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Efficient one-step production of microencapsulated hepatocyte spheroids with enhanced functions

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Abstract

Hepatocyte spheroids microencapsulated in hydrogels can contribute to liver research in various capacities. Conventional approach of microencapsulating spheroids produces variable number of spheroids per microgel and requires an extra step of spheroid loading into the gel. Here, we report a microfluidics technology bypassing the step of spheroid loading and controlling the spheroid characteristics. Double-emulsion (DE) droplets were used to generate microencapsulated homotypic or heterotypic hepatocyte spheroids (all as single spheroids <200 μm in diameter) with enhanced functions in 4 hours. The composition of the microgel is tunable as demonstrated by improved hepatocyte functions during 24 day culture (albumin secretion, urea secretion and cytochrome P450 activity) when alginate-collagen composite hydrogel was used instead of alginate. Hepatocyte spheroids in alginate-collagen also performed better than hepatocytes cultured in collagen-sandwich configuration. Moreover, hepatocyte functions were significantly enhanced when hepatocytes and endothelial progenitor cells (used as a novel supporting cell source) were co-cultured to form composite spheroids at an optimal ratio of 5 to 1, which could be further boosted when encapsulated in alginate-collagen. This microencapsulated-spheroid formation technology with high yield, versatility and uniformity is envisioned to be an enabling technology for liver tissue engineering as well as biomanufacturing.

Keywords

Microdroplets; microfluidics; encapsulation; tissue engineering; hepatocyte spheroids

1. Introduction

The past decades have witnessed the exciting advances of cell microencapsulation technology in the medical field - beginning with the use of alginate-polylysine capsules for islet transplantation.^[1] The envelopment of tissues or cells in microcapsules and microgels with semi-permeable membrane can protect the enclosed cells from the host immune system upon transplantation, facilitating the use of xenogeneic cell sources in clinical

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

applications.^[2] Besides, given that many cell types are fragile and highly vulnerable to shear forces, the microcapsule/microgel layer can shield the enclosed cells from shear damage in bioreactor culture.^[3] Lastly, the encapsulation material itself or entrapped growth factors provide localized delivery of mechanical and biochemical cues to support or stimulate the functions or differentiation of the encapsulated cells.^[4, 5, 6]

The intrinsic characteristics associated with microcapsules/microgels such as short diffusion distance and high surface-to-volume ratio have spurred interests to use microencapsulated cells to treat various diseases.^[2, 7] One notable example is the development of bioartificial liver. Most of the extracorporeal bioartificial liver systems currently examined in clinical trials involve a hollow fiber design where individual hepatocytes are immuno-isolated via hydrogel entrapment or membrane compartmentalization.^[8] While the membrane offers protection from immune attack and shear force, it also creates a diffusion barrier to mass exchange.^[9] In addition, flow rates within the bioreactors are low (100–200 mL/min) compared with those of *in vivo* perfusion (~1,500 mL/min) due to resistance within the fibers.^[10] Entrapment of hepatocytes into microgels has been shown to preserve hepatocyte functions when they were cultured in bioreactor.^[11] A fluidized or packed bed bioreactor containing microencapsulated hepatocytes appears promising in overcoming the limitations encountered with current liver support systems.^[12, 13] In addition, injection of encapsulated hepatocytes intraperitoneally has been proposed to treat liver-associated inborn metabolic disease and acute liver failure as a better alternative to intrahepatic delivery of hepatocyte in suspension.^[14, 15] The microgel layer (e.g. alginate) provides anchorage and protection against host immune attack for hepatocytes, leading to better cell viability and functions in the engraftment site. Maintaining functional longevity of hepatocytes *in vitro* to more closely reflect the characteristics of liver *in vivo* is also the key to a more effective drug-screening platform.^[16]

It is particularly attractive to encapsulate hepatocyte spheroids owing to the improved cellular functions mediated by cell-cell interactions.^[17, 18, 19] Traditionally, hepatocyte spheroids are generated before loading into microdroplets of hydrogel solution followed by polymerization.^[20] To avoid clogging of spheroids in the nozzle or needle where microdroplet is generated, there exists a minimum diameter requirement of the nozzle/needle which leads to diffusional limitations and large transplant/device volume imposed by the size of capsule generated (500-1000 μm in diameter).^[21] In addition, non-uniform distribution of spheroid in capsule following Poisson equation is observed, resulting in empty capsules and possible agglomeration of multiple spheroids.^[22] Since billions of hepatocytes are required to recapitulate liver function in the case of liver failure^[8], the challenge of generating millions of hepatocyte spheroids and subsequently encapsulating them in a well-controlled and reproducible manner would be a hurdle to satisfy the Good Manufacturing Practice (GMP) for clinical translation.

Microfluidics has emerged as a high-throughput platform for biochemical assays and bioprocessing.^[23, 24] Employing microfluidics to generate tiny monodisperse emulsion droplets creates microscale bioreactor and can be leveraged for scalable biomanufacturing of microencapsulated spheroids.^[25] Earlier reports usually relied on formation of solid microgel or microcapsule encapsulating individual cells before the cells proliferated to form

spheroids, which could take up to 5 days.^[26, 27] In this study, we report a high-throughput DE (water-in-oil-in-water) platform that promotes cell assembly within the droplet in 4 hours and subsequently induces the polymerization of the inner hydrogel phase to generate microencapsulated hepatocyte spheroids (Figure 1a). We have previously shown that DE droplets can serve as platform for cell culture.^[28, 16] Without any restriction on nozzle/needle size, the diameter of the microgel can be readily reduced to below 200 μm . Importantly, the inner phase of each droplet polymerizes individually to generate microgels all containing single spheroids. We demonstrate that the biochemical composition of the inner phase can be tuned to deliver appropriate cues for controlling hepatocyte behavior.

Besides matrix microenvironment, hepatocytes are surrounded by various types of cells such as endothelial cells, Kupffer cells and stellate cells in liver. In order to recapitulate the different types of heterocellular interactions present in liver, many attempts have focused on co-culturing hepatocytes with other cell types such as liver sinusoidal endothelial cells (LSEC), umbilical vein endothelial cells (HUVEC), kupffer cells, stellate cells, or even fibroblasts and mesenchymal stem cells.^[29, 30, 31, 32] In general, hepatocyte functions are enhanced and sustained with co-culture. Among the supporting cell types tested, endothelial cells are attractive as co-culture of hepatocytes and endothelial cells emulates the *in vivo* situation where the two cell types form a continuous lining along the sinusoid separated by the space of Disse.^[33] Endothelial cells also contribute to vascularization of *ex vivo* engineered constructs to increase oxygen and nutrients supply,^[34] and to mimic human physiological system.^[35, 36, 37] In view of the invasive collection or limited supply of LSEC and HUVEC, we explored the feasibility of using EPC as an alternative supporting cell source. EPC are highly proliferative, able to differentiate into endothelial cells and available from umbilical cord as well as adult peripheral blood.^[38, 39] Moreover, EPC express liver morphogen such as hepatocyte growth factor (HGF) which has been shown to stimulate albumin production in hepatocytes.^[40, 41] Therefore, we hypothesized that co-culture of hepatocytes and EPC could enhance hepatocyte functions just like a conducive microenvironment does. To our best knowledge, this is the first study which researched into the co-culture of hepatocytes and EPC.

2. Results

2.1. Generation of DE droplet and hepatocyte spheroid-encapsulated microgel

Monodisperse DE droplet (~200 μm in inner phase diameter) was generated (>20 Hz) using two connected microfluidics flow-focusing devices (200 μm in channel width and depth) made from polydimethylsiloxane (PDMS) (Figure 1b): the first device produced aqueous (cells suspended in hydrogel solution) droplets in an oil phase and the second device with hydrophilic chip surface produced droplet with aqueous core and oil shell dispersed in an external aqueous phase (Figure 1c). The size of the droplet is tunable by altering the device design or flow rates of liquid (Figure S1). Primary rat hepatocytes at 6 million cells/mL could be encased in the DE droplets (~4 nL/droplet) in accordance with Poisson distribution, giving rise to around 30 cells in each droplet (Figure 2a). At 4 hours post droplet formation, monodisperse spheroids were formed (Figure 2b). The oil shell could be removed by pipetting the droplets on top of a cell strainer then the oil would evaporate after transient

contact with air (Figure S2). The inner phase (1% alginate) could be induced to polymerize upon oil shell removal and exposure to calcium chloride solution, generating microgels with single spheroids. The average size of the microgels was around 200 μm , the same as the DE droplets (Figure 2c). The microgel size and hence the thickness of the microgel layer is tunable as demonstrated by the production of smaller gel (122 μm) containing spheroids of ~80 μm diameter using a device with a reduced channel width and depth (100 μm) (Figure S3).

The biochemical composition of the droplet inner phase could be precisely controlled to modulate hepatocyte functions. To demonstrate, 0.25 mg/mL rat tail collagen I was added to the alginate solution, which was applied as the inner phase of droplet. Collagen I is one of the native extracellular matrix components and can be found in tissues like tendons, ligaments and skin. It maintains hepatocyte functions efficiently when used as a substrate matrix during culture,^[42] and can also serve as an immune-protective material.^[19] Immunostaining of the spheroids encapsulated in microgels after their formation verified the presence of collagen fibrils around both human HEK-293 and rat hepatocyte spheroids, suggesting the collagen fibrils did not originate from the rat hepatocytes and could supply biochemical and mechanical cues in microscale range (Figure 2d).

2.2. Characterization of hepatocytes cultured in 2D and 3D

Maintaining long-term hepatocyte functions has been a challenge of liver tissue engineering. Collagen sandwich is the current gold standard of culturing hepatocytes in 2D^[42]. In a collagen-sandwich configuration, hepatocytes were situated between two layers of collagen gel and exposed to microenvironment cues in a pseudo-3D configuration where they displayed normal cubic cell shape with tight cell-cell junctions after 7 days of culture, a feature that was lost when the top collagen layer was absent (Figure 3a). Using hepatocytes cultured in the collagen sandwich configuration (Col-sandwich) as a 2D control, the performance of the microencapsulated hepatocyte spheroids with different microscale niche was compared. After 24 days of culture, hepatocyte spheroids maintained their compact, spherical morphology and viability in alginate-collagen (Alg-col) microgels, whereas the two features were lost in hepatocyte spheroids encapsulated in alginate (Alg) microgels (Figure 3b). Hepatocytes cultured in Col-sandwich could also maintain the morphology and viability over the course of 24 days. 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) staining indicated the activity of the MRP-2 transporter and formation of bile canaliculi in all three cases. In the case of Col-sandwich, channel-like canaliculi were observed in some regions, whereas the spheroid samples displayed patchy and increased amount of signals. Functional assessments showed that the amount of albumin synthesized by hepatocytes per day in Alg-col was higher than the other two cases on day 12, 16 and 18 (Figure 4a), and the cumulative amount of albumin synthesized was also significantly higher than the other two cases (Figure 4b). Hepatocytes encapsulated in Alg-col synthesized significantly more urea on a few days but there was not significant difference in the cumulative level of release (Figure 4c & 4d). Lastly, cytochrome P450 3A4 (CYP3A4) luminogenic assay revealed that the basal level of CYP3A4 activity of hepatocytes in Alg-col was ~4-5 fold & ~15 fold higher than the those in Alg and Col-sandwich at day 16 and 24 respectively (Figure 4e). The induction of CYP3A4 activity (3-5 fold) upon 10 μM

dexamethasone exposure of all three cases conformed with results reported in literature (Figure 4f).^[43] Overall, the results of this experiment showed that hepatocytes in Alg-col exhibited the highest functions, while those in Alg and Col-sandwich performed similarly but inferiorly. The results also implied that both 3D culture configuration with extensive cell-cell interactions and a conducive matrix microenvironment are important to maintaining hepatocyte functions. Given that other proteins such as fibronectin and hyaluronic acid can be incorporated in our system readily, our technology holds promise to screen for optimal matrix microenvironment for spheroid culture in many applications.

2.3. Investigation of the co-culture of EPC and Hep

As a first step of the co-culture experiment, different media formulations were tested to determine the optimal cell culture conditions for both types of cells. EGM-2, the culture medium for EPC, was mixed with hepatocyte medium at assorted ratios for screening. The proliferation and viability of EPC decreased as the fraction of hepatocyte medium increased while hepatocyte viability was poor when they were cultured in EGM-2. The cumulative albumin and urea secretions were not significantly different when the hepatocyte medium constituted 50 or 66% of the co-culture medium (Figure S4). Consequently, a 1:1 mixture of EGM-2 and hepatocyte medium was selected to best preserve the functions and viability of EPC and hepatocytes respectively.

Next, various ratios (5:1, 3:1, 1:1, 1:3) of hepatocytes and EPC were mixed and encased into DE droplets to generate co-culture spheroids encapsulated in alginate microgels. Before loaded into droplets, the two cell types were labelled with different celltracker markers to assess their organization in the composite spheroid (Figure 5a). Analysis on the fluorescent images taken with the spheroids showed that at low hepatocyte to EPC ratio, EPC tended to envelop individual hepatocytes. When their numbers were approximately equal, the two cell types distributed evenly. As hepatocyte fraction increased, hepatocytes preferentially aggregated, leaving the EPC on the periphery. Functional assessments showed that EPC improved hepatocyte performance (syntheses of albumin and urea, basal activity of CYP3A4) significantly when the ratio of hepatocyte to EPC was 5:1 (Figure 5b – 5d). The performance declined as EPC fraction increased. Whilst our data confirmed that EPC could support hepatocyte functions in a co-culture spheroid configuration, the influence was only observed at certain cell-to-cell ratio. At some other ratios (1:1 and 1:3) the effect was even opposite where hepatocyte performance was below that of control. One possible explanation is that, as EPC fraction increased, homocellular interactions among hepatocytes were disrupted which could not be substituted with heterocellular influence from EPC. This might have a larger effect on albumin secretory ability of the cells than production of urea and cytochrome activity, as seen by the significantly lower level of albumin secretion in the case of 1:3. Further research will be needed to understand the mechanisms of co-culture effect and prove the hypothesis.

2.4. Combination of heterocellular influence and conducive matrix cue

Finally, we aimed at investigating whether conducive matrix cues would complement heterocellular interactions to further increase hepatocyte performance synergistically. Hepatocyte spheroids encapsulated in Alg-col (Hep in Alg-col), co-culture spheroids (5:1

ratio) encapsulated in Alg (HepEPC in Alg) and Alg-col (HepEPC in Alg-col) were generated and analyzed (Figure 6a). The spheroids were immunofluorescently stained to discern the two distinct types of cells. Staining for albumin and von Willibrand factor (vWF) indicated strong staining from heptaocytes and the presence of EPC. Sign of angiogenesis was observed in some spheroids encapsulated in either alginate or alginate-collagen (Figure S5). The viability of all three cases was well preserved at day 14 and formation of bile canaliculi was also observed. The amount of cumulative albumin synthesized were comparable between Hep in Alg-col and HepEPC in Alg and significantly higher in HepEPC in Alg-col (Figure 6b) while the difference in urea secretion was not significantly different (Figure 6c). For basal CYP3A4 activity, the levels of three cases were not significantly different at day 8, however HepEPC in Alg-col recorded significantly higher level of activity (~2 times of other groups) at day 16 (Figure 6d). Applying 10 μ M dexamethasone to HepEPC in Alg-col induced CYP3A4 activity by 5 fold which was again consistent with literature data (Figure 6e).^[43] Overall, our data suggested that matrix cues from collagen exerted a similar supporting effect to co-culture with EPC on hepatocytes, and hepatocytes responded to both types of influence when they were supplied simultaneously. This example is a clear manifestation of the capability of our technology in optimizing both cell-cell and cell-extracellular matrix interactions for hepatocyte spheroid encapsulation.

3. Discussion

Microencapsulated hepatocyte spheroids can be applied in the fluidized bed of bioreactor for bioartificial liver, direct injection into the peritoneal space or drug screening platform.^[44, 11, 31] Our technology, in particular, offers certain benefits over traditional microencapsulation technology. First of all, the microgel size is smaller than 200 μ m containing a spheroid of ~80 μ m. This is hard to achieve with existing technology as the needle/nozzle size would be restrained by the size of spheroids encapsulated and shear force exerted on cells, resulting in a size range of 500 – 1000 μ m of the microgel/microcapsule generated.^[21] Reducing the microgel/microcapsule size would definitely be advantageous as the hydrogel layer has proven a significant barrier to the diffusion of large molecules. Literature data showed that it took 15 min versus 1 hr for the release of bovine albumin serum to reach equilibrium from alginate gels of 400 μ m and 1 mm in diameter respectively.^[45] Our data showed that it took longer, though not significantly, for albumin to diffuse out from the 300 μ m microgels than the 122 μ m ones, demonstrating microgels with larger surface-to-volume ratio enables faster diffusion (Figure 7). Since many of the liver's substrates for detoxification and synthetic products are large molecules, the presence of a diffusion barrier associated with the immobilization materials may partially explain why so few, if not none, of the existing bioartificial livers have attained satisfactory results in clinical trials.^[9] From a clinical point of view it is also important to decrease the total device volume by using smaller microgels in order to reduce patients' extracorporeal plasma compartment and hence prevent hypotension occurring in patients.^[46] Secondly, perhaps more importantly, our technology produces microgels containing single spheroid, in contrast to a Poisson-distributed fashion. The latter scenario will lead to possible agglomeration of multiple spheroids within a single microgel/microcapsule, compromising molecular transport to and from a fused spheroid. There is wide consensus that necrosis in spheroid

core will occur if the spheroid size is larger than $\sim 150\text{ }\mu\text{m}$,^[47] which is likely to happen in the event of spheroid fusion within the microgel/microcapsule. Our high-throughput technology also circumvents the need to purify the microencapsulated spheroids from empty capsules. This would be crucial if a sufficient number (in the order of million/billion) of microgels/microcapsules are to be generated with high yield and uniformity to satisfy GMP for clinical applications. Last but not least, the DE technology we embraced provides one-step generation of microencapsulated hepatocyte spheroids whereas other technologies require formation of spheroid before microencapsulation.^[22] The biochemical composition of the encapsulation materials and cell composition in spheroid could be flexibly tuned to enhance and maintain hepatocyte functions. In the current study, the alginate and alginate-collagen used exhibit different biochemical as well as mechanical properties.^[27] It is difficult to uncouple the effects of each cue. In future work, we will explore the effect of mechanical property alone such as using alginate of various concentrations on hepatocyte functions and viability. The use of EPC as a novel supporting cell type to enhance hepatocyte functions opens up new opportunities in maintaining long-term hepatocyte functions by co-culture with cell types that are not immortalized or obtained via invasive means. Nevertheless, the fact that at certain co-culture ratios the beneficial effect was lost means that further research should be devoted to studying the mechanism of the phenomenon and optimizing the process. The potential of forming co-culture spheroids in DE droplets could also facilitate the high-throughput generation of miniaturized, injectable liver-bud for use in liver replacement therapy.

4. Conclusion

In this study, we demonstrated the efficient one-step production of microencapsulated hepatocyte spheroids with high yield, versatility and uniformity via the generation of microfluidics DE droplet. We showed that the incorporation of collagen in the encapsulation materials and EPC (hepatocyte to EPC ratio = 5:1) as a novel supporting cell source would enhance the long-term performance of hepatocytes. We envision the technology can be adopted to screen for optimal matrix environment and cell composition, and can be the platform for biomanufacturing of microencapsulated spheroids for various liver tissue engineering and medical applications.

5. Experimental Section

Cell culture

Fresh primary rat (Sprague-Dawley) hepatocytes (Hep) were purchased from Triangle Research Labs (Durham, NC) and cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY), $0.02\text{ }\mu\text{g/mL}$ EGF (Life Technologies, Grand Island, NY), $7.14\text{ }\mu\text{g/mL}$ glucagon (Sigma Aldrich, St. Louis, MO), $17.36\text{ }\mu\text{g/mL}$ insulin (Sigma Aldrich, St. Louis, MO), 7.5 ng/mL hydrocortisone (Sigma Aldrich, St. Louis, MO) and 100 U/mL penicillin/ streptomycin (Life Technologies, Grand Island, NY). For collagen sandwich culture, neutralized $20\text{ }\mu\text{g/cm}^2$ Type I Bovine dermal collagen (BD Biosciences, San Jose, USA) was coated on culture well for 1 h for cell seeding. Another layer of collagen gel was overlaid 24 h after initial cell

seeding. Human umbilical cord blood-derived endothelial progenitor cells (EPC) were isolated as previously described.^[48] Umbilical cord blood was obtained from the Carolina Cord Blood Bank. All patient identifiers were removed prior to receipt. The protocol for the collection and the usage of human blood in this study was approved by the Duke University Institutional Review Board. EPC were cultured in EGM-2 BulletKit (Lonza, Walkersville, MD) supplemented with 10% FBS and used within passage 3-5. HEK-293 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/ streptomycin. To determine the optimal co-culture medium composition, the Hep and EPC media were mixed at various ratios (1:0, 1:1, 2:1) and used to culture Hep and EPC respectively. The viability and von Willibrand factor (vWF) expression of EPC were analyzed while the albumin and urea secretions of Hep were assessed to determine the optimal culture conditions for both types of cells.

Microfluidics device fabrication and DE generation

Two flow-focusing microfluidics devices with a channel width and height of 200 μm or 100 μm were fabricated according to a reported protocol.^[16, 49] The procedure of generating DE was described in the main text. Briefly, cell culture medium or alginate (PRONOVA SLG100, Novamatrix, Norway) dissolved in cell culture medium (1%) was used as the inner aqueous phase. The oil phase used was HFE-7500 (Miller-Stephenson Chemical Co. Inc., Danbury, CT) supplemented with Pico-Surf TM 2 surfactant (1%) (Dolomite Microfluidics, Charlestown, MA). The external aqueous phase comprised culture medium supplemented with Pluronic® F-127 (2.5 wt%). The flow rates of inner aqueous phase (4 mL/min), middle oil phase of HFE7500 (3 M, St. Paul, MN) (12 mL/min) and external aqueous (24 mL/min) were controlled by a Harvard Apparatus PHD 2000 Syringe Pump. To create alginate microgel after droplet formation, DE containing alginate solution were dispersed onto a cell strainer with pore size of 70 μm . 200 mM calcium chloride solution was pipetted onto the droplets for a few times. After the oil phase was washed through the strainer and microgels were formed and trapped on the strainer, the strainer was flipped upside down before 150 mM sodium chloride solution was pipetted on top to wash the microgels down for collection. The microgels were washed with 150 mM sodium chloride solution for a few times to remove excess calcium chloride solution.

Microencapsulated spheroid generation and characterization

Hep or HEK-293 cells at a density of 8 million cells/ mL were loaded into droplets containing 1% alginate or 0.8% alginate/ 0.25 mg/mL collagen I solution (Rat tail collagen I, Life Technologies, Grand Island, NY). Collagen I was neutralized with sodium hydroxide solution (Sigma Aldrich, St. Louis, MO) before mixing with alginate solution. To generate co-culture spheroids composed of Hep and EPC, Hep and EPC were mixed at various ratios (1:0, 5:1, 3:1, 1:1, 1:3) to make up to 8 million cells/ mL and loaded into droplet. At 4 h after droplet formation, microgels encapsulating Hep spheroid were produced as described above. The sizes of DE, spheroids and microgels were quantified from at least three bright field images consisting of > 30 samples using ImageJ software. The viability of cell culture samples were evaluated by staining with 3 μM calcein AM and 2.5 μM propidium iodide solution for 30 min before imaged using the inverted confocal microscope (Zeiss LSM 510)

available at Duke Light Microscopy Core Facility. To track the organization of co-culture cell types in the composite spheroid, EPC and Hep were labelled with CellTracker™ Green CMFDA Dye and CellTracker™ Red CMTPX Dye respectively before loaded into DE to form composite spheroids. The microencapsulated spheroids were imaged using the inverted confocal microscope at day 0 and 1.

MRP-2 transporter activity

5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) (Sigma Aldrich, St. Louis, MO) was used as the substrate to detect MRP-2 transporter activity and thus bile canaliculi formation. The samples were incubated with 5 μ M CDFDA for 30 min. Thereafter, dye solution was aspirated and the cells were washed with PBS for a few times before imaged using the inverted confocal microscope.

Immunostaining

For immunostaining against collagen I, the microgels were first added to 50 mM sodium citrate solution for 5 min to dissolve the alginate layer before stained with collagen I primary antibody (PA1-27397) and the corresponding secondary antibody (31573). For immunostaining against albumin and vWF, the microgels were again treated with sodium citrate before permeabilized with 0.1 % Triton X-100 solution for 30 min. The antibodies used were vWF antibody (PA5-16634) and the corresponding secondary antibody (31670), and albumin antibody conjugated with FITC (PA1-86695). All antibodies were purchased from Thermo Scientific (Waltham, MA). The samples were then fixed in FluoroGel mounting medium (Electron Microscopy Sciences, Hatfield, PA) before imaged using the inverted confocal microscope (Zeiss LSM 510) available at Duke Light Microscopy Core Facility.

Albumin ELISA quantification

The single and co-culture microgels were cultured in Hep and the optimized co-culture media respectively. Hep in Col-Sandwich condition were cultured in Hep medium. The culture media were changed and collected once every two days. The samples were analyzed using rat albumin ELISA quantification kit (Bethyl Laboratories, Montgomery, TX) following the manufacturer's protocol and the absorbance was determined with the FLUOstar OPTIMA microplate reader (BMG Labtech, Germany).

Urea assay

The samples were analyzed for urea secretion using Urea Nitrogen (BUN) Test (Stanbio Laboratory, Boerne, TX) following the manufacturer's protocol and the absorbance was determined with the FLUOstar OPTIMA microplate reader (BMG Labtech, Germany).

Cytochrome P450 3A4 (CYP3A4) assay and induction

At day 8, 16 and 24, the cell culture samples were treated with CYP3A4 assay substrate (Promega, Madison, WI) following the manufacturer's protocol before the luminescence was analyzed with the FLUOstar OPTIMA microplate reader (BMG Labtech, Germany). Dexamethasone (Dex) is a known inducer for CYP3A4 enzymatic activity. To perform the

induction, the cell culture samples were pretreated with 10 μ M Dex for 72 h prior to analysis.

Statistical analysis

All results (single-/co-culture experiments) were reported as the mean $\pm$ S.E.M. for three independently performed experiments. Statistical significance was determined using one or two way ANOVA followed by Tukey's Post Test (Prism 5.0, GraphPad Software, La Jolla, CA). The albumin, urea and CYP3A4 results were reported as amount per 10^6 cells where the number of cells in each sample was determined by counting cells after trypsination or Accutase (Life Technologies, Grand Island, NY) treatment. In the case of co-culture experiment, the results were normalized with the number of hepatocytes present in the spheroid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Schematic diagram of the process of generating microencapsulated hepatocyte spheroid using DE droplet. In the 1st device, water-in-oil (cell suspension: red; oil: blue) emulsion droplets were generated, which were transferred to the 2nd device where an external water phase (green) wrapped around the single emulsion to produce double emulsion droplets. b) Microfluidics devices assembled on a glass microscope slide (75mm X 25 mm). c) DE droplet generation (Left: Aqueous-oil droplets generated in 1st Device. Middle: Aqueous-oil-aqueous droplets generated in 2nd Device. Right: Appearance of droplets after collection). (Scale bar = 200 μ m)

Figure 2.

a) Microencapsulated spheroid production (Left & middle: Bright field image taken at time 0 & 4 h. Right: Microgel formation after oil removal). (Scale bar = 200 μm) b) Size distribution of spheroid and microgel. c) Size distribution of DE droplet. d) Immunostaining of rat collagen I of spheroid encapsulated in different materials. (Scale bar = 50 μm)

Figure 3.

a) Characterization (morphology and live-dead staining) of hepatocytes cultured in collagen sandwich configuration or on collagen coating only for 7 days. (Scale bar = 100 μ m)
 Hepatocytes cultured in Col-sandwich exhibited cubic cell shape while the ones cultured on a single collagen coating displayed elongated, fibroblast-like shape. b) Characterization (morphology, live-dead staining at Day 24 and staining for bile canaliculi) of hepatocytes cultured in different conditions. (Scale bar = 50 μ m)

Figure 4.

a) Daily albumin release (#p<0.05 between Alg and Col-Sandwich, **p<0.01 between Alg-col and Col-Sandwich). b) Cumulative albumin release (*p<0.05). c) Daily urea secretion (#p<0.05 between Alg and Col-Sandwich, *p<0.05 between Alg-col and Col-Sandwich). d) Cumulative urea secretion. e) Basal CYP3A4 activity measured by a luminogenic assay. f) Induction of CYP3A4 activity after treatment with 10 μM Dex for 72 h. (Data represent mean $\pm$ S.E.M. n=3)

Figure 5.

a) Tracking of cell organization in the composite spheroids at different co-culture ratios. (Scale bar = 50 μm) b) Cumulative albumin release (** $p < 0.01$ between 1:5 and 1:1/3:1/0:1). c) Cumulative urea secretion (* $p < 0.05$ between 1:5 and 1:1/3:1/0:1). d) Basal CYP3A4 activity measured by a luminogenic assay (** $p < 0.01$ between 1:5 and 0:1). The figure legends denote co-culture cell ratios (EPC: hepatocytes). (Data represent mean $\pm$ S.E.M. $n=3$)

Figure 6.

a) Characterization (immunostaining against hepatocyte (albumin) and EPC (vWF) markers, live-dead staining and staining of bile canaliculi) of single or co-culture spheroids cultured in different conditions. (Scale bar = 50 μm) b) Cumulative albumin release. c) Cumulative urea secretion. d) Basal CYP3A4 activity measured by a luminogenic assay (* $p < 0.05$ between HepEPC in Alg-col and HepEPC in Alg). e) Induction of CYP3A4 activity after treatment with 10 μM Dex for 72 h. (Data represent mean $\pm$ S.E.M. $n=3$)

Figure 7. Relative intensity of albumin-FITC encapsulated in microgels of different size. The total intensity of the gel at each time point was subtracted with the intensity at equilibrium (5 days) and normalized with the intensity at time 0. Gaussian nonlinear fitting was shown on the graph. The top and bottom figure on the right shows the fluorescent images of small and large gel. (Scale bar = 200 μm)