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A novel ultrathin collagen nanolayer assembly for 3-D microtissue engineering: Layer-by-layer collagen deposition for long-term stable microfluidic hepatocyte culture

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Abstract

The creation of stable hepatocyte cultures using cell-matrix interactions has proven difficult in microdevices due to dimensional constraints limiting the utility of classic tissue culture techniques that involve the use of hydrogels such as the collagen “double gel” or “overlay”. To translate the collagen overlay technique into microdevices, we modified collagen using succinylation and methylation reactions to create polyanionic and polycationic collagen solutions, and deposited them layer-by-layer to create ultrathin collagen nanolayers on hepatocytes. These ultrathin collagen layers covered hepatocytes in microdevices and 1) maintained cell morphology, viability, and polarity, 2) induced bile canaliculi formation and actin reorganization, and 3) maintained albumin and urea secretions and CYP activity similar to those observed in hepatocytes in collagen double gel hepatocytes in plate cultures. Beyond the immediate applications of this technique to create stable, *in vitro* microfluidic hepatocyte cultures for drug toxicity testing, this technique is generally applicable as a thin biomaterial for other 3D microtissues.

INNOVATION

We report the assembly of ultrathin collagen nanolayers using layer-by-layer deposition of modified collagen solutions. Using these nanolayers, we demonstrate stable hepatocyte morphology and function in microdevices over 14 days. Translating successful techniques for stable hepatocyte cultures using cell-matrix interactions, such as the collagen double gel method, has proven difficult in microdevices due to dimensional constraints. We overcame these limitations by combining the layer-by-layer thin film deposition technique with polyanionic and polycationic collagen solutions, to create ultrathin collagen nanolayer assemblies. Due to its natural abundance, biocompatibility, robust cell attachment sites, and ubiquity in the extracellular matrix, collagen is one of the most frequently used proteins for tissue engineering and biomaterial applications. These ultrathin collagen nanolayers may be useful for many biomedical engineering applications, including for cell micropatterned and microfluidic culture, precise cell layering, and imaging applications requiring high optical

clarity. Here, we demonstrate their use in stabilizing hepatocyte morphology and function in microdevices.

INTRODUCTION

Primary hepatocytes in culture provide a first-order approximation of an *in vitro* liver tissue that researchers have utilized for over 40 years. Successful *in vitro* models of the liver must replicate the major liver-specific functions over a prolonged period to allow for both acute and chronic studies. Extending *in vitro* hepatocyte culture duration, while maintaining *in vivo*-like phenotype and liver-specific functionality, has proven difficult given the tendency of primary hepatocytes to lose function over time in culture. Freshly isolated hepatocytes in suspension culture maintain several short-term (~4 hrs) functions^{1–3}, but lose many important characteristics⁴, including polarity, junction formation, and bile production.

Several culture techniques using cell-matrix and heterotypic cell-cell contact have been developed to extend the *in vitro* function of hepatocytes, but few can be applied to microfluidic systems. Microfluidics allows for exquisite control of the cellular microenvironment, and is a powerful tool for mimicking and investigating liver physiology. Monolayer hepatocyte culture can be used successfully for short-term (1–3 day) culture^{4–6}, but is limited by adaptation and loss of function. Spheroid^{3,7–12} techniques can extend the functionality of hepatocyte cultures out several weeks, though imaging is difficult, as is scaling the system down for microfluidic applications. Matrix encapsulation methods^{13–17} show similar long term function, and in one case have been adapted for short-term use in micro-fluidic devices¹⁸. Heterotypic cell-cell interactions to maintain hepatocyte function^{19,20} have also been used for micropatterned culture²¹, but require cumbersome assembly for introducing fluid flow^{22,23}, which is likely an important parameter for mimicking liver physiology.

Matrix sandwich and matrix immobilization methods for hepatocyte culture significantly increase their usable time-scale, out to >4 weeks. Sandwiching hepatocytes between two layers of extracellular matrix, typically collagen or Matrigel™ basement membrane matrix, leads to development of stable hepatocyte polarity and albumin, urea, transferrin, fibrinogen, and bile salt secretions in 6–24-well culture plates^{24–26}. Stability of hepatocyte phenotype in these configurations is likely due to the induction of polarity, as seen by actin and integrin staining²⁶. Such polarity, including basal surfaces induced by the extracellular matrix layers and apical surfaces by cell-to-cell contact, leads to bile canalicular network development²⁷ and bile secretion²⁸, as well as maintenance of biotransformation activities over periods of many weeks²⁹.

Translating the collagen double gel technique to microfluidic devices has proven difficult. The collagen sandwich configuration for hepatocytes relies on self-assembling gelled extracellular matrices with typical thicknesses of hundreds of microns that are too bulky and imprecise to fit without clogging closed devices with small (~10 µm) features and channel heights (50–100 µm). An alternative matrix assembly approach may allow translation of the collagen double gel method to microfluidic devices. Such a technology would facilitate high

throughput drug screening assays over longer times by maintaining parallelizable channels of hepatocyte monolayers, forming arrays of test conditions with fluid flow.

Thin extracellular matrix layers can be created through layer-by-layer (LBL) deposition of polyelectrolyte multilayers³⁰. In this technique, alternating layers of cationic and anionic polymers are deposited via electrostatic attraction on charged surfaces or on top of cells. LBL has been used with natural and synthetic³¹ polyelectrolytes to cover hepatocytes in plate culture^{32–35}, and as a seeding layer for hepatocyte co-cultures in microfluidic devices^{36,37}. Here, we report the deposition of an ultrathin, pure collagen nanolayer on hepatocytes in microfluidic devices using the LBL technique with methylated and succinylated collagens. The results clearly demonstrate that hepatocyte morphology and function is maintained over 14 days at similar levels as those observed with the traditional hepatocyte sandwich technique in plate cultures, as well as inducing formation of bile canaliculi, secretion of albumin and urea, and maintenance of CYP activity.

METHODS

Modification of collagen side chains and verification

Native, soluble type I collagen was prepared from rat tails through acetic acid extraction, serial salt precipitation, and dissolution in HCl. To create solutions of net positively charged collagen, native collagen was methylated^{38,39}. Briefly, pH-precipitated collagen was added to methanol with 0.1N HCl and allowed to react at room temperature with stirring for 4 days. Methylated collagen was pelleted at 3,000 g for 20 min and reconstituted at 3 mg/mL in phosphate buffered saline (PBS) at pH 7.3. To create solutions of net negatively charged collagen, native collagen was succinylated^{38,40}. Briefly, the pH of the collagen solution was increased to pH 9–10 with NaOH, and 1/20th the collagen solution volume of acetone with 0.4 mg succinic anhydride per mg collagen was slowly added while the pH was maintained at 9–10. The reaction was allowed to continue at room temperature with stirring for 2 hrs, and then succinylated collagen was precipitated by reducing the solution pH to 4.0 with HCl. Succinylated collagen was pelleted at 3,000 g for 20 min and reconstituted at 3 mg/mL in PBS pH 7.3 and stored at 4°C until use within 4 weeks.

The charge characteristics of the modified collagens were verified using hydrogen ion titration and the 2,4,6-trinitrobenzenesulfonic acid (TNBA) colorimetric method. Hydrogen ion titration of 1 mg/mL native and modified collagens, with pH 7.3 as the reference point, was used to determine the net molecular charges on the methylated and succinylated collagens compared to native collagen. The relative charge per amino acid was calculated following the methods of Tanford⁴¹. The modified collagens were also assessed by TNBA assay^{42,43} to determine the relative number of amino groups blocked by succinylation.

Microfluidic device fabrication and preparation

Microfluidic chambers were fabricated by replica molding PDMS from photolithographically defined SU-8 masters on silicon. PDMS was cast on the molds to a thickness of 1–2 mm and baked at 70°C. Cell culture chambers were 100 µm high, 0.4–3 mm wide, and 10 mm long. The narrow channels were used to show proof-of-concept in

small dimensions, while the wider channels allowed for enough cells such that the mass of their secretions was easily quantified. Inlet and outlet ports were punched into the devices using a 0.75 mm dermal punch, and they were then plasma cleaned, bonded to microscope slides, and baked (70°C, 20 min). Devices were UV-sterilized (15 min) and coated with fibronectin (50 µg/mL, 37°C, 45 min) in PBS before cell seeding.

Hepatocyte isolation, culture, and layer-by-layer deposition

Rat hepatocytes were freshly isolated and human cryopreserved hepatocytes were purchased. Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 180–200 g, by a modified procedure of Seglen⁴⁴, as described previously²⁶. Routinely, 200–300 million cells with 90–95% viability were isolated, as determined by trypan blue exclusion. Rat hepatocytes were cultured in Dulbecco's modified eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 µg/mL hydrocortisone, 200 U/mL penicillin, 200 µg/mL streptomycin, and 50 µg/mL gentamycin. All data reported here were collected using rat hepatocytes. However, we also performed proof-of-concept pilot studies using human hepatocytes that showed similar morphological effects. Human cryopreserved hepatocytes were purchased (Triangle Research Labs, Research Triangle Park, NC, USA), and thawed according to the vendor's instructions (post-thaw viability: 89–94%), and cultured in the vendor's proprietary serum-free media. Rat and human hepatocytes were seeded into devices (20 µL at 14 M cells/mL), allowed to attach for 3–4 hours, and then rinsed with fresh media. Cells were incubated at 37°C in humidified air with 5% (human) or 10% (rat) CO₂. As controls, rat and human hepatocytes were also seeded into 6-well tissue culture plates and covered with a collagen gel, following the methods of Dunn⁴⁵.

LBL deposition of the modified collagen solutions was performed on devices 24 hrs after seeding. Alternating methylated and succinylated collagen solutions (20 µL each) were introduced into the device, waiting 1 min between solution changes, until each solution had been introduced 10 times (10 bilayers), as was done in a prior LBL study on hepatocytes in plates³². Based on pilot experiments, 3 biplayers was too few to see the morphological effects. Negative controls (NoTop) devices were prepared in the same way, except PBS was introduced instead of the modified collagen solutions. Additional controls were tested where only a single solution (i.e. either methylated or succinylated collagen) was used instead of altering the solutions to verify the necessity of the charge interaction (see **Supplementary Methods**). Cells were then rinsed twice in media and incubated for the various experiments, with daily culture media changes. Proof-of-concept experiments were also performed using single-pass fluid flow at 20 µL/hr, which translated to an average channel velocity of 18 µm/s and hepatocyte shear stress of <0.1 dynes/cm².

Hepatocyte TEM, morphology, and function

Devices of cells were fixed in ½ Karnovsky's fixative for 1–2 hrs and then rinsed in 0.1M cacodylate buffer until further processing for transmission electron microscopy (TEM). Briefly, fixed cells were dehydrated, embedded in resin, sectioned perpendicular to the growing surface, ultra-thin sectioned onto grids, and imaged. The average thickness of the

visible matrix layer covering COL LBL cells was measured using image analysis tools (ImageJ, NIH, Bethesda, MD, USA). For each image, a region of interest (ROI) including the upper cell surface was manually selected and the histogram of greyscale values in the ROI was used to determine threshold values for excluding the background and main cell body. The thickness of the layer was calculated by dividing the pixel area remaining by the cell length. For the NoTop samples, which should ideally show no layer thickness, the thickness value indicates the variability of this analysis method due to imaging artifacts and cellular debris.

Hepatocyte morphology was assessed by phase microscopy, viability, and bile canaliculi staining at days 2, 7, and 14. Hepatocyte viability and cell numbers were assessed using a mammalian LIVE/DEAD® kit (Life Technologies) according to the manufacturer's instructions, with DAPI counterstain. Average viability was calculated by subtracting the number of ethidium homodimer-stained nuclei from the total number of DAPI-stained nuclei and normalizing to the total number of DAPI-stained nuclei using ImageJ's built-in particle analysis tool. Bile canaliculi were stained using CellTracker™ Green CMFDA at 2 μ M in Live Cell Imaging Solution (Life Technologies), which remains in the cytosol in the absence of canaliculi, and is excreted by hepatocytes into canaliculi when they are present. Hepatocyte cytoskeletal structure was assessed by actin staining (Alexa 488 phalloidin, Life Technologies).

Hepatocyte function was assessed by albumin and urea secretion, and cytochrome P450 (CYP) induction. Conditioned media was collected from devices and assessed for albumin content by sandwich ELISA (Abcam, Cambridge, MA, USA) and urea content by colorimetric assay (Stanbio Laboratory, Boerne, TX, USA). Native and induced CYP1A1/2, one of the main inducible human xenobiotic and drug-metabolizing CYPs⁴⁶, activities were assessed by induction with 3-methylcholanthrene (3-MC) at 2 μ M in media or vehicle control (0.1% DMSO) for 24 hrs. Conversion of ethoxyresorufin to resorufin was determined by fluorescence using resorufin standards after 25 min exposure to cells and the activity reported as fold-induction by normalizing the induced values to the vehicle control values.

Statistical analysis

The data are presented as mean $\pm$ SEM for $n = 6-8$ devices (or wells), derived from at least 3 different rat isolations. The effects of the collagen modification reactions on the titration equivalence points were compared using F-tests to assess differences between Gaussian fit means. The effects of culture type (COL GEL, COL LBL, or NoTop) and time (days in culture) on albumin and urea secretions were assessed using 2-way repeated measures ANOVAs and on CYP activity using a 2-way ANOVA. Tukey post-hoc tests were used to determine differences between groups where significant ($P < 0.05$) effects were found.

RESULTS

Methylation and succinylation of collagen

Starting from acidified solutions of rat tail collagen, we created net positively (methylated collagen, MC) and negatively (succinylated collagen, SC) charged polymer solutions (**Fig. 1a**) for use in the LBL deposition process. These polymers were soluble at >4 mg/mL in PBS at pH 7.3 and did not precipitate or gel at room temperature. Succinylation capped $91 \pm 3\%$ ($n = 4$ different solution batches) of the ϵ -amino groups relative to native collagen, while methylation had no effect on free amino groups, as measured by the TNBA assay. To determine the relative charges of the polymer solutions, we performed hydrogen ion titrations and plotted the derivative of the pH change as a function of bound or released hydrogen ions (**Fig. 1b**). Shifts in the numbers of H^+ ions required to lower the pH in the pKa regions associated with ϵ -amino and carboxyl groups between native collagen, MC, and SC were apparent after modification. In the ϵ -amino region, MC was not different than native collagen ($P = 0.59$), but SC was shifted right ($P < 0.01$), indicating a loss of ϵ -amino groups. In the carboxyl region, MC was shifted left ($P < 0.001$) and SC was shifted right ($P < 0.001$), indicating a loss and gain, respectively, of carboxyl groups. We quantified and combined those shifts to show that MC is net positively charged and SC is net negatively charged (**Fig. 1c**).

LBL collagen deposition on hepatocytes in a microdevice

We deposited ultrathin layers of collagen using the polyanionic SC and polycationic MC solutions onto hepatocytes cultured in microdevices (**Fig. 2a,b**). TEM imaging of NoTop (see **Methods**) control hepatocytes after 2 days in culture showed typical *in vitro* hepatocyte morphology (**Fig. 2c**), including a flattened cell shape ($4\text{--}5$ μm height), defined nucleus, and cell body containing many mitochondria, similar to previous reports^{27,47}. Following LBL deposition, we observed a thin, diffuse matrix layer covering the hepatocytes (**Fig. 2d, arrows**). The thickness of the matrix layer per cell was quantified using image analysis. The matrix thickness was 130 ± 25 nm (mean $\pm$ SD, $n = 20$ cells), significantly higher ($P < 0.01$) than any residual debris found over NoTop control cells (**Fig. 2e**).

Collagen LBL maintains hepatocyte morphology and function in microdevices

To determine the long-term effects of COL LBL on hepatocytes in microdevices, we cultured cells for up to 14 days. Although hepatocytes seeded as confluent layers in microdevices with no top matrix layer appear healthy at day 2 (**Fig. 3a**), by day 7 the cells begin to contract and lift off the surface (**Fig. 3b**) becoming pure debris by day 14 (**Fig. 3c**). Similar results were found for the single solution controls up to day 7 (**Fig. S2**). In contrast, hepatocyte morphology was maintained in 6–24-well TC plates using the collagen double gel technique for over 14 days here (**Fig. 3d–f**), and for up to 6 weeks in previous reports²⁶. Similarly, after COL LBL deposition, the morphology of hepatocytes in microdevices was maintained for 14 days (**Fig. 3g–i**), with clearly defined nuclei, distinct cell borders, polygonal shape, and optically dense cytoplasm.

In addition to cell morphology, COL LBL deposition maintained hepatocyte viability and induced bile canaliculi formation. LIVE/DEAD™ staining indicated $91 \pm 2\%$ hepatocyte

viability after 14 days (**Fig. 4a–c**). We assessed bile canalicular formation using CMFDA, a cell tracker dye. In the absence of canaliculi (hepatocyte apical domains), the dye remains within the cell body (**Fig. 4d**). However, by day 7 (**Fig. 4e**), most cells contained discrete apical domains, and even some short canalicular structures, since very few cell bodies were visible. At day 14 (**Fig. 4f**), we observed a more extensive branching canalicular network.

Collagen LBL deposition caused a reorganization of the hepatocyte cytoskeleton. Without a top matrix layer, the actin filaments in seeded hepatocytes were diffuse or radially oriented around the nucleus (**Fig. 4g**). In contrast, hepatocyte actin fibers reorganized along the cell-to-cell borders in cells in plates covered with a top collagen gel layer (**Fig. 4h**) and in cells in microdevices covered with the ultrathin collagen layer (**Fig. 4i**). Similar results were reported previously for the collagen double gel technique⁴⁸.

We also assessed hepatocyte function after collagen LBL deposition in microdevices. Albumin secretion from hepatocytes into the media started low and increased steadily until reaching a plateau at day 10 for the COL LBL devices, following a similar shape (though delayed by ~2 days) as the collagen double gel hepatocyte secretion (**Fig. 5a**). The magnitude of albumin secretion in the COL LBL devices was similar, though slightly less than, that of the cells in the collagen double gel configuration. Albumin secretion by hepatocytes with no top matrix or only a single charged solution (**Fig. S3**) remained low.

Urea production by hepatocytes in the COL LBL devices began high at day 4 and decreased over time until reaching a plateau at day 8–10 (**Fig. 5b**). Hepatocytes in the collagen double gel configuration also showed a decline in urea production over time, though it was more gradual than in the COL LBL devices, and had not reached a plateau by day 14. The NoTop negative controls initially produced urea at a similar rate as the COL LBL hepatocytes, but secretion quickly dropped down to low levels as the cells lost their differentiated function and lifted off the device (**Fig. 3a–c, 5b**).

Collagen LBL deposition maintained CYP1A1/2 inducibility out to 14 days. In microdevices with COL LBL, hepatocyte CYP activity was low at day 2, but was significantly increased by day 7 ($P < 0.01$) and maintained at that level at day 14 ($P = 0.58$), compared to NoTop negative controls that only showed minimal CYP activity at day 7 (**Fig. 5c**). Hepatocytes in wells cultured using the collagen double gel technique maintained high inducibility of CYP activity at days 2, 7 and 14.

DISCUSSION

These results demonstrate that LBL deposition of an ultrathin collagen matrix onto hepatocytes in microdevices maintains their morphology and function for 14 days. We modified native collagen through methylation and succinylation reactions to create polycationic and polyanionic collagen solutions stable at neutral pH (**Fig. 1**), and used them to deposit ultrathin collagen layers on hepatocytes (**Fig. 2**). These thin layers maintained hepatocyte morphology (**Fig. 3**) and function (**Fig. 5**), similar to the collagen sandwich technique, as well as viability, and induced polarization and bile canalicular formation, presumably through cytoskeletal reorganization (**Fig. 4**).

The maintenance of hepatocyte morphology and function presented here were determined using rat hepatocyte under static and flow conditions. The large body of published experiments and data using rat hepatocytes makes their use for developing new methods desirable, as it allows for detailed comparisons between new results and previously published work. For specific applications, such as toxicity testing or the creation of normal and pathological liver-on-a-chip platforms, the use of human hepatocytes may be preferred. We verified that our collagen LBL technique is also applicable to human cryopreserved hepatocytes by culturing them in microdevices and assessing their morphology (**Fig. S1a**). Similarly, the most direct comparison can be made between hepatocyte culture in tissue culture plates, where the media is typically changed daily at most, and hepatocyte culture in microdevices without flow. Our results indicate the strength of this comparison and culture technique, as hepatocyte morphology and function are very similar between the two methods. To ensure that fluid flow did not remove the effect of the collagen nanolayer on hepatocytes, some devices were cultured under flow. The morphology results (**Fig. S1b**) clearly demonstrate that, even under flow, cell morphology was maintained over 14 days. Currently, we are expanding these proof-of-concept studies on human hepatocyte function under flow, which will be the substance of a follow-on paper.

We scaled up the hepatocyte functional data based on cell number to compare the microfluidic devices (~20,000 cells) and tissue culture plates ($\sim 1 \times 10^6$ cells). The rates of albumin and urea secretion were similar between culture configurations, as well as being consistent with previously published static, single day culture secretion rates normalized to 1 million cells^{26,45}. These data comparisons suggest that functional hepatocyte recovery by collagen LBL deposition in microdevices is delayed by a few days compared to recovery in plates using the collagen sandwich method. Both initial albumin secretion and CYP activity are more depressed in microdevices than in collagen sandwich plate cultures (**Fig. 5a,c**). The albumin secretion rates nearly catch up after a few days, but the CYP activity remains slightly less inducible in the microdevices. There are several possible explanations for these differences, including differences in the matrix layer to which the hepatocytes are attached: fibronectin in microdevices, and a thick collagen gel in the sandwich plate configuration. Given the importance of cell-matrix interactions indicated by the hepatocyte morphological changes without a top matrix layer (**Fig. 3a-c**), it is not surprising that the seeding matrix would also cause differences in function. Differences in the media volume per cell ratio (~1 nL/cell in plates and ~0.15 nL/cell in devices) could also contribute. Another difference is that the ultrathin collagen layer is likely a more permeable barrier against the loss of newly synthesized matrix components than a thick hydrogel, leading to the delay in the polarization and functional recovery observed (**Fig. 5a,c**). Finally, differences in the mechanical properties of collagen nanolayers and gels likely also contribute to functional magnitude and recovery time.

Hepatocytes in the liver are arranged in plates along sinusoids, interacting mainly with other hepatocytes and the matrix of the space of Disse. These different surfaces facilitate the polarization of hepatocytes, with development of apical regions between hepatocytes leading to canalicular development, and basolateral surfaces exposed to extracellular matrix. One advantage of matrix overlay techniques is that they mimic this arrangement. Collagen LBL

deposition and the sandwich techniques, polarize hepatocytes through both cell-to-cell and matrix-cell contacts, similar to their physiological state. This leads to the development of bile canaliculi through the reorganization of actin filaments to the hepatocyte borders with cell-to-cell contacts. The thickness of the space of Disse is estimated as 0.2–1.0 μm ⁴⁹, composed of fibrillar collagens and basement membrane matrix molecules, which could be created using this LBL deposition technique. Although we cultured the cells on fibronectin here to isolate the effects of the top matrix layer, pre-coating the device bottom with an ultrathin collagen layer would further mimic the Space of Disse and hepatocyte matrix interactions *in vivo*. Hepatocytes robustly attach to most native, and even denatured, collagens⁵⁰, as well as to methylated and succinylated collagens⁵¹. The reorganization of actin filaments after collagen deposition likely indicates a polarization response to the cell-matrix attachments — either to the collagen fibers themselves, or to other matrix molecules secreted by hepatocytes and kept in the area by the collagen layer⁵².

The deposition of ultrathin, pure collagen layers has many potential applications. Although numerous polyelectrolyte pairs for LBL deposition have been reported, including a number using collagen as one of the pairs^{53–55}, to our knowledge this is the first report of deposition of pure collagen layers with nanometer thickness. Due to its natural abundance, biocompatibility, robust cell attachment sites, and ubiquity in the extracellular matrix, collagen is one of the most frequently used proteins for tissue engineering and biomaterial applications⁵⁶. As with other polyelectrolytes used in LBL, the collagen polymers could be further functionalized or other proteins could be introduced within the layers⁵⁷, depending on the application. In addition to the chemical properties of the layer, the physical thinness of the collagen layer may increase optical clarity for imaging applications and provide a more physiologically relevant barrier to transport than thick hydrogels, which are hundreds of microns in height. Although we did not determine the porosity or density of the layers, the appearance of albumin, a relatively large protein, in the media at expected *in vitro* levels indicates that the thin collagen layers are indeed porous. Another potential application making use of the general, robust cellular attachment to collagen is the creation of precise cell layering, using thin collagen matrix deposition between the layers to hold them together and facilitate cell attachment.

In summary, we report the creation of ultrathin collagen matrix layers using LBL deposition of modified collagen solutions. Using this procedure, we demonstrated the maintenance of hepatocyte morphology and function in microdevices for 14 days, suggesting that this method translates the benefits of the collagen double gel culture method for hepatocytes in plate culture into microdevices. Beyond the immediate applications of this technique to the creation of stable, *in vitro* microfluidic hepatocyte cultures for drug toxicity and liver pathology testing, this technique is generally applicable as a thin biomaterial for many other tissue engineering and biomedical applications.

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Figure 1.

Rat tail collagen solutions were chemically modified to create net positively or net negatively charged polymers. (a) Schematic representations of the succinylation and methylation reactions that modify carboxyl and ϵ -amino groups, respectively. (b) Hydrogen titration curve derivatives showing shifts in the relative numbers of H^+ ions needed to alter solution pH after collagen modification. The SC ϵ -amino and carboxyl peaks were different ($P < 0.01$, $P < 0.001$) than the respective collagen peaks, and the MC carboxyl peak was also different than collagen peak ($P < 0.001$). (c) The net charges (mean $\pm$ SD) of the modified collagen solutions calculated from the H^+ shifts in (b) normalized to native collagen. MC: methylated collagen. SC: succinylated collagen. AA: amino acid residues.

Figure 2. Layer-by-layer deposition of modified collagen solutions created a thin collagen matrix layer on top of the hepatocytes. (a) 3D rendering of the PDMS device (gray) and fluidic space (blue). (b) Schematic representation of hepatocytes seeded on fibronectin on a microdevice and incubated with alternating methylated and succinylated collagen solutions. (c) TEM cross-section of control hepatocytes after 2 days of culture. (d) TEM cross-section of hepatocytes covered with COL LBL. arrows: LBL collagen layer. N: nucleus. Scale bar: 2 μ m. (e) COL LBL layer thickness (mean $\pm$ SD) per cell measured from TEM images (n = 20 total cells from 4 different devices). In the classic double collagen gel technique, the gel thickness above hepatocytes in tissue culture plates is typically 100–500 μ m.

Figure 3.

The COL LBL technique maintained hepatocyte morphology in microdevices over 14 days in culture. Hepatocytes with no top layer in microdevices (negative control) contract and lift off the surface as debris over time (**a–c**). In 6–24-well TC plates, the double collagen gel method (positive control) maintains hepatocyte morphology, including well-defined nuclei, distinct cell borders, and optically dense cytoplasm over time (**d–f**). Similar to the collagen double gel, LBL deposition of modified collagens maintains hepatocyte morphology in microdevices over time (**g–i**). Image scale bar: 100 µm. Inset scale bar: 50 µm.

Figure 4.

Collagen LBL deposition maintains hepatocyte viability and induces bile canaliculus formation by reorganizing actin filament organization, similar to the collagen double gel method. LIVE/DEAD stain with DAPI counterstain (**a–c**) indicates that COL LBL maintained hepatocyte viability at $95 \pm 2\%$ (**a**), $92 \pm 5\%$ (**b**), and $91 \pm 2\%$ (**c**) over the 14 days in culture. CMFDA is a generic cell marker that remains in the cytoplasm of non-polarized hepatocytes (**d**). The development of bile canaliculi can be seen by the excretion of the fluorophore into canaliculus spaces (**e**) that develop around the cells over time (**f**). COL LBL induces a reorganization of hepatocyte actin, changing from diffuse actin staining (**g**) to strong staining at the cell borders, similar to the effects of the collagen double gel (**h,i**). Image scale bar: 100 μm . Inset scale bar: 50 μm .

Figure 5.

COL LBL supports normal *in vitro* hepatocyte albumin and urea secretions and CYP activity over 14 days in culture. Albumin secretion by hepatocytes in COL LBL started low and increased with time until reaching a plateau at day 10, similar to (though slightly delayed from) the collagen double gel curve (a). Urea secretion by COL LBL hepatocytes decreased with time until reaching a plateau at day 10 at a slightly lower magnitude than the collagen double gel, which appeared to still be decreasing (b). The induction of CYP1A activity was high at all times using the collagen double gel. With COL LBL, the activity was low at day 2, but significantly increased at day 7 and maintained through day 14 (c). Data are presented as mean $\pm$ SEM, $n = 6-8$ devices (or wells). Significant effects of culture type (NoTop vs. COL LBL vs. COL GEL) and time (days) were found for albumin ($P < 0.001$, $P < 0.001$) and urea ($P < 0.001$, $P < 0.001$) secretions and CYP activity ($P < 0.001$, $P < 0.001$).