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ARTICLE

Methods For Advanced Hepatocyte Cell Culture In Microwells Utilizing Air Bubbles

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Flat, two-dimensional (2D) cell culture substrates are simple to use but offer little control over cell morphologies and behavior. In this article, we present a number of novel and unique methods for advanced cell culture in microwells utilizing air bubbles as a way to seed cells in order to provide substantial control over cellular microenvironments and organization to achieve specific cell-based applications. These cell culture methods enable controlled formation of stable air bubbles in the microwells that spontaneously formed when polar solvents such as cell culture media are loaded. The presence of air bubbles (air bubble masking) enables highly controllable cell patterning and organization of seeded cells as well as cell co-culture in microwells. In addition, these cell culture methods are simple to use and implement, yet versatile, and have the potential to provide a wide range of microenvironments to improve *in vivo*-like behavior for a number of cell types and applications. The air bubble masking technique can also be used to produce a micron thick layer of collagen film suspended on top of the microwells. These collagen film enclosed microwells could provide an easy way for high throughput drug screening and cytotoxicity assays as different drug compounds could be pre-loaded and dried in selected microwells and then released during cell culture.

Introduction

Since the introduction of the first cell culture flask in 1923 by Alexis Carrel,[1] cells have been routinely cultured on hard, flat, two-dimensional (2D) substrates that offer simplicity and ease of use. However, 2D culture substrates do not provide a tissue or *in vivo*-like microenvironment for the cells. As a result, there is often a substantial gap between the physiological behavior of cells *in vivo* versus their biological response in the 2D artificial environments created by flat culture substrates. Although a number of alternative cell culture formats have been developed, such as hollow fibers, membranes, roller bottles, spinner flasks, wave bags, and continuously stirred tanks and airlift bioreactors, these formats were not designed to offer control over cellular behavior and morphologies, but rather they only provide throughput and scale-up. In recent years, other technologies have been applied to cell culture systems to provide more control over local microenvironments, for example, by using microfluidic technologies to provide well defined diffusion gradients and precise control over metabolites.[2-12] Although microfluidic devices were able to provide biologically relevant data, microfluidic cell culture devices have not been widely used, which is likely due to the complexity and training required for their operation.

In this article, we present several novel and unique methods for advanced cell culture utilizing air bubbles that are capable of directing cellular behavior, organization, morphology, and function in a number of distinct formats. In order to demonstrate these novel cell culture methods, we chose hexagonal "honeycomb" microwells as an example to help demonstrate the versatility of using air bubbles to control cell function. Although other microwell formats such as round or flat bottomed circular microwells could also be used, honeycomb microwells are simple to fabricate with high packing and surface areas. In addition, we designed the honeycomb microwells to be compatible with standard microplate format which allows them to be used with standard laboratory equipment and conventional biological protocols with little to no training required. Our "air bubble" approach can be exploited to present cells with a number of physical microenvironments, substrate moduli, surface chemistries and patterned cell-attachment/signaling molecules on cell-relevant length scales to achieve desired control over cell behavior. Furthermore, these novel cell culture methods could be used to easily modulate the organization and activities of a wide range of cell types such as primary cells, stem cells, induced pluripotent stem cells (iPSCs), cancer cells and cell lines.

Experimental

1. Microwell microplate fabrication, assembly and sterilization

Arrays of honeycomb microwells were fabricated out of poly(dimethylsiloxane) (PDMS) and polystyrene substrates by soft lithography using a photoresist-patterned silicon mold and by hot embossing using a patterned PDMS mold,[13] respectively (Fig. 1). Briefly, features on a plastic photo mask were first transferred onto a silicon wafer using the standard

photolithographic process. The photoresist-defined silicon wafer was then used as a mold for casting a PDMS prepolymer (10 : 1 w/w) (Sylgard® 184, Dow Corning Corporation, Midland, MI, USA). After curing at room temperature for at least 24 hours to minimize shrinkage, the PDMS replica was carefully peeled away from the silicon wafer. Next, the PDMS replica was attached to the bottom of a standard flat bottom 96-well holey (bottomless) microplate (Corning Incorporated, Corning, NY, USA) using a double-sided pressure sensitive adhesive (PSA) sheet (ARcare 90106®, Adhesive Research, Inc., Glen Rock, PA, USA). For polystyrene-based honeycomb microwell substrates, the PDMS replica was used as a mold to fabricate a reverse PDMS honeycomb microwell mold. The reverse PDMS honeycomb microwell mold was then used to hot emboss a polystyrene substrate as previously described.[13] Next, the embossed polystyrene substrate was attached to the bottom of a standard flat bottom 96-well holey microplate using the same double-sided PSA sheet as the PDMS-based honeycomb microwell microplate. For some cell culture experiments, the honeycomb microwells were treated with oxygen plasma after assembly. Finally, before each cell culture experiment, we sterilized each individual honeycomb microwells of the assembled microplates with 300 µl of 70 % ethanol for 30 minutes, followed by washing twice with 300 µl of sterile deionized water and air drying the assembled microplates in a biosafety cabinet for 15 minutes.

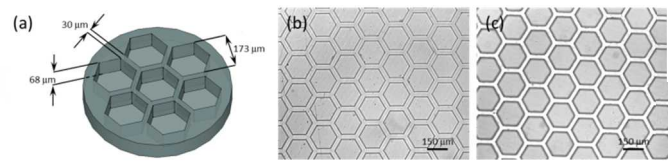

Fig. 1 (a) Schematic diagram of a honeycomb microwell substrate. Images of honeycomb microwell substrate fabricated out of (b) PDMS and (c) polystyrene by soft lithography using a photoresist-patterned silicon mold and by hot embossing using a patterned PDMS mold, respectively.

2. Air bubble mask cell seeding methods

Various cell culture methods presented here utilized the formation and/or removal of air bubbles in the honeycomb microwells (Fig. 2). In the presence of live cells, honeycomb microwells spontaneously filled in with cell culture media and cells within a few hours (Fig. 2a). Control experiments were performed using honeycomb microwells loaded with only cell culture media. The cell culture media loaded honeycomb microwells were incubated inside a 37 °C incubator with and without 5 % CO_2. In all control experiments, air bubbles remained inside the honeycomb microwells even after 72 hour incubation. Alternatively, honeycomb microwells can be forcibly filled before cell seeding by first filling the microwells with an absolute ethanol solution followed by flushing with water, buffer or cell culture media. In this way, honeycomb microwells were filled without any formation of air bubbles. Ethanol was chosen based on its interfacial free energy (≈ 22.4 dyn/cm) and advancing and receding contact angles (31 ° ± 10 ° and 20 ° ± 10 °, respectively) on PDMS (interfacial free energy ≈ 21 dyn/cm) that could fill the honeycomb microwells effectively.[14] Also, although PDMS is swollen by many organic solvents,[15] it is unaffected by other polar solvents such as alcohols. In addition, we believe that the discontinuous dewetting technique demonstrated by Jackman *et al.* could also be used to selectively fill the honeycomb microwells.[14]

On the other hand, because of the design of the honeycomb microwell dimensions (173 µm in diameter and 68 µm in height with 30 µm spacing), air bubbles were spontaneously formed within honeycomb microwells by adding a weakly polar solvent, such as cell culture media, or hydrogel solutions, such as collagen gel solution, onto the honeycomb microwells (Fig. 2b). These air bubbles act as a mask to mask off the honeycomb microwells while leaving the top honeycomb microwell walls exposable to extra-cellular matrix (ECM) protein and/or cells containing cell culture media. The air bubbles can also be used to suspend a solution of ECM, such as collagen gel solution, which can be dried at 37 °C to form a micron thick suspended ECM film on the top of the honeycomb microwells enclosing the honeycomb microwells (Fig. 2c).

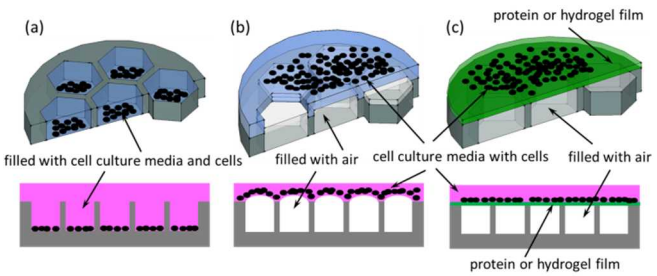

Fig. 2 Schematic diagrams of different honeycomb microwell cell seeding methods. Top and bottom panels are three-dimensional (3D) and cross-sectional views, respectively. (a) Filled microwells: cell culture media and cells are seeded in honeycomb microwells. (b) Air bubble masked: air bubbles act as a mask for patterning and seeding cells. (c) Film covered: gel film formed on top of honeycomb microwells with the aid of air bubble mask and cells can be seeded on the gel film.

2.1 Filled microwells

2.1.1 Uniform cell spheroid (aggregate) formation in microwells

We first pipetted 100 µl of absolute ethanol into each well of the 96-well honeycomb microwell-patterned microplate to fill the honeycomb microwells. Ethanol was then aspirated out of the microplate wells and the microplate wells were washed twice with cell culture media (DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin) before seeding HepG2/C3A cells at a concentration of 30,000 cells per microplate well in 150 µl of cell culture media (Fig. 2a). After cells were cultured for several days at 37 ºC and 5 % CO_2, they were stained with 2 µM calcein-AM (Invitrogen Corporation, Carlsbad, CA, USA) in cell culture media for 15 minutes, washed with phosphate buffered saline (PBS) solution and imaged using a Zeiss Axiovert Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) to visualize their viability. HepG2/C3A hepatocyte cells were chosen as a model cell line to demonstrate the different culture formats in the honeycomb microwells because these cells are capable of adherent cell growth, growth in clusters and hepatocytes are of particular interest in pharmacokinetic studies.

2.1.2 Uniform cell spheroid formation in microwells by gel encapsulation

HepG2/C3A cells were encapsulated inside polymerized gels such as collagen using the honeycomb microwell filling method similar to the one described in Section 2.1.1. We first pre-filled the microplate wells with absolute ethanol, washed twice with cell culture media and then seeded each microwell with 30,000 cells suspended in 50 µl of chilled Chemicon collagen I solution neutralized in DMEM media (EMD Millipore, Billerica, MA, USA) according to the manufacturer's specifications. After centrifuging at 100 g for 5 minutes, the seeded microplate was incubated at 37 ºC and 5 % CO_2 for 1 hour to polymerize the collagen gel. After collagen polymerization, an additional 100 µl of serum containing DMEM cell culture media was added to each microplate well and the cells were cultured for several days before viability staining and visualization.

2.2 Air bubble masked microwells

2.2.1 Cell patterning on the top walls of microwells

HepG2/C3A cells were patterned on the top walls of honeycomb microwells using the air bubble masking technique (Fig. 2b). First, the honeycomb microwell-patterned microplate wells were washed twice with cell culture media (DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin) before adding 50 µl of chilled Chemicon collagen I solution. In this case, since honeycomb microwells were not pre-filled with absolute ethanol, air bubbles were instantaneously formed and masked off the honeycomb microwells only exposing the top walls to the collagen solution. Next, the collagen loaded microplate wells were placed on ice for 15 minutes before the collagen solution was completely aspirated out. HepG2/C3A cells were then immediately seeded at a concentration of 30,000 cells per microplate well in 150 µl of cell culture media. Finally, cells were cultured for 24 hours at 37 ºC and 5 % CO_2 before viability staining and visualization.

2.2.2 Co-culture using microwells

The first cell type was first seeded and cultured on the top walls of the honeycomb microwells as described in Section 2.2.1. Because of the presence of live cells and after overnight cell culture, the air bubbles that were used to mask and pattern the first cell type on the top walls of the honeycomb microwells spontaneously dissolved and all the honeycomb microwells were filled with cell culture media. We observed that this air bubble removal process was very reproducible in the presence of live cells. Next, a second cell type was added to the well and centrifuged at 100 g for 5 minutes. Freshly added cells were seeded at the bottom of each microwell filled with cell culture media for co-culturing with the first cell type that was already attached on the top walls of the honeycomb microwells. In order to demonstrate this co-culture method, HepG2/C3A cells that were cultured on the top walls of the honeycomb microwells were first nuclear stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) after 24 hours of cell culture. Then, freshly trypsinized HepG2/C3A cells were viability stained with calcein-AM (LIVE/DEAD® Viability/Cytotoxicity Assay Kit, Molecular Probes, Inc., Eugene, OR, USA) and seeded at a concentration of 30,000 cells per microplate well into the honeycomb microwells as described above.

2.2.3 Cell culture on suspended collagen film

The air bubble masking technique can also be used to produce a micron thick layer of collagen film suspended on top of the honeycomb microwells for cell culture applications (Fig. 2c). In this case, 100 µl of chilled Chemicon collagen I gel solution was first added to each honeycomb microwell-patterned microplate well and 70 – 80 µl of collagen gel solution was immediately removed from each filled microplate well. The excess collagen gel solution was needed initially to ensure uniform distribution of the collagen gel solution in each microplate well. Next, the honeycomb microwell-patterned microplate was allowed to dry at 37 ºC for overnight. After overnight drying, a micron thick layer of collagen film was formed on the top of the honeycomb microwells enclosing the honeycomb microwells. HepG2/C3A cells were then seeded at a density of 30,000 cells per microplate well in 150 µl of cell culture media on the collagen film covered honeycomb microwells. Cells were cultured for additional 24 hours at 37 ºC and 5 % CO_2 before viability staining and visualization.

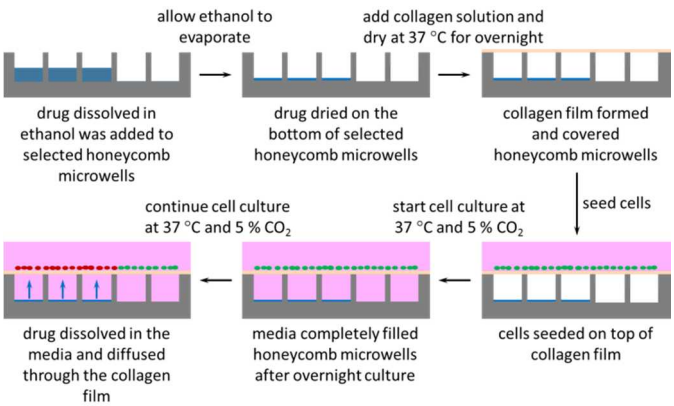

Fig. 3 Schematic diagrams depicting the working principle of drug release using suspended collagen film on honeycomb microwells.

2.2.4 Drug release using suspended collagen film

The collagen film covered honeycomb microwells can also be used for high throughput drug screening and cytotoxicity assays. In this case, a drug compound, such as Nefazodone (Sigma-Aldrich, St. Louis, MO, USA), was first dissolved in ethanol and added to the honeycomb microwells (Fig. 3). Because of the presence of ethanol, the honeycomb microwells were spontaneously filled without any air bubbles. Next, the drug mixture was allowed to evaporate and dry on the bottom of the honeycomb microwells. After the drug mixture was completed dried out, a collagen film was added on the top of the honeycomb microwells to enclose the honeycomb microwells as described in Section 2.2.3. Next, HepG2/C3A cells were seeded on the collagen film. Cells were then

cultured for 24 hours at 37 ºC and 5 % CO_2 before viability staining and visualization.

3. Viability cell staining

Cell viability assays were performed using the calcien AM substrate in combination with DAPI which stains all cell nuclei. Briefly, cells were incubated with the fluorescent dye mixture according to manufacturer's instructions for 15 minutes at 37 °C inside a cell culture incubator followed by the PBS buffer wash. Fluorescent live and dead staining images were collected using a Zeiss Axiovert 200 inverted fluorescence microscope equipped with an epifluorescence condenser and camera system (Carl Zeiss MicroImaging, Inc.).

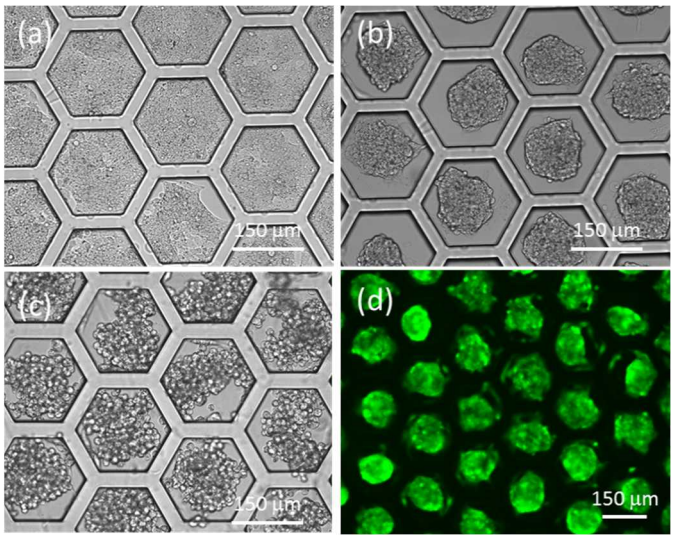

Fig. 4 (a) – (c) Bright field and (d) fluorescent images of HepG2/C3A cells on the bottom of the honeycomb microwells after 72 hours of cell culture. Cell cultured in the polystyrene honeycomb microwells (a) that were treated and (b) non-treated with oxygen plasma. (c) Cells embedded in a collagen gel were cultured in the non-oxygen plasma treated PDMS honeycomb microwells. (d) Fluorescent image of cells cultured in (b) stained with calcein-AM to study their viability. Cell seeding density was 30,000 cells per microplate well in all cases.

Results and Discussion

Microwell materials, surface treatment and design parameters

It is well-known that materials, surface treatments and surface coatings of cell culture substrates are important factors for modulating cell morphologies and/or functions because of the different properties such as stiffness, surface energy, chemistry, roughness and propensity to adsorb or absorb biomolecules. Thus, the choice of cell culture substrate materials, surface treatments or surface coatings is important for achieving the desired morphology and function. In this study, by way of example, we fabricated honeycomb microwells out of PDMS and polystyrene treated or non-treated with oxygen plasma (Fig. 1). As expected, cells cultured in honeycomb microwells fabricated out of polystyrene treated or non-treated with oxygen plasma resulted in two different morphologies. A 2D cell monolayer formed when cells were cultured in oxygen plasma treated polystyrene honeycomb microwells (Fig. 4a) whereas cells self-assembled into highly uniform cell spheroids when cultured in non-oxygen plasma treated polystyrene honeycomb microwells (Fig. 4b and 4d). Non-oxygen plasma treated polystyrene substrate has a low cell attachment property which hinders cell spreading and favors cells to form three-dimensional (3D) spheroidal cell aggregates. In contrast, oxygen plasma treated polystyrene substrate has a strong cell adhesion property which allows cells to spread easily resulting in a 2D cell monolayer. However, the choice of cell culture substrate treatment may have less influence when cross-linked hydrogels such as collagen are used during cell seeding. When cells were first embedded in a collagen gel solution and then cultured in PDMS (which also has a low cell attachment property) honeycomb microwells without oxygen plasma treatment, similar cell spheroids could be achieved due to the physical encapsulation by the collagen gel (Fig. 4c).

Air bubble formation and removal in microwells during liquid filling depend on the interfacial free energies of the substrate and the liquid of interest, and the topology of the surface.[14, 16] Jackman *et al.* presented experimental insights and a discontinuous dewetting technique to fill large arrays of circular microwells while Kawai and Nagata studied the effect of roughness on the wettability using patterned square-shaped microwells. Jackman *et al.* demonstrated that the discontinuous dewetting technique could be used to fill large arrays of microwells with aspect ratios (depth : width) between 1 : 5 and 1 : 1 using different liquids. Diameters (and depths) of their microwells were ranged between 2 μm (~ 1 μm deep), 10 μm (1 – 2 μm deep), 50 μm (1 – 50 μm deep), and 1000 μm (40 – 200 μm deep. Thus, both Jackman *et al.* and Kawai and Nagata's work could be used as a guideline for air bubble formation and removal in microwell design for the air bubble masking technique.

The dimensions of the microwells are important not only for air bubble formation and removal but also for enabling cell culture. For example, in this study, we chose the honeycomb microwell diameter to be approximately 200 µm based on fluid diffusional limitations while providing an effective separation of cells. It is well understood that cell spheroids above 200 µm in diameter begin to form necrotic centers as oxygen and nutrients have limited diffusion through densely packed cells of over 100 µm.[17, 18] Thus, the chosen diameter effectively limits cell spheroid sizes, creating uniform cell spheroids with minimum necrosis (Fig. 4b – 4d). Smaller diameter honeycomb microwells have been successfully used as well (data not shown), however, smaller diameter honeycomb microwells may hinder cell seeding and air bubble removal when seeding cells onto the bottom of the honeycomb microwells. When hydrophobic 2D flat culture substrates are used for spheroidal culture, poorly attached cell spheroids are often washed away during cell culture media exchanges. Thus the depth of honeycomb microwells was chosen to be approximately 70 µm, in order to allow cell culture media and buffer exchanges with minimum cell loss. Although deeper honeycomb microwells may be more effective in retaining cells during cell culture media exchanges, it makes the honeycomb

microwell fabrication more difficult and less reliable due to the higher aspect ratio of the honeycomb microwell. The spacing between individual honeycomb microwells was chosen to be 30 μm wide for optimal separation of individual honeycomb microwells as it minimizes the risk of cells bridging across individual honeycomb microwells making top wall-bottom microwell co-culture difficult (Fig. 5).

Air bubble mask cell seeding methods and patterning

By combining air bubble mask cell seeding methods in microwells with appropriate surface treatments and materials, one has the potential to create unique microenvironments to control cell morphologies and behaviors. In this study, we used HepG2/C3A cells as a cell model to demonstrate that air bubble masking can be used in combination with microwells to control cell seeding and cell morphology. For example, HepG2/C3A cells were patterned on the top walls of PDMS honeycomb microwells coated with collagen by using the air bubble masking technique to prevent adsorption of collagen on the bottom of microwells (Fig. 5a). This technique led to the growth of liver cells into cord-like structures reminiscent of normal liver architecture. Moreover, since air bubbles were subsequently dissolved, a second cell type may be seeded into the microwells to create a co-culture model system. In the present study, the feasibility of preforming co-cultures was demonstrated by plating HepG2/C3A cells preloaded with the viability stain calcein AM into the microwells (Fig 5b).

Although we only used HepG2/C3A cells as the cell model to demonstrate the versatility of the air bubble mask cell seeding methods in the honeycomb microwells, other cell types such as primary human cells, pluripotent and adult stem cells can also be used. Also, air bubble mask cell seeding methods are not limited to flat-bottomed honeycomb microwells and can be applied to other shapes of microwells with flat or rounded-bottom microwells.

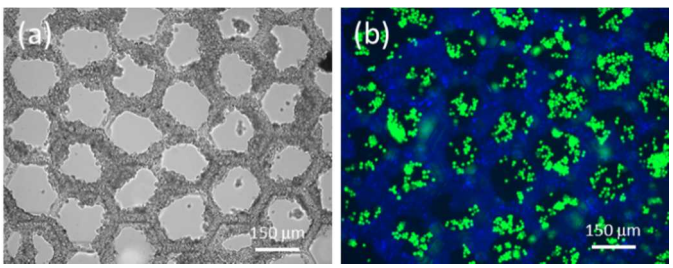

Fig. 5 (a) Bright field image of HepG2/C3A cells cultured on collagen coated top walls of PDMS honeycomb microwells for 24 hours. (b) After 24 hours top wall-patterned HepG2/C3A cells were nuclear stained with DAPI (blue) and the HepG2/C3A cells preloaded with the viability stain, calcien AM (green) were cultured inside the honeycomb microwells. Top walls of the honeycomb microwells were patterned with collagen using the air bubble masking technique. Cell seeding density for each cell seeding step was 30,000 cells per microplate well.

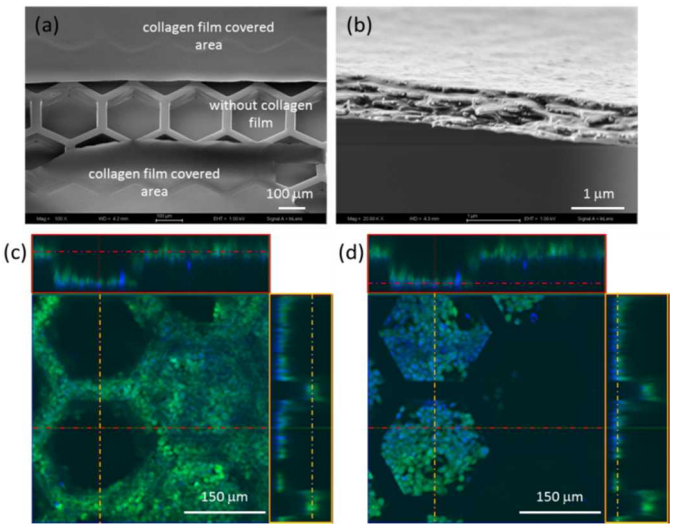

Fig. 6 Scanning electron microscope (SEM) images of (a) collagen film covered PDMS honeycomb microwells and (b) a portion of the collagen film. (c) and (d) Confocal images of HepG2/C3A cells stained with calcein-AM (green) and DAPI (blue). Cells were cultured (c) on top of collagen film and (d) on the bottom of the PDMS honeycomb microwells after 24 hours of cell culture. Cell seeding density was 30,000 cells per microplate well.

Drug release using suspended collagen film

One of the novel and unique advantages of the air bubble mask cell seeding method is that it provides an easy way to create a micron thick collagen film to enclose the microwells and then cells can be cultured on the collagen film (Fig. 6) for high throughput drug screening and cytotoxicity assays as different drug compounds could be pre-loaded and dried in selected microwells, for example, using a non-contact nano and/or microliter liquid handling devices (BioFluidix GmbH, Freiburg, Germany) or using the discontinuous dewetting technique demonstrated by Jackman *et al.*,[14] before enclosing the microwells with the collagen film. As a proof-of-concept experiment, Nefazodone, a drug compound that is toxic to HepG2/C3A cells, was first loaded and dried in the left half of the honeycomb microwells (Fig. 3 and 7a) before enclosing all the honeycomb microwells with the collagen film. After seeding cells on the collagen film and culturing overnight, all the honeycomb microwells were filled with cell culture media while cells were still culturing on the collagen film above. As a result, Nefazodone was dissolved in the cell culture media in the honeycomb microwells and gradually diffused through the collagen film to the cells above. After 24 hours of additional cell culture, the viability cell staining clearly revealed that most cells on the left half (drug loaded area) of the honeycomb microwells were dead while cells on the right half (drug free area) of the honeycomb microwells were still alive (Fig. 7b and 7c) demonstrating the novel and unique drug release concept using the air bubble mask cell seeding methods together with the microwells.

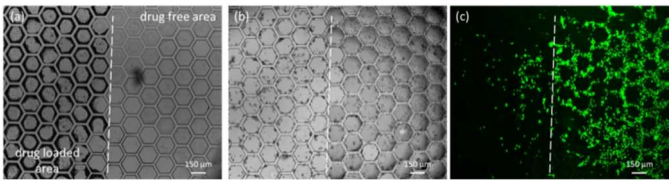

Fig. 7 (a) Bright field image of honeycomb microwells after loading and drying of Nefazodone (the drug) on the bottom of selected honeycomb microwells. (b) Bright field and (c) viability stained fluorescent images of HepG2/C3A cells after 24 hours of cell culture with dried Nefazodone on the bottom of selected honeycomb microwells. The white dashed lines are the boundary between the drug loaded (left) and drug free (right) areas. Cell seeding density was 10,000 cells per microplate well.

Conclusions

In this study, we demonstrated several novel and unique air bubble mask cell seeding methods for advanced cell culture utilizing air bubbles together with microwells to direct cell behavior, organization, morphology, and function in a number of distinct formats. The air bubble masking technique can also be used to produce a micron thick layer of collagen film suspended on top of the microwells. These collagen film enclosed microwells could provide an easy way for high throughput drug screening and cytotoxicity assays as different drug compounds could be pre-loaded and dried in selected microwells and then released during cell culture.

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