased from Evonik Industries (Essen, Germany). Chloroform (CHCl_3) and dimethylformamide (DMF) were purchased from Fisher Scientific

(Waltham, MA, USA). *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinamide (NHS), 2-ethanesulfonic acid (MES Buffer), NaOH, and Pluronic® F-108 were purchased from Sigma Aldrich (St. Louis, MO, USA). Rat tail type I collagen and fibronectin, plasma, were purchased from Life Technologies (Carlsbad, California, USA).

2.2. Synthesis of PLGA-ECM scaffolds

2.2.1. Preparation of PLGA nanofibers by wet electrospinning—PLGA was dissolved at 8% in a solvent mixture of 7:3 (v:v) of CHCl₃ and DMF. The PLGA solution was electrospun with the electrospinning apparatus NANON-01A (MECC, Fukuoka, Japan) at a flow rate of 0.5 mL/h and a voltage of 18 kV through a 13.4 gauge blunt needle. The fibers were collected using a mold immersed in a bath containing 7:3 (v:v) IPA and deionized (DI) water, with 0.05 wt% Pluronic® F-108 used as a surfactant. The fibers were washed thoroughly with DI water for 30 min to remove the surfactant and were then freeze-dried to retain the scaffold dimensions.

2.2.2. Preparation of PLGA nanofibers by conventional electrospinning—A conventional 2D electrospun fibrous mat was prepared by dissolving 8% PLGA in 3:1 CHCl₃ and DMF solution. Electrospinning was performed at a flow rate of 0.5 ml/h with an applied voltage of 20 kV. Fibers were collected on an aluminum foil that was removed for subsequent analysis of scaffold properties and cell culture.

2.2.3. Surface modification of PLGA scaffolds—Surface modification of the scaffolds was carried out using EDC/NHS. Briefly, the fiber surface was hydrolyzed by immersing freeze-dried fibers in 0.01 N NaOH solution for 30 min at 25 °C. After surface hydrolysis, scaffolds were washed with DI water three times to remove additional NaOH. A solution containing EDC (40 mM) and NHS (80 mM) in 50 mM MES buffer (pH = 6) was added to the hydrolyzed scaffolds and kept at 4 °C for 4 h. The activated fibers were washed with DI water three times to remove the unreacted solution. The fibers were then treated with two different concentrations of either collagen solutions (100 µg/mL and 50 µg/mL) or fibronectin solutions (50 µg/mL and 25 µg/mL) prepared in phosphate buffered saline (PBS), followed by incubation at 4 °C for 12 h. Relative amounts of type I collagen and fibronectin were used to mimic the level of each protein type within the acellular fraction of normal liver, which was found to be ~60 wt % collagens and ~30 wt % of non-collagenous proteins. [19] After incubation, excess protein solution was removed, and scaffolds were frozen at –80 °C for 30 min followed by lyophilization.

2.3. Characterization of PLGA-ECM scaffolds

2.3.1. Fourier transform infrared spectroscopy (FTIR)—The surface chemistry of the fibers was investigated with a FTIR-Frontier spectrometer (Perkin Elmer Inc., Shelton, USA). PLGA fibers with different surface modifications were encapsulated in the KBr pellet and scanned within a range of 200–4000 cm⁻¹.

2.3.2. Scanning electron microscopy (SEM) of scaffolds before cell seeding—Scanning electron microscopy (SEM, JEOL JSM-6340F) was used to examine the

morphology of the fibers. Average fiber diameter and pore size of the PLGA scaffolds were determined by collecting data from 100 different locations within SEM images, and ImageJ was used for subsequent measurements and analysis.

2.3.3. Biconinic acid assay (BCA Assay)—The efficiency of the protein immobilization was determined using the BCA assay. The scaffolds were dissolved in CHCl_3 and the proteins were extracted using PBS. The solution mixture was centrifuged and the supernatant was used for BCA analysis.

2.4. Cell Culture

2.4.1. Cells and culture conditions—Primary human hepatocytes expanded in triple knockout $\text{Fah}^{-/-}/\text{Rag2}^{-/-}/\text{Il2rg}^{-/-}$ mice were purchased fresh from Yecuris Corporation (Portland, OR) and shipped fresh.[20] Hepatocytes were cultured with Hepatocyte Culture Medium (HCM, Lonza Walkersville Inc., Walkersville, MD).

2.4.2. Cell seeding—Each scaffold (diameter = 6 mm, thickness = 3 mm) was placed into a well of a 96-well plate. Hepatocytes were seeded by pipetting a 40 μL cell suspension (10,000 cells/ μL) onto each scaffold. The culture plate was placed on a rocker plate inside an incubator for 20 min to allow attachment of the cells to the scaffold. An additional volume of culture medium (160 μL) was then added to each scaffold to bring the total working volume to 200 μL . The hepatocyte-seeded scaffolds were kept in an incubator at 37 °C with a humidified atmosphere of 5% CO_2 . The medium was changed daily. A type I collagen-Matrigel sandwich culture of hepatocytes was used as a control system. A type I collagen-coated culture plate was seeded with 100 μL of hepatocytes (1,000 cells/ μL). The next day, 0.25 mg/mL growth factor-reduced Matrigel Matrix (Corning) was placed over the cells. The sandwich-seeded hepatocytes were kept in an incubator at 37 °C with a humidified atmosphere of 5% CO_2 . The culture medium for the sandwich culture control group was changed daily.

2.4.3. Resazurin Assay—The number of cells in each culture environment was determined using the resazurin reduction assay, as previously described by us.[21] Briefly, a 44 μM stock solution of resazurin sodium salt (Sigma, USA) diluted in PBS was prepared and then diluted 1:10 in HCM to prepare a resazurin working solution on the same day as each time point (day 2, 7, 11, or 14). After aspirating the media, the resazurin working solution (100 μL) was added to each scaffold and incubated at 37 °C with a humidified atmosphere of 5% CO_2 for 1 h. The partially reduced resazurin solutions were pipetted into black, opaque 96-well plates and the fluorescence was measured using a spectrophotometer (Cytation 3, BioTek Instruments, Inc.; excitation: 540 nm; emission: 590 nm). Completely reduced resorufin solutions were used as positive controls. A standard curve was generated using fresh primary hepatocytes plated at serially-diluted densities. After 1 h incubation with resazurin working solutions, a linear regression equation was generated that related the cells:volume ratio (10^6 cells/mL) to the measured fluorescence intensity (FI). This linear regression was used to calculate the number of cells present in each scaffold after performing the resazurin assay at each time point.

2.4.4. Hematoxylin and eosin staining and SEM analysis of cells on scaffolds

—At day 14, the PLGA unmodified scaffold and the PLGA-ECM scaffolds were fixed in 10% formalin, embedded and processed in paraffin, cut in 5 μm sections, and stained with hematoxylin and eosin (H&E). For SEM analysis, samples were collected at day 14, washed with PBS, and fixed with 2.5% glutaraldehyde in PBS overnight at 4 °C. The samples were washed with PBS, followed by fixation in 1% osmium tetroxide for 1 h. The samples were then subjected to progressive dehydration with ethanol (25%, 50%, 75%, 95%) for 15 min each and 100% EtOH for 3 times, 15 min each. The samples were dried using a Samdri®-795 CO₂ critical point dryer (Tousimis, Rockville, MD, USA), followed by osmium coating using an OPC-60A Osmium Plasma Coater. The morphology of primary hepatocytes cultured on PLGA-ECM and PLGA unmodified scaffolds was examined by SEM using a Hitachi S-3400N-II (Hitachi High Technologies America, Inc.).

2.4.5. Albumin secretion assay—Albumin secretion was measured using a commercially available human ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) to assess hepatocyte synthetic function. The medium was collected at days 2, 7, 11, and 14 and stored at –20 °C until used.

2.4.6. Urea secretion assay—Urea secretion was measured using a commercially available urea assay kit (Abcam, Cambridge, MA) to evaluate hepatocyte metabolic function. The medium was collected at days 2, 7, 11, and 14 and stored at –20 °C until used.

2.4.7. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)—The gene expression of the hepatocytes in sandwich and 3D culture was measured using qRT-PCR. After 14 days of culture, total RNA was isolated using TRIzolTM Reagent (Thermo Fisher Scientific Life Sciences, USA). DNase (DNA-freeTM Kit, Thermo Fisher Scientific Life Sciences, USA) was then used to treat the isolated RNA. Reverse transcription was performed using iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, USA). qRT-PCR was carried out using SYBR Green PCR (Bio-Rad Laboratories, USA) on a QuantStudioTM 7 System (Thermo Fisher Scientific Life Sciences, USA). Gene transcription was evaluated using the CT method, and was normalized to the sandwich culture gene expression data and to fresh (day 0) primary hepatocyte gene expression data using cyclophilin as a housekeeping gene.

2.4.8. Measurement of cytochrome P450 enzyme activities for CYP2C9 and CYP3A4—Primary hepatocytes seeded in sandwich and 3D culture were treated with or without an induction agent (25 μM rifampicin) 48 h prior to the P450 activity testing. Promega P450 bioluminescence enzymatic assays were used to measure CYP metabolites in solution because they are more sensitive than, and correlate with, LC-MS, a technique used historically before the development of the newer bioluminescent assays.[22, 23] Cells were subsequently incubated with 100 μM Luciferin-H (CYP2C9) for 4 h and 3 μM Luciferin-IPA (CYP3A4) for 1 h at 37 °C (Promega, Madison, WI). After incubation, the luciferin medium (25 μL) was added to a 96-well white opaque plate with the luciferin detection reagent (25 μL). The luminescence signal was measured on a Synergy2 microplate reader (BioTek,

USA). After measuring the activity, the number of cells was determined using the resazurin assay.

2.4.9. Statistical analysis—All samples were run as biological triplicates and expressed as mean $\pm$ standard deviation. For all two-way comparisons, a *t*-test analysis was used and *p* < 0.05 was considered statistically significant.

3. Results

3.1. Preparation and Characterization of PLGA Scaffolds

3D nanofibrous scaffolds were prepared by the wet electrospinning technique to investigate pore size, and compare the resulting morphology to scaffolds constructed by conventional electrospinning. Fiber diameter and porosity of PLGA scaffolds prepared by each method were measured from digital images acquired by SEM. We found that conventional electrospinning led to scaffolds with a tighter overall morphology and a high degree of compact layering of fibers (Figure 1a). Pores within these scaffolds ranged from 5.2 μm to 19.7 μm , and the average pore size was $10.9 \pm 3.6 \mu\text{m}$ (Figure 1c), which is smaller in size than primary hepatocytes (range 15–30 μm). [24] PLGA scaffolds prepared by wet electrospinning technique were made up of comparatively larger pores in all directions (range of pore size, 9.7 to 44.5 μm , Figure 1b) and the average pore size was $27.5 \pm 7.5 \mu\text{m}$ (*p* < 0.001 compared to the traditional technique, Figure 1d), which is large enough to effectively accommodate incorporation of primary human hepatocytes into the scaffold, and approximates the average pore size of acellular human liver tissue, $\sim 30 \mu\text{m}$. [25] Though pore size was significantly increased in scaffolds produced using the wet electrospinning technique, this method did not significantly alter the average diameter of individual PLGA fibers that were $870 \pm 300 \text{ nm}$ (range 500 to 1,400 nm, Figure S1, Supplementary Data) compared to an average diameter of $1,000 \pm 250 \text{ nm}$ (*p* = 0.07) using conventional electrospinning. Pore size tracked with infiltration and growth of primary hepatocytes within the nanofibrous scaffolds produced by each method. Specifically, 2- to 4-fold more cells (*p* < 0.05, *p* < 0.01) remained by day 2 within wet electrospun scaffolds, whose pore size closely matches the size of primary hepatocytes, compared to scaffolds with smaller pores made by the conventional electrospinning method (Figure S2a, Supplementary Data). Moreover, SEM imaging depicts few cells in scaffolds produced by the traditional method (Figure S2b, Supplementary Data), and cross-sectional imaging of H&E-stained scaffolds demonstrates that cells were largely unable to penetrate the matrix and were only located on the surface (Figure S2c, Supplementary Data). Taken together, conventional electrospinning produced layers of fibers that resulted in scaffolds with high fiber density and smaller pore size, which severely limited cell infiltration. Moving forward to develop scaffolds with the minimal essential ECM components for hepatocyte growth, we employed the wet electrospinning technique for the remainder of the study because of the higher degree of cell growth within these scaffolds that was afforded by its high porosity (>90%) and pores that are equivalent in size to human hepatocytes.

3.2. Immobilization of ECM Proteins on Electrospun PLGA Scaffolds

ECM-associated proteins (type I collagen or fibronectin) were subsequently coupled to PLGA fibers after electrospinning by direct chemical modification with NHS/EDC using two different concentrations of each ECM protein (PLGA-C_{high}: collagen at 100 µg/mL or PLGA-C_{low}: collagen at 50 µg/mL; PLGA-F_{high}: fibronectin at 50 µg/mL or PLGA-F_{low}: fibronectin at 25 µg/mL) as previously described.[26] SEM imaging was used to ensure that the porous structure of the scaffolds and fiber morphology were not altered or deformed by the chemisorption process. All ECM-modified PLGA scaffolds retained large pores in both horizontal and vertical directions (Figure S3a–d, Supplementary Data). FTIR analysis confirmed the presence of protein, fibronectin or collagen, on each scaffold. Peaks at 1637 cm⁻¹, 1562 cm⁻¹, and 1240 cm⁻¹ in the FTIR spectra specifically correspond to N-H bending from primary amines in the PLGA-C_{high} and PLGA-F_{high} scaffolds (Figure 2a), which indicate the presence of each added protein conjugated to PLGA fibers. These peaks are absent from the FTIR spectra of the unmodified PLGA scaffold. The concentration of immobilized protein on PLGA scaffolds was measured by the BCA assay and confirms a range of protein surface concentrations for each protein enabling us to evaluate both the type and amount of ECM components on each scaffold. The amount of protein in PLGA-C_{high} and PLGA-C_{low} was 4.4 ± 0.3 µg/mg PLGA and 2.4 ± 0.5 µg/mg PLGA, respectively (Figure 2b). The level of protein in PLGA-F_{high} and PLGA-F_{low} was 1.4 ± 0.5 µg/mg PLGA and 0.9 ± 0.4 µg/mg PLGA, respectively (Figure 2b). All chemisorbed PLGA scaffolds and unmodified control PLGA scaffolds made by wet electrospinning had equivalent pore size, fiber diameter and porosity.

3.3. Primary Hepatocyte Culture within Wet Electrospun PLGA-ECM Scaffolds

A 14-day time course experiment was subsequently carried out to investigate the capacity of the ECM-modified wet electrospun PLGA scaffolds to maintain viability and support function of primary human hepatocytes. Presence of viable cells within 3D scaffolds was measured by the degree of resazurin reduction in mitochondria on days 2, 7, 11, and 14 (Figure 3), which is a non-invasive method that we optimized to enumerate the amount of living cells within 3D scaffolds [21]. A color change in the media resulting from reduction of resazurin is quantified non-invasively via a spectrophotometer without disturbing growing cells. Primary, terminally differentiated hepatocytes rarely proliferate and our serial determination of cell number during the study was aimed to compare cell viability and investigate non-inferiority of the ECM-linked PLGA against the control unmodified PLGA scaffold and the type I collagen-Matrigel sandwich. First, seeding efficiency was determined by the number of cells present at day 2 as a percentage of cells added to each scaffold and was highest for hepatocytes seeded on PLGA-C_{high}, PLGA-C_{low}, and PLGA-F_{low} scaffolds ($3.11 \pm 0.62 \times 10^4$, $2.75 \pm 0.64 \times 10^4$, and $2.43 \pm 0.88 \times 10^4$ cells per scaffold, respectively) compared to unmodified PLGA and PLGA-F_{high} scaffolds ($1.64 \pm 0.04 \times 10^4$ and $1.68 \pm 0.38 \times 10^4$ cells per scaffold) (Figure 3). Second, cell enumeration revealed that all scaffolds have an initial drop off between day 2 to day 7 (PLGA-C_{high}= 60% cells remaining, PLGA-C_{low} = 58% cells remaining, PLGA-F_{high}: 83% cells remaining, PLGA-F_{low} = 63% cells remaining, and PLGA = 77% cells remaining). Viability was then maintained during extended culture for days 7 to 14 (PLGA-C_{low} p = 0.24; PLGA-F_{high} p = 0.15) or days 11 to 14 (PLGA-C_{high} p = 0.51; PLGA-F_{low} p = 0.21; PLGA p = 0.27). The

amount of cells within the type I collagen-Matrigel sandwich culture was stable throughout the time course study. Decrease in cell number early in the culture period, such as on day 2 in 3D scaffolds, may be attributed to non-adherent cells that fall from scaffolds onto the surrounding non-adherent wells, and are removed during daily media changes. This is in comparison to the ECM sandwich culture that spans the type I collagen-coated tissue culture well and is bound by the Matrigel layer covering all hepatocytes. Nonetheless, the number of cells in the type I collagen-Matrigel sandwich culture was largely comparable to the number of cells maintained in PLGA-based scaffolds, and demonstrated equivalency at the termination of the 14-day study.

SEM imaging was used to delineate the morphology of primary hepatocytes within the wet electrospun nanofibrous PLGA scaffolds at day 14. Aggregates of hepatocytes and clusters of cells with close cell-cell contacts are evident in all PLGA-ECM scaffolds, but are absent in unmodified PLGA scaffolds (Figure 4). Some hepatocytes exhibit a rounded shape, which indicates a differentiated phenotype.[27, 28] Hepatocytes grown within ECM-modified scaffolds, particularly those linked to collagen, exhibited spreading along and across the nanofibers of PLGA-ECM scaffolds and appear to wrap around nanofibers of the PLGA- C_{high} scaffold (Figure 4b). H&E staining of scaffolds in cross-section further depict cell spreading along the PLGA- C_{high} nanofibers (Figure 4b, **inset**). Notably, SEM imaging confirms greater abundance of cells within PLGA-ECM scaffolds compared to unmodified scaffolds, indicating that grafting of ECM constituents onto PLGA scaffolds support cell infiltration into the matrix and reinforces the non-invasive cell enumeration data obtained by the resazurin assay. Hepatocytes also establish cell-cell contacts (Figure S4, Supplementary Data) when grown in a monolayer within ECM sandwich culture, even though they lack a 3D structure found in electrospun scaffolds. In comparison, H&E stained cross-sections of cell-laden scaffolds reveals cell penetration into the 3D matrix (Figure 4). Cell-cell contacts were more abundant in PLGA-ECM scaffolds compared to unmodified PLGA scaffolds. PLGA- C_{high} scaffolds had the highest degree of cell-cell contacts among the 3D PLGA-ECM scaffolds (Figure 4b).

3.4. Expression of Liver-Specific Genes

Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) enumerated the transcription level of liver-specific genes in primary hepatocytes within each culture configuration at day 14. Gene transcription in each environment was normalized against transcriptional levels in the ECM sandwich assay (Figure 5a) or against fresh primary hepatocytes (Figure 5b). As a measurement of hepatocyte synthetic function, expression of albumin mRNA was the highest in hepatocytes cultured within PLGA- C_{high} scaffolds normalized against both controls (Figure 5a,b). Specifically, expression of albumin mRNA was 3.5-fold higher within PLGA- C_{high} scaffolds compared to unmodified PLGA scaffolds ($p < 0.001$), demonstrating the importance of type I collagen to enhance hepatocyte synthetic function at the transcriptional level. Furthermore, PLGA- C_{high} scaffolds showed a 4-fold increase compared to fresh primary hepatocytes ($p < 0.01$, Figure 5b), and a 1.5-fold increase in expression compared to the ECM sandwich culture control ($p < 0.01$, Figure 5a). Hepatocytes cultured on PLGA- F_{low} scaffolds also exhibited a ~1.5-fold increase in albumin expression when compared to the sandwich culture control group ($p < 0.01$).

Expression of P450 isoforms was investigated to examine the influence of the ECM-modified scaffolds on the transcriptional levels of catalytic enzymes. The P450 enzyme CYP3A4 metabolizes ~50% of drugs that are cleared by the liver [29], and thus transcription of the CYP3A4 gene in hepatocytes was examined in each 3D wet electrospun culture system. Both PLGA-C_{high} and PLGA-C_{low} scaffolds led to the expression of CYP3A4 in a manner that was dependent upon the concentration of collagen on the scaffold. In comparison, expression of CYP3A4 was undetectable in PLGA-F_{high}, PLGA-F_{low}, and unmodified PLGA scaffolds (Figure 5a,b). Specifically, hepatocytes cultured on PLGA-C_{high} scaffolds revealed a 4-fold ($p < 0.01$, Figure 5a) increase in expression of CYP3A4 compared to the ECM control sandwich culture. Furthermore, a corresponding increase in CYP3A4 expression in hepatocytes cultured on PLGA-C_{high} (1.6-fold) and PLGA-C_{low} (1.5-fold) was also identified in comparison to fresh (day 0) primary hepatocytes as an additional control (Figure 5b).

The expression of CYP2C9, which catalyzes warfarin, was likewise highest in hepatocytes grown within PLGA-C_{high} scaffolds. Specifically, transcription of CYP2C9 was significantly higher, by 3-fold ($p = 0.019$), compared to hepatocytes grown in sandwich culture and was undetectable within unmodified PLGA scaffolds (Figure 5a,b). Taken together, data on both the expression of synthetic and catalytic genes strongly suggest that type I collagen is a component of the micro-environment that stimulate transcription of liver-specific genes in 3D PLGA electrospun scaffolds.

3.5. Albumin and Urea Secretion from Primary Hepatocytes within Electrospun PLGA-ECM Scaffolds

Albumin and urea secretion, normalized to cell number on the day of sampling, were measured to assess synthetic function of hepatocytes over 14 days. A sample measurement at 48 hours (day 2) was added to establish the baseline production of albumin and urea in fresh human hepatocytes in each culture environment and to provide adequate time for protein synthesis and excretion. The level of albumin released from hepatocytes within PLGA-C_{high} scaffolds increased from a baseline at day 2 ($24.36 \pm 4.35 \mu\text{g}/10^6 \text{ cells/day}$) to day 14 ($34.56 \pm 2.54 \mu\text{g}/10^6 \text{ cells/day}$, $p = 0.044$, Figure 6a). Conversely, the level of albumin secreted from hepatocytes cultured within unmodified scaffolds without ECM components decreased from day 2 ($7.06 \pm 2.34 \mu\text{g}/10^6 \text{ cells/day}$) to day 14 ($3.27 \pm 0.83 \mu\text{g}/10^6 \text{ cells/day}$, $p = 0.02$). Importantly, albumin secretion was significantly higher for hepatocytes grown within PLGA-C_{high} scaffolds compared to unmodified scaffolds at all time points (day 2, 7, 11, and 14: $p < 0.001$). Furthermore, on day 14 albumin secretion from hepatocytes cultured within PLGA-C_{high} scaffolds was significantly higher ($34.56 \pm 2.54 \mu\text{g}/10^6 \text{ cells/day}$) compared to the ECM sandwich culture control ($11.59 \pm 3.07 \mu\text{g}/10^6 \text{ cells/day}$, $p < 0.01$).

The rate of urea secretion increased over time in PLGA-C_{high} scaffolds and was likewise higher at day 14 ($49.68 \pm 1.63 \mu\text{g}/10^6 \text{ cells/day}$) compared to unmodified PLGA scaffolds that decreased over time and led to the lowest urea production ($12.32 \pm 5.8 \mu\text{g}/10^6 \text{ cells/day}$ at day 14, $p < 0.001$, Figure 6b). Additionally, urea synthesis on day 14 was also higher in PLGA-C_{high} scaffolds compared to the sandwich control culture ($20.49 \pm 2.31 \mu\text{g}/10^6 \text{ cells/day}$).

day, $p < 0.01$, Figure 6b). These results demonstrate improvement in liver-specific synthetic function of primary hepatocytes when cultured on 3D PLGA ECM-modified nanofibers compared to the control sandwich culture. Additionally, the higher level of protein expression in PLGA-C_{high} scaffolds reinforces the significant trends in gene transcription noted earlier and demonstrating again that type I collagen is a critical factor leading to increased synthesis of liver-specific proteins.

3.6. Cytochrome P450 Activity

The cytochrome P450 activity of CYP3A4 and CYP2C9 were gauged to further assess the catalytic function of primary hepatocytes within the 3D wet electrospun scaffolds at days 7 and 14 (Figure 7). P450 activity was measured with and without induction by rifampicin, a drug commonly used to induce the catalytic activity of CYP3A4 and CYP2C9, which is a specific characteristic of primary human hepatocytes. P450 activity of fresh (day 0) primary human hepatocytes was also measured as a control to represent the standard function of human hepatocytes *in vivo*. A known trait of primary hepatocytes is that P450 catalytic activity decreases over time when cultured *in vitro*. This is shown by the decrease in activity between human hepatocytes at day 7 to 14 grown in the sandwich control culture that recover, to a degree, after pharmacologic induction.[30, 31] CYP2C9 and CYP3A4 activity in human hepatocytes likewise decreased from day 7 to day 14 within all ECM-modified PLGA scaffolds, including those in the control ECM sandwich culture (Figure 7). However, PLGA-C_{high} scaffolds show significantly higher CYP2C9 activity (day 7 induced: $p < 0.001$; day 14: PLGA undetectable) and CYP3A4 activity (day 7 induced: $p < 0.01$; day 14 induced: $p < 0.01$) compared to unmodified PLGA scaffolds, demonstrating the specific effect of type I collagen to potentiate hepatocyte activity at both time periods. In comparison to the ECM sandwich culture, uninduced CYP2C9 activity of hepatocytes in the PLGA-C_{high} scaffolds and the sandwich culture were equivalent ($p = 0.96$) on day 7. Pharmacologic induction of CYP2C9 activity with rifampicin in primary hepatocytes grown within PLGA-C_{high} scaffolds on day 7 (Figure 7a) and 14 (Figure 7c) were also equivalent to the induced CYP2C9 activity observed in ECM sandwich culture (day 7: $p = 0.91$; day 14: $p = 0.88$).

Rifampicin induction of CYP3A4 activity on day 7 and day 14 was higher for all culture conditions compared to the non-induced activity (Figure 7b,d), which was largely undetectable by 14 days in PLGA-ECM scaffolds and negligible within the ECM sandwich control. Pharmacologic induction of P450 activity is a specific trait of primary hepatocytes, and is similar to the trend seen by others in which induction led to higher P450 activity.[32] Induced CYP3A4 activity was greatest in the PLGA-C_{high} scaffolds compared to all other PLGA scaffolds at both day 7 and 14 (Figure 7c,d), again showing the importance of type I collagen as a critical ECM constituent.

4. Discussion

In this study we describe the development of a synthetic PLGA-based scaffold system that contains the minimal essential ECM components of the liver micro-environment to test the contribution of each to *in vitro* hepatocyte function. The ability to produce scaffolds conjugated with individual ECM components, or defined combinations thereof, would widen

the use of hepatocytes cultured *in vitro* and would potentially enable their use as a clinical cellular therapy by dispensing with ECM materials produced by transformed cell lines, such as Matrigel that is commonly used in ECM sandwich culture.

The 3D scaffolds that we describe here resemble the pore structure of the normal liver tissue matrix, contain critical components of the natural liver micro-environment, and are a synthetic, standardizable, and easy to implement culture system to grow human hepatocytes *in vitro*. To investigate essential ECM components for these PLGA-based synthetic matrices, we synthesized scaffolds using a new wet electrospinning technique that resulted in a highly porous, nanofibrous structure that replicates the pore size of the acellular liver parenchyma. ECM proteins (type I collagen or fibronectin) were then chemically linked to scaffolds via amine coupling. We demonstrate that hepatocyte synthetic function (e.g., protein production) is preserved for two weeks within these scaffolds and potentiated beyond the level seen in primary human hepatocytes grown in unmodified PLGA scaffolds or within ECM sandwich culture. Moreover, in contrast to unmodified scaffolds, we demonstrate the importance of type I collagen to maintain, and at times potentiate, hepatocyte function. Hepatocytes cultured within PLGA-ECM scaffolds, specifically those modified with type I collagen, exhibited higher synthetic and inducible catalytic function compared to unmodified PLGA scaffolds, supporting our hypothesis that the ECM, and specifically type I collagen, is a critical component of the liver micro-environment that influences hepatocyte function. Additionally, hepatocytes cultured within PLGA-C_{high} scaffolds demonstrated higher synthetic and inducible catalytic function in a type I collagen-concentration dependent manner and were consistently higher in comparison to PLGA scaffolds modified with varying concentrations of fibronectin, despite near equivalent cell viability between each ECM component tested. Hepatocytes cultured within PLGA-C_{high} scaffolds expressed higher levels of liver-specific genes and secreted higher levels of albumin and urea compared to hepatocytes in sandwich culture. Taken together, these results demonstrate the benefit of utilizing the highly porous, 3D wet electrospun scaffold system modified with type I collagen compared to the planar type I collagen-Matrigel culture system, where hepatocytes grow within a monolayer and are bound by ECM on either side.

We previously demonstrated the use of the new wet electrospinning technique for the production of polymer scaffolds for chronic wound repair. [26] In the present study, we utilized a similar electrospinning approach to develop a liver-like matrix using the wet electrospinning technique that produces scaffolds with a comparatively larger pore size, compared to traditional electrospinning, and enables incorporation of hepatocytes into the matrix. Electrospinning, as a general method, has been used for liver tissue engineering applications though the wet electrospinning technique has not been used to recapitulate the porosity of the liver matrix in synthetic scaffolds. Recently, one study from Rajendran and coworkers reported the fabrication of fibronectin-linked chitosan nanofibers using the traditional electrospinning approach to culture rodent hepatocytes.[33] They report a rapid loss of albumin secretion from primary rat hepatocytes that contrasts with our results in which primary human hepatocytes on PLGA-F_{low} exhibited equivalent albumin secretion compared to ECM sandwich culture over 14 days. In addition to variations in biomaterial type and cell source (rat versus human), the higher albumin production in our study may be due to the use of the wet electrospinning technique that result in a larger pore size in our

study. The pores of the chitosan nanofiber scaffolds formed in the Rajendran study were smaller ($2.2 \pm 0.4 \mu\text{m}$ [33]) compared to the pore size using the wet electrospinning technique we describe here ($27.5 \pm 7.5 \mu\text{m}$). The higher porosity and lower density of the PLGA fibers produced by the wet electrospinning technique in our study enabled infiltration of hepatocytes into the scaffold as well as the ability of hepatocytes to form cell-cell contacts, as shown in SEM micrographs and cross-sectional H&E images of hepatocytes within PLGA- C_{high} scaffolds on day 14 (Figure 4).

Type I collagen is one of the most abundant proteins found in liver ECM, and our study implicates type I collagen as a critical ECM component for the *in vitro* culture of primary human hepatocytes that leads to increased synthetic and catalytic function. Moreover, our findings reinforce the observation of others using different, though related, scaffolding systems for *in vitro* hepatocyte culture. Kim and co-workers reported on the fabrication of a 3D poly(ethylene-glycol, PEG)-based hydrogel modified with type I collagen and investigated its effect on transformed Huh 7.5 hepatocytes.[34] Specifically, the presence of the type I collagen in these scaffolds led to a 4-fold increase in albumin secretion compared to bare scaffolds over a 14-day period using the Huh 7.5 hepatocarcinoma cell line, demonstrating a similar trend to our results using primary human hepatocytes. The group also demonstrated an increase in expression of liver-specific genes within Huh 7.5 cells compared to the unmodified PEG hydrogels without collagen. Hepatoma-derived cell lines such as HepG2 and Huh7 are frequently used for *in vitro* studies due to their stability in culture compared to primary hepatocytes, which are more fragile and are susceptible to dedifferentiation. Additionally, primary hepatocytes are preferable for cell therapy or pharmacology applications because they have not been altered by genetic mutations that lead to cancer, and because they are the actual cell-type within the normal liver that catalyze exogenous medications.[35] To further provide context for our results, other groups, such as Li et al., used PLGA scaffolds for the culture of fresh primary human hepatocytes.[36] In contrast to our methodology, type I collagen was added to PLGA scaffolds produced using a freeze-extraction process. Primary hepatocytes cultured on the collagen-PLGA scaffolds led to an increased albumin secretion of $1.63 \pm 0.23 \text{ pg/cell/h}$ ($39.12 \pm 5.52 \mu\text{g}/10^6 \text{ cells/day}$) over 13 days, which is similar to the rate of albumin secretion by human hepatocytes within PLGA- C_{high} scaffolds in our study at day 14 ($34.56 \pm 2.54 \mu\text{g}/10^6 \text{ cells/day}$). Further demonstrating the preservation of hepatocyte-specific function, this level of albumin secretion was higher when compared to the *in vivo* albumin production levels of 0.742 pg/cell/h ($17.81 \mu\text{g}/10^6 \text{ cells/day}$).[37–39]

Fibronectin is another ECM protein that has a beneficial effect on hepatocytes grown *in vitro* by mediating hepatocyte adhesion and expression of hepatic genes.[40–42] Our findings revealed a benefit to the presence of fibronectin compared to the unmodified PLGA scaffold, specifically leading to increased albumin and urea production and gene expression. Nonetheless, type I collagen-modified scaffolds were superior to scaffolds modified by fibronectin (at both concentrations) and in all tested indices of standard primary hepatocyte function (albumin and urea secretion, gene expression analysis, and cytochrome P450 activities).[43] Wang and coworkers also demonstrated a positive benefit for fibronectin incorporated into a 3D inverted colloidal crystal (ICC) scaffold, relative to bare scaffolds that did not include ECM constituents.[44] They also reported that albumin secretion was

~80% higher from Huh-7.5 hepatocytes cultured on the fibronectin-modified scaffolds compared to cells cultured on type I collagen-modified scaffolds. Differences in cell type used to evaluate the 3D scaffolds may be a reason for the contrasting findings, as Wang and coworkers used Huh-7.5 cells which have been reported to show functional differences compared to primary hepatocytes.[35] Another study by the same group revealed that including both type I collagen and fibronectin in ICC hydrogel scaffolds resulted in improved expression of liver-specific genes, such as albumin and HNF4A, and albumin secretion compared to scaffolds modified with type I collagen.[45]

While our study represents a proof-of-concept that 3D nanofibrous polymer-ECM scaffolds can be used as an *in vitro* culture system for primary hepatocytes and that type I collagen specifically stimulates function of primary hepatocytes, the system is not without limitations as P450 activity continued to decay in primary hepatocytes cultured within ECM-modified PLGA scaffolds and in the ECM control sandwich culture. Nonetheless, the findings of this study together indicate that type I collagen is an important contributor to primary hepatocyte synthetic function *in vitro* and make-up one of the ECM-based components that should be retained in *in vitro* culture systems to maximize hepatocyte function. Though our goal for this study is to identify minimal essential ECM components of the liver micro-environment that preserve hepatocyte function *in vitro*, grafting multiple combinations of different ECM components onto the PLGA scaffold, such as type I collagen and fibronectin, may further potentiate the catalytic function of hepatocytes *in vitro* leading to higher P450 function.

5. Conclusions

The work presented here demonstrates that PLGA wet electrospun 3D nanofibrous scaffolds modified with type I collagen augment primary human hepatocyte synthetic activity and may serve as a biosynthetic starting material for long term culture of primary hepatocytes. After modification, the highly porous nanofibrous electrospun scaffold developed by the wet electrospinning technique mimics the *in vivo* ECM structure, which is essential for interaction and function of the hepatocytes. This porous micro-environment is completely absent in the ECM sandwich culture, which is limited by its production in transformed cells and potentially inconsistent protein composition. We demonstrate that incorporation of essential ECM components into biosynthetic 3D scaffolds leads to improved *in vitro* performance of primary human hepatocytes stimulated specifically by type I collagen. In future studies, this system, either alone or in combination with other ECM molecules, may serve as a platform to investigate the individual components needed to further optimize hepatic function for biomedical applications such as pharmacology screening, disease-based testing and human cellular therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

J.H.B. and P.D. contributed equally to this work. J.H.B. wrote the manuscript. J.H.B., P.D., M.D.D., and D.I. performed experiments for this work. J.H.B., P.D., M.D.D., L.P.T., and J.A.W. contributed to experimental design

and all authors edited the manuscript. We thank the NTU-Northwestern Institute for Nanomedicine (NNIN) for funding this work. Research reported in this publication was also supported in part by the McCormick Foundation and the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award number K08DK101757 to J.A.W, T32DK077662 to J.H.B., and TL1TR001423 to M.D.D. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This work made use of the EPIC facility of Northwestern University's NUANCE Center, which received support from the Soft and Hybrid Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205), the MRSEC program (NSF DMR-1121262) at the Materials Research Center, the International Institute for Nanotechnology (IIN), the Keck Foundation, and the State of Illinois, through the INN. Imaging work was performed at the Northwestern University Center for Advanced Microscopy generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. This work was supported by the Northwestern University Pathology Core Facility and a Cancer Center Support Grant (NCI CA060553).

References

1. Hu C, Li L. In vitro culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. *Protein Cell*. 2015; 6(8):562–574. [PubMed: 26088193]
2. Rowe C, Gerrard DT, Jenkins R, Berry A, Durkin K, Sundstrom L, Goldring CE, Park BK, Kitteringham NR, Hanley KP, Hanley NA. Proteome-wide analyses of human hepatocytes during differentiation and dedifferentiation. *Hepatology*. 2013; 58(2):799–809. [PubMed: 23526496]
3. Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Bottger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gomez-Lechon MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Haussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhutter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stober R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol*. 2013; 87(8):1315–530. [PubMed: 23974980]
4. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J*. 1989; 3(2):174–7. [PubMed: 2914628]
5. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol*. 1992; 116(4):1043–53. [PubMed: 1734019]
6. Foster E, You J, Siltanen C, Patel D, Haque A, Anderson L, Revzin A. Heparin hydrogel sandwich cultures of primary hepatocytes. *Eur Polym J*. 2015; 72:726–735.
7. Berthiaume F, Moghe PV, Toner M, Yarmush ML. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *FASEB J*. 1996; 10(13):1471–84. [PubMed: 8940293]
8. Wang X, Ding B, Li B. Biomimetic electrospun nanofibrous structures for tissue engineering. *Mater Today*. 2013; 16(6):229–241.
9. Lim SH, Mao HQ. Electrospun scaffolds for stem cell engineering. *Adv Drug Delivery Rev*. 2009; 61(12):1084–1096.
10. Bhardwaj N, Kundu SC. Electrospinning: A fascinating fiber fabrication technique. *Biotechnol Adv*. 2010; 28(3):325–347. [PubMed: 20100560]
11. Kitsara M, Agbulut O, Kontziampasis D, Chen Y, Menasché P. Fibers for hearts: A critical review on electrospinning for cardiac tissue engineering. *Acta Biomater*. 2017; 48:20–40. [PubMed: 27826001]
12. Chen N, Tian L, He L, Ramakrishna S. Nanobiomaterials for neural regeneration. *Neural Regener Res*. 2016; 11(9):1372–1374.

13. Rezvani Z, Venugopal JR, Urbanska AM, Mills DK, Ramakrishna S, Mozafari M. A bird's eye view on the use of electrospun nanofibrous scaffolds for bone tissue engineering: Current state-of-the-art, emerging directions and future trends. *Nanomedicine*. 2016; 12(7):2181–2200. [PubMed: 27247186]
14. Dias JR, Granja PL, Bártolo PJ. Advances in electrospun skin substitutes. *Prog Mater Sci*. 2016; 84:314–334.
15. Khorshidi S, Solouk A, Mirzadeh H, Mazinani S, Lagaron JM, Sharifi S, Ramakrishna S. A review of key challenges of electrospun scaffolds for tissue-engineering applications. *Journal of tissue engineering and regenerative medicine*. 2016; 10(9):715–38. [PubMed: 25619820]
16. Nosar MN, Salehi M, Ghorbani S, Beiranvand SP, Goodarzi A, Azami M. Characterization of wet-electrospun cellulose acetate based 3-dimensional scaffolds for skin tissue engineering applications: influence of cellulose acetate concentration. *Cellulose*. 2016; 23(5):3239–3248.
17. Yokoyama Y, Hattori S, Yoshikawa C, Yasuda Y, Koyama H, Takato T, Kobayashi H. Novel wet electrospinning system for fabrication of spongiform nanofiber 3-dimensional fabric. *Mater Lett*. 2009; 63(9–10):754–756.
18. Hiraoka Y, Kimura Y, Ueda H, Tabata Y. Fabrication and biocompatibility of collagen sponge reinforced with poly(glycolic acid) fiber. *Tissue Eng*. 2003; 9(6):1101–12. [PubMed: 14670098]
19. Baiocchi A, Montaldo C, Conigliaro A, Grimaldi A, Correani V, Mura F, Ciccocanti F, Rotiroli N, Brenna A, Montalbano M, D'Offizi G, Capobianchi MR, Alessandro R, Piacentini M, Schinina ME, Maras B, Del Nonno F, Tripodi M, Mancone C. Extracellular Matrix Molecular Remodeling in Human Liver Fibrosis Evolution. *PloS one*. 2016; 11(3)
20. Azuma H, Paulk N, Ranade A, Dorrell C, Al-Dhalimy M, Ellis E, Strom S, Kay MA, Finegold M, Grompe M. Robust expansion of human hepatocytes in Fah(−/−)/Rag2(−/−)/Il2rg(−/−) mice. *Nat Biotechnol*. 2007; 25(8):903–910. [PubMed: 17664939]
21. Uzarski JS, DiVito MD, Wertheim JA, Miller WM. Essential design considerations for the resazurin reduction assay to noninvasively quantify cell expansion within perfused extracellular matrix scaffolds. *Biomaterials*. 2017; 129:163–175. [PubMed: 28343003]
22. MacArthur R, Leister W, Veith H, Shinn P, Southall N, Austin CP, Inglese J, Auld DS. Monitoring Compound Integrity With Cytochrome P450 Assays and qHTS. *Journal of Biomolecular Screening*. 2009; 14(5):538–546. [PubMed: 19483146]
23. Meisenheimer PL, Uyeda HT, Ma D, Sobol M, McDougall MG, Corona C, Simpson D, Klaubert DH, Cali JJ. Proluciferin Acetals as Bioluminescent Substrates for Cytochrome P450 Activity and Probes for CYP3A Inhibition. *Drug Metabolism and Disposition*. 2011; 39(12):2403–2410. [PubMed: 21890735]
24. Higuchi A, Tsukamoto Y. Cell separation of hepatocytes and fibroblasts through surface-modified polyurethane membranes. *Journal of biomedical materials research Part A*. 2004; 71(3):470–9. [PubMed: 15386485]
25. Uygün BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygün K. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nature medicine*. 2010; 16(7):814–20.
26. Chen HZ, Peng Y, Wu SC, Tan LP. Electrospun 3D Fibrous Scaffolds for Chronic Wound Repair. *Materials*. 2016; 9(4)
27. Arterburn LM, Zurlo J, Yager JD, Overton RM, Heifetz AH. A morphological study of differentiated hepatocytes in vitro. *Hepatology*. 1995; 22(1):175–187. [PubMed: 7601410]
28. Olsavsky Goyak KM, Laurenzana EM, Omiecinski CJ. Hepatocyte differentiation. *Methods in molecular biology (Clifton, NJ)*. 2010; 640:115–38.
29. Smith HS. Opioid Metabolism. *Mayo Clin Proc*. 2009; 84(7):613–624. [PubMed: 19567715]
30. Vorriink SU, Ullah S, Schmidt S, Nandania J, Velagapudi V, Beck O, Ingelman-Sundberg M, Lauschke VM. Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2017; 31(6):2696–2708. [PubMed: 28264975]

31. Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson P, Koch P, Antonian L, Wagner G, Yu L, Parkinson A. Effects of Prototypical Microsomal Enzyme Inducers on Cytochrome P450 Expression in Cultured Human Hepatocytes. *Drug Metabolism and Disposition*. 2003; 31(4):421–431. [PubMed: 12642468]
32. Haque A, Gheibi P, Gao Y, Foster E, Son KJ, You J, Stybayeva G, Patel D, Revzin A. Cell biology is different in small volumes: endogenous signals shape phenotype of primary hepatocytes cultured in microfluidic channels. 2016; 6:33980.
33. Rajendran D, Hussain A, Yip D, Parekh A, Shrirao A, Cho CH. Long-Term Liver-Specific Functions of Hepatocytes in Electrospun Chitosan Nanofiber Scaffolds Coated with Fibronectin. *J Biomed Mater Res, Part A*. 2017; 105A:2119–2128.
34. Kim MH, Kumar SK, Shirahama H, Seo J, Lee JH, Cho NJ. Phenotypic regulation of liver cells in a biofunctionalized three-dimensional hydrogel platform. *Integr Biol*. 2016; 8(2):156–166.
35. Soars MG, McGinnity DF, Grime K, Riley RJ. The pivotal role of hepatocytes in drug discovery. *Chem-Biol Interact*. 2007; 168(1):2–15. [PubMed: 17208208]
36. Li J, Li L, Yu H, Cao H, Gao C, Gong Y. Growth and metabolism of human hepatocytes on biomodified collagen poly(lactic-co-glycolic acid) three-dimensional scaffold. *ASAIJ*. 2006; 52(3):321–7. [PubMed: 16760723]
37. Török E, Lutgehetmann M, Bierwolf J, Melbeck S, Düllmann J, Nashan B, Ma PX, Pollok JM. Primary human hepatocytes on biodegradable poly(l-lactic acid) matrices: A promising model for improving transplantation efficiency with tissue engineering. *Liver Transplantation*. 2011; 17(2): 104–114. [PubMed: 21280182]
38. Martini WZ, Wolf SE, Chinkes DL, Chung KK, Dubick MA, Blackburn L, Yu YM. Enhanced albumin synthesis in severely burned adults. *Shock*. 2010; 34(4):364–8. [PubMed: 20160662]
39. Sohlenius-Sternbeck AK. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicol In Vitro*. 2006; 20(8): 1582–6. [PubMed: 16930941]
40. Sawada N, Tomomura A, Sattler CA, Sattler GL, Kleinman HK, Pitot HC. EFFECTS OF EXTRACELLULAR-MATRIX COMPONENTS ON THE GROWTH AND DIFFERENTIATION OF CULTURED RAT HEPATOCYTES. *In Vitro Cell Dev Biol*. 1987; 23(4):267–273. [PubMed: 2883170]
41. Sawada N, Tomomura A, Sattler CA, Sattler GL, Kleinman HK, Pitot HC. EXTRACELLULAR-MATRIX COMPONENTS INFLUENCE DNA-SYNTHESIS OF RAT HEPATOCYTES IN PRIMARY CULTURE. *Exp Cell Res*. 1986; 167(2):458–470. [PubMed: 3490392]
42. Sanchez A, Alvarez AM, Pagan R, Roncero C, Vilaro S, Benito M, Fabregat I. Fibronectin regulates morphology, cell organization and gene expression of rat fetal hepatocytes in primary culture. *J Hepatol*. 2000; 32(2):242–250. [PubMed: 10707864]
43. Song W, Lu Y-C, Frankel AS, An D, Schwartz RE, Ma M. Engraftment of human induced pluripotent stem cell-derived hepatocytes in immunocompetent mice via 3D co-aggregation and encapsulation. *Sci Rep*. 2015; 5:16884. [PubMed: 26592180]
44. Wang Y, Kim MH, Shirahama H, Lee JH, Ng SS, Glenn JS, Cho NJ. ECM proteins in a microporous scaffold influence hepatocyte morphology, function, and gene expression. *Sci Rep*. 2016; 6
45. Wang Y, Lee JH, Shirahama H, Seo J, Glenn JS, Cho NJ. Extracellular Matrix Functionalization and Huh-7.5 Cell Coculture Promote the Hepatic Differentiation of Human Adipose-Derived Mesenchymal Stem Cells in a 3D ICC Hydrogel Scaffold. *ACS Biomater Sci Eng*. 2016; 2(12): 2255–2265.

Statement of Significance

Culturing primary hepatocytes within a three-dimensional (3D) structure that mimics the natural liver environment is a promising strategy for extending the function and viability of hepatocytes *in vitro*. In the present study we generate porous PLGA nanofibers, that are chemically modified with extracellular matrix proteins, to serve as 3D scaffolds for the *in vitro* culture of primary human hepatocytes. Our findings demonstrate that the use of ECM proteins, especially type I collagen, in a porous 3D environment helps to improve the synthetic function of primary hepatocytes over time. We believe the work presented within will provide insights to readers for drug toxicity and tissue engineering applications.

Figure 1.

Porosity of conventional and wet electrospun PLGA nanofibers. A) SEM micrograph of an electrospun scaffold collected from the conventional flat platform with a smaller pore size compared to B) scaffolds developed using wet electrospinning. Distribution of pore sizes (μm) within scaffolds developed by C) conventional electrospinning and D) wet electrospinning; $n = 3$; scale bar = 10 μm.

Figure 2.

Chemical analysis of chemisorbed PLGA nanofibers. A) Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) analysis of PLGA, PLGA-F_{high}, and PLGA-C_{high} scaffolds. The peaks at 1637 cm⁻¹, 1562 cm⁻¹, and 1240 cm⁻¹ in the FTIR spectra correspond to N-H bending from primary amines in the PLGA-C_{high} and PLGA-F_{high} scaffolds. B) Table of pore size, fiber diameter, porosity, and amount of immobilized protein (μg) for scaffolds made from each PLGA chemisorbed nanofiber or unmodified (blank) PLGA nanofibers; *n* = 3.

Figure 3.

Amount of metabolically active (viable) primary hepatocytes present within PLGA-ECM electrospun scaffolds during 14 days of culture. Viability of primary hepatocytes was determined over time by resazurin reduction on days 2, 7, 11, and 14, ($n = 6$ for each group), ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

SEM micrographs of primary human hepatocytes within electrospun PLGA scaffolds after 14 days of culture at 1,000X, scale bar = 50 μm. A) unmodified PLGA, B) PLGA-C_{high}, C) PLGA-C_{low}, D) PLGA-F_{high}, E) PLGA-F_{low}. Images depict more cells within PLGA-ECM scaffolds compared to unmodified scaffolds. Insets: Cross-sectional images of hepatocytes within the same PLGA scaffolds by hematoxylin and eosin staining at 63X, scale bar = 20 μm. Cross-sectional views show cell spreading along the PLGA-C_{high} nanofibers.

Figure 5.

Gene expression of liver-specific proteins albumin, CYP2C9, and CYP3A4 in primary human hepatocytes cultured within each 3D PLGA electrospun scaffold or the ECM sandwich control environment on day 14. A) normalized to the sandwich control culture ($n = 3$ for each group), * $p < 0.05$, ** $p < 0.01$, # = statistics not able to be calculated due to lack of expression, and B) normalized to fresh (day = 0) primary hepatocytes as a control for ideal *in vivo* hepatocyte function, ($n = 3$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # = statistics not able to be calculated due to lack of PLGA expression, ND = not detected.

Figure 6.

Protein production and synthetic function during the 14-day time course experiment within each PLGA scaffold or control ECM sandwich culture. A) Albumin secretion of primary hepatocytes on days 2, 7, 11, and 14 ($n = 4$ for each sample). B) Urea synthesis of primary hepatocytes on days 2, 7, 11, and 14. Day 2 albumin and urea production represents the synthetic function of fresh hepatocytes as a control. ($n = 3$ for each sample); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7.

CYP450 activity of primary hepatocytes within each PLGA scaffold and ECM sandwich culture. A) CYP2C9 and B) CYP3A4 activity at day 7 and C) CYP2C9 and D) CYP3A4 activity at day 14, with and without induction by rifampicin. Fresh (day 0) human hepatocytes are included as a control. ($n = 3$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ND = not detected.