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Matrix Softness-Mediated 3D Zebrafish Hepatocyte Modulates Response to Endocrine Disrupting Chemicals

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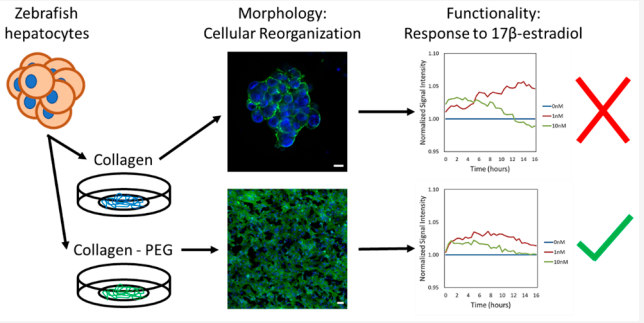
ABSTRACT: Endocrine disrupting chemicals (EDC) include synthetic compounds that mimic the structure or function of natural hormones. While most studies utilize live embryos or primary cells from adult fish, these cells rapidly lose functionality when cultured on plastic or glass substrates coated with extracellular matrix proteins. This study hypothesizes that the softness of a matrix with adhered fish cells can regulate the intercellular organization and physiological function of engineered hepatoids during EDC exposure. We scrutinized this hypothesis by culturing zebrafish hepatocytes (ZF-L) on collagen-based hydrogels with controlled elastic moduli by examining morphology, urea production, and intracellular oxidative stress of hepatoids exposed to 17β-estradiol. Interestingly, the softer gel drove cells to form a cell sheet with a canaliculi-like structure compared to its stiffer gel counterpart. The hepatoids cultured on the softer gel exhibited more active urea production upon exposure to 17β-estradiol and displayed faster recovery of intracellular reactive oxygen species level confirmed by gradient light interference microscopy (GLIM), a live-cell imaging technique. These results are broadly useful to improve screening and understanding of potential EDC impacts on aquatic organisms and human health.



INTRODUCTION

For the last few decades, endocrine disrupting chemicals (EDCs) have been extensively used in various industrial and household products and medicine. It is well documented that the majority of endocrine disruption effects in fish field populations are due to exposure to natural and synthetic steroidal estrogens and their breakdown products such as 17β-estradiol.[1] Certain industrial chemicals also have shown an estrogenic activity. For example, bisphenol A is a precursor of polycarbonate plastics and resins.[2] Another chemical, di(2-ethylhexyl) phthalate, is used as a plasticizer of food packaging and medical devices.[3] The subsequent increase in EDC levels in the river and potential drinking water prompted efforts to understand their impacts on the human endocrine system. EDCs may disrupt endocrine pathways through mimicking naturally occurring hormones or by blocking receptors. As they are transported into the liver for metabolic degradation, chronic exposure can result in nonalcoholic fatty liver disease which severely impacts metabolism, potentially leading to cirrhosis or cancer.[4] Thus, a platform must be developed to test chemical substitutes for their potentially adverse effects on liver morphology and function.

One common way to examine the potential toxicity of EDCs is through the examination of the zebrafish (*Danio rerio*), a standard testing fish species for biomonitoring due to their small size, easy cultivation, and transparent embryos. As EDCs are accumulated in the liver, the zebrafish liver would be an ideal organ to develop into an *in vitro* platform. Zebrafish sensitivity to toxins in the water is evaluated by examining damage in the DNA or chromosomes, P450 detoxification activity, and endocrine activity at the RNA level.[5] Endocrine disruption is not species-specific; therefore, EDC effects are widespread and often irreversible.[6,7] Many studies report the zebrafish liver would make a useful model for accurate prediction of both endocrine effects and reproductive toxicity.[5,8,9]

With the movement away from animal models due to ethical reasons and tight regulations on their captivity, an alternative way to test the potential toxicity of current and newly developed EDCs without using the "live" zebrafish is necessary,[14] especially for the cosmetic industry due to the complete ban of animal testing in the European Union since 2013.[15] Consequently, efforts have been increasingly made to engineer an *in vitro* 3D hepatic platform that recapitulates anatomical and physiological

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function of liver to test the potential impacts of chemical compounds on liver function. In particular, spheroids formed from primary trout hepatocytes were found to present canalicular structures and have similar gene expression profiles as *in vivo* trout livers, as long as the spheroids matured for 25 days.[16] Additionally, there have been efforts to create *in vitro* liver models that closely predict human metabolism and match *in vivo* clearance rates when exposed to common pharmaceuticals like propanolol.[17] Despite challenges such as extended culture periods, these results show promising advances in replacing fish models *in vitro*.

Many approaches of 2D cell culture were focused on culturing primary liver cells or cell lines into clusters using an ultra-low-adhesion dish or the hanging drop method. Other methods culture the cells on a flat surface like tissue culture plastic or glass coated with extracellular matrix proteins. However, cells isolated from tissues lose their phenotypes quickly through uncontrolled dedifferentiation.[10] It is suggested that liver cells would exhibit a reasonable reorganizational period by placing cells into a physiologically similar microenvironment. Ultimately, cells would form a more cohesive structure and well-defined functional units during the first couple of days. These insights have not been widely tested for assembling zebrafish liver hepatoids used for EDC toxicology studies.

However, studies made with hydrogel coupled with cell-adherent proteins have shown positive effects of substrate softness on cell viability and metabolic secretions in primary cells as compared to the unmodified gel.[11−13] In particular, rat primary hepatocytes cultured on heparin-PEG hydrogels with an elastic modulus of 2.3 kPa showed higher cell viability, albumin secretion, and urea secretion than cells cultured on stiffer heparin-PEG hydrogels.[11] In addition, mouse primary hepatocytes on collagen-polyacrylamide hydrogels with an elastic modulus of 140 Pa showed significantly higher albumin production and HNF4α expression, a transcriptional regulatory factor that is critical in normal liver development and maintaining normal liver functions, than those cultured on a stiffer hydrogel.[13] These studies state that the elastic modulus of the substrate on which hepatocytes are cultured is an extracellular factor of modulating hepatocyte function and structure in an *in vitro* culture.

In this study, we hypothesized that the softness of a matrix modulates whether zebrafish hepatocytes can regulate their intercellular organization. In turn, the response of the resulting "hepatoid-like cluster" (hepatoid) to EDCs can be tuned through modulation of cell-matrix interactions. The matrix softness would mitigate the effects of EDCs on cellular detoxification activity through the urea cycle. We examined this hypothesis using 17β-estradiol as a model EDC,[18,19] and studied the morphology and metabolic activity of zebrafish hepatocytes cultured on collagen-based gels with two different elastic moduli of 14 and 256 Pa. We assessed cellular oxidative stress from 17β-estradiol by monitoring the reactive oxidative species activity in real-time using a fluorescent probe. The extent to which 17β-estradiol affects the detoxification activity of cells is monitored by the urea cycle activity of liver cells exposed to different concentrations of 17β-estradiol. Overall, this study aims to develop an advanced *in vitro* zebrafish liver and use it to understand the impact of EDCs on the physiological activities of urea and vitellogenin synthesis.

MATERIALS AND METHODS

ATCC Zebrafish Cell Culture. Zebrafish hepatocytes [ZF-L] (ATCC CRL-2643) were cultured in 50% Leibovitz's L-15, 35% Dulbecco's Modified Eagle Medium high glucose, and 15% Ham's F12, all without sodium bicarbonate and supplemented with 0.15 g/L sodium bicarbonate, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.01 mg/mL bovine insulin, 5% heat-inactivated fetal bovine serum, and 0.5% trout serum (Caisson Laboratory). Cells were expanded in T-75 flasks and incubated at 28 °C and atmospheric carbon dioxide in a tabletop, low temperature incubator (Fisherbrand). We used cells with a passage number of 3 to 5 for hepatoid formation on gels with controlled elastic moduli. Media was formulated in the University of Illinois Cell Media Facility, except for the trout serum which was added separately, and the completed medium was not sterile filtered. Cells were cultured in T-75 flasks until about 80% confluent and cryopreserved using the culture medium supplemented with 10% heat-inactivated fetal bovine serum and 5% DMSO. Cells were cryopreserved in vapor phase liquid nitrogen.

Collagen-Polyethylene Glycol (PEG) Hydrogel Formation. Collagen-polyethylene glycol (collagen-PEG) hydrogels were prepared in 96-well plates via *in situ* cross-linking between collagen molecules by mixing PEG (Sigma, MW 7500) at mass ratios of 0 (pure collagen) and 10 to bovine Type I collagen (Advanced Biomatrix) in an equal volume of the zebrafish medium. Reconstitution solution containing 0.26 M sodium hydrogen carbonate, 0.2 M HEPES, and 0.04 M sodium hydroxide (Sigma) was added to modulate the pH of the gel to initiate gel formation. Hydrogels were then incubated at 37 °C and 5% CO_2 for 30 min. Cells were plated directly onto hydrogels at a cell density of 1.0×10^6 cells/mL and cultured for 14 days at 28 °C and atmospheric carbon dioxide. The media was replaced every two to three days.

Mechanical Analysis. The elastic modulus of the hydrogels was measured using a rheometer (DHR-3, TA Instruments). Collagen and collagen-PEG gels were prepared as previously described and loaded onto the parallel plate (diameter = 22 mm). The gap between the two plates was kept constant at 200 μm. The hydrogel was left for 20 min at 37 °C to gel. Then, the hydrogel was oscillated at 0.1% strain while varying frequency from 0.1 to 10 Hz. The resulting stress was measured to calculate elastic and loss moduli at individual frequency. The test was conducted in triplicate.

Scanning Electron Microscopy (SEM). Collagen and collagen-PEG hydrogels were formed in a 96-well plate as previously described. Then, they were removed with a spatula and slowly dehydrated in 30%, 50%, and 70% w/w ethanol−water solution for at least 1 h each and 100% ethanol overnight. The dehydrated gels were dried using the critical point dryer (Tousimis 931). SEM (Hitachi S4700) imaging was performed directly after drying the samples. First, the samples were mounted using copper tape and coated with a 6−8 nm layer of gold (EMITECH 575). Images were taken with an accelerating voltage of 2 kV, a working distance of 8−9 mm, and the emission current was adjusted to reduce sample damage.

The pore area and fiber diameter were measured using ImageJ software. For the pore area, an automatic threshold was applied to remove the background and the particle analyzer tool was used to measure pores that were larger than 1 um^2. The average pore area was calculated by dividing the total pore area by the area of collagen. Fiber diameter was directly measured, and at

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Environ. Sci. Technol. 2020, 54, 13797−13806

least 20 measurements were taken from each image. The statistical significance was calculated using the ANOVA test.

Immunofluorescence Imaging. Cells were cultured for two weeks on collagen or collagen-PEG hydrogels installed on glass bottom dishes (Cellvis). Then they were fixed with 1:1 v/v methanol and acetone at −20 °C for 20 min. Then, the samples were washed twice with room temperature phosphate buffered saline (PBS, Corning) for 5 min each. Cells were blocked with 2% bovine serum albumin for 1 h. Cells fixation using this method does not need an extra permeabilization step as permeabilization is obtained by acetone and methanol.

The cells were stained with phalloidin-Alexa 488 (Invitrogen) overnight at 4 °C (1:250) for the imaging of actin filaments and washed twice with PBS for 5 min each. 4′,6-Diamidino-2-phenylindole (DAPI) was incubated at room temperature for 1 min (1:500) and washed twice for 1 min each immediately before imaging.

For β1-integrin staining, the primary antibody P5D2 (Abcam) was incubated overnight at 4 °C. The sample was then washed twice with PBS for 5 min each. Then, the secondary antibody antimouse conjugated AF555 (Cell Signaling Technology, 4409S) was incubated for 4 h at room temperature. Then, the samples were washed with PBS twice for 5 min each. Finally, nuclei of the cells were imaged with DAPI immediately before imaging. Although the P5D2 antibody is noted as only reactive with human cells, according to the National Center for Biotechnology Information, zebrafish and humans are orthologs for β1 integrin which would suggest that the antibody will have cross-species reactivity.[20]

The images were taken using a four laser, point scanning confocal microscope (Zeiss LSM 700). The 10x/0.3 or the 20x/0.8 air objectives were used. Images of the cells were obtained using the tile scan feature. The pinhole for all channels was set to the size of one Airy unit for the DAPI channel. The line averaging was set to 8 times, and the pixel dwell time was set to 1.58 μs. Image acquisition and review were done through the Zeiss Zen (Black and Blue, respectively) programs.

Bicinchoninic Acid (BCA) Protein Assay. The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific 23225) was used to determine the total protein concentration of the zebrafish hepatoid samples. The microplate protocol was followed using the instructions provided with the assay kit. Samples were tested in triplicate. Samples were collected from the culture on days 1, 7, and 14.

First, diluted albumin standards were made so that there was a range of standard from 0 to 2000 μg/mL of BSA concentration. Then, the working reagent was prepared by mixing 50:1 BCA reagent A to B. The solution was mixed to reduce turbidity to yield the clear, green color as described. The working reagent was prepared fresh, immediately before starting the assay.

Following the microplate procedure, 25 μL each of the standard and unknown were pipetted into a 96-well plate. Then, 200 μL of the working reagent was pipetted into each well and mixed thoroughly by pipet. The plate was covered, protected from light, and placed into an incubator at 37 °C for 30 min. Finally, the plate was cooled to room temperature and read at 562 nm wavelength on a plate reader (BioTek).

17β-Estradiol and Bisphenol A Treatment. Zebrafish hepatocytes were plated at a density of 1.0×10^6 cells/mL and cultured in the conditions as described above for 14 days. A stock solution of 17β-estradiol (E2) or bisphenol A (BPA) was prepared with a DMSO solution, 0.1 wt % in water. Then, for 24 h, the cells were exposed to 0, 1, or 10 nM of 17β-estradiol (E2) or bisphenol A (BPA) in cell culture media. The cells were analyzed after the 24-h exposure period.

Urea Colorimetric Assay. Cell culture media was collected from each condition and stored at −20 °C for short-term storage until testing. The low concentration urea assay kit (BioVision K375) was used to determine the urea cycle activity. The media samples and kit components were first warmed to room temperature. Then, in a 96-well plate, the standard curve and samples were prepared according to the manufacturer's instructions. Each condition was tested in triplicate. The colorimetric output was read at 570 nm using a plate reader (BioTek). Then, the data was analyzed with an ANOVA test to determine significance.

The media was collected from each condition and stored at −5 °C until it was used for experiments. Urea synthesis was assayed in cell culture medium using the urea assay kit (MAK006, Sigma-Aldrich, Steinheim, Germany). A standard curve was created to generate 0, 1, 2, 3, 4, and 5 nmol urea/well. Then, 50 uL of the media was applied to the 96 wells for urea quantification according to the manufacturer's instruction. The colorimetric product was measured at wavelengths of 570 nm using a microplate reader (TECAN, Männedorf, Switzerland). Each condition was tested in triplicate, and statistical significance was measured using an ANOVA test.

Vitellogenin (VTG) Measurements and Immunofluorescent Staining. Vitellogenin was measured in cell pellets using an ELISA kit (10004995; Cayman Chemical, Ann Arbor, MI, USA). A cell concentration of 1.0×10^6 cells/mL was seeded on collagen and collagen-PEG coated well plates and cultured for 14 days. Then, the cells were exposed to 1 nM of 17β-estradiol (Sigma-Aldrich) and 10 μM of BPA (Sigma-Aldrich) for 48 h, respectively. The cell pellets were collected in 1.5 mL tubes and centrifuged for 5 min at 125 × g, and the supernatants were discarded and washed with cold PBS. The pellets were lysed with passive lysis buffer (Promega, Mannheim, Germany) after recentrifugation. The supernatants were used for the vitellogenin quantification according to the manufacturer's instructions (Cayman Chemical). Each sample was normalized by total protein content, which was calculated using the BCA protein assay. The ANOVA test was run to determine significance of the results for the hepatoids cultured on the collagen-PEG hydrogel.

For vitellogenin staining, the primary antibody vitellogenin (LifeSpan, LS-C76845−100) was incubated overnight at 4 °C. The sample was then washed twice with PBS for 5 min each. Subsequently, the secondary antibody antimouse conjugated FITC (Thermo, 31232) was incubated for 2 h at room temperature. Then, the samples were washed with PBS three times every 5 min. Finally, nuclei of the cells were stained with DAPI immediately before imaging.

Live Cell Imaging with 17β-Estradiol. Glass bottom dishes (29 mm diameter), the well (14 mm diameter), #0 glass, and the glass top (D29-14-0-TOP, Cellvis) were first coated with poly-D-lysine (Sigma) for 20 min and then washed twice with culture media to remove excess chemical. Zebrafish hepatocytes (ATCC CRL-2643) were seeded at 5000 cells per dish and allowed to adhere for 10 min before being coated by collagen or collagen-PEG hydrogel as described above. Cells were cultured in this manner because of the short working distance of the microscope objective. Therefore, cells needed to be cultured as close to the glass coverslip as possible. After the hydrogel was formed, 3 mL of culture media was added and the cells were incubated in the conditions previously described. The

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Environ. Sci. Technol. 2020, 54, 13797−13806

cells were then cultured for 2 days prior to the live cell imaging experiment to ensure that the cells were interacting with the hydrogel and each other without forming large clustered structures. Thus, individual cell ROS levels could be analyzed.

Before imaging, zebrafish culture medium with 0, 1, and 10 nM of 17β-estradiol (Sigma-Aldrich) replaced the media and 6 μL/3 mL of CellROX Green Reagent (Thermo Fisher Scientific) was added to each sample. Co-localized fluorescence and phase imaging was performed on an Axio Observer Z1 (Zeiss) with GLIM Pro add-on module (Phi Optics). Gradient light interference microscopy is an upgrade to differential interference contrast microscopy that uses phase-shift shifting to improve image quality by separating unwanted amplitude information from high detail phase information.[21] Here, we use a 10x/0.3 objective and sCMOS camera (Prime BSI, Photometrics). Imaging began 20 min after addition of the CellRox dye with a total of 18 representative fields of view (1150 × 1150 μm) acquired every 30 min for 16 h.

The images were then analyzed with ImageJ (Fiji) to measure signal intensity from the CellROX reagent as well as to overlay the GLIM and fluorescent images. To measure the intensity, images from the same sample were loaded into ImageJ as a sequence in chronological order. Then, a region of interest (ROI) was enclosed using an oval, ensuring that the entire cell cluster or single cell was within the boundaries of the ROI for the entire set of images. Using the stacks menu, the Z-axis profile was plotted, and the values were exported to Excel for further analysis. The Z-axis profile plots the mean gray signal of the ROI. At least 10 ROI were chosen per sample. In Excel, the data was averaged and normalized to the control sample of 0 nM 17β-estradiol for each hydrogel modulus, respectively.

RESULTS AND DISCUSSION

Increasing the pH of the pre-gelled collagen solution and the collagen-polyethylene glycol (PEG) mixture from 2.0 to 7.4 resulted in the collagen gel and collagen-PEG gel, respectively. Both hydrogels are made with interconnected fibrous networks as confirmed by scanning electron microscope images (Figure 1). The PEG in the collagen-PEG gel altered the diameter and spacing of collagen fibers minimally compared with the pure collagen gel (Table 1). In contrast, the elastic modulus of the PEG-collagen gel was 14 Pa while that of the pure collagen gel was 256 Pa. This result confirms that PEG modulates the mechanical stiffness of the collagen gel without altering the microstructure significantly.

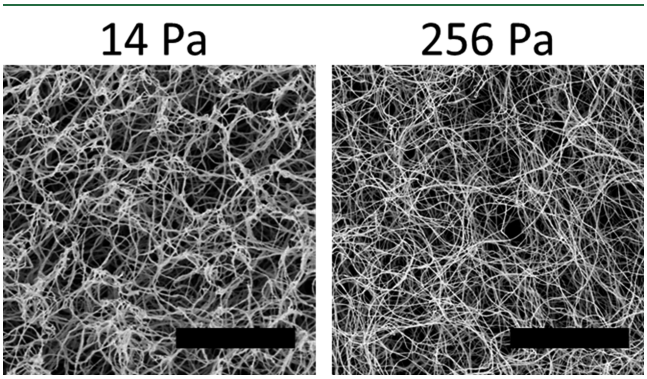

Figure 1. Two hydrogels were formulated; pure collagen and collagen mixed with polyethylene glycol (PEG) and imaged with scanning electron microscopy. The black scale bars represent 5 μm.

Table 1. Collagen Hydrogel Shear Moduli and Microstructure Properties[a]

PEG/Collagen (m/m)	Shear modulus (Pa)	Fiber diameter (μm)	Pore area (μm^2)
10:1	14 ± 1	0.10 ± 0.02	053 ± 0.21
0:1	256 ± 132	0.09 ± 0.02	0.38 ± 0.19

[a]The shear modulus was decreased from 256 to 14 Pa with the addition of PEG at a mass matio of PEG to collagen being 10:1. The concentration fo collagen was held constant for both hydrogel formulations at 1.5 ng/ mL. The asterisk indicates statistical significance of the difference of values between two conditions (*p < 0.02).

This collagen-PEG hydrogel system allows for the control of the elastic modulus without significantly altering other matrix parameters like the pore area and fiber diameter. Microscopic images of the gel confirm that there is no significant difference in microstructure, independent of the change in softness. As analyzed previously,[22] it is likely that PEG depletes the hydrogen bonds between water molecules and collagen fibers, which are major components to generate the elastic properties of the gel. As the free water molecules are depleted, the fibrillogenesis of the collagen fibers is hindered, resulting in a softer hydrogel. Such change of the intermolecular association at the molecular scale may influence the stiffness of individual collagen fibers but may not impact the pore size of the gel. This strategy to control gel softness is different from other formulations, in that the PEG act as spacers between the collagen fibers instead of being used to chemically cross-link collagen molecules.[23,24]

The zebrafish hepatocytes were seeded onto the gels with controlled elastic moduli. Then, cell growth was monitored for two weeks by measuring the total protein concentrations with a BCA protein assay kit (Figure 2a). The total protein concentration was increased at a comparable rate regardless of the elastic modulus of the gel. In contrast, the elastic modulus of the gel influenced cellular organization. Cells cultured on the pure collagen gel with an elastic modulus of 256 Pa proliferated independently or in small clusters (Figure 2c-iv). However, cells cultured on the softer collagen-PEG gel with an elastic modulus of 14 Pa aggregated to form a large cell sheet (Figure 2c-i). More interestingly, the cells self-organized on the collagen-PEG gel to form a hollow lumen, or canaliculus (Figure 2c-ii,iii). In addition, actin molecules were localized on the cell membrane more significantly than cells cultured on the stiffer pure collagen gel. Furthermore, cells cultured on the softer collagen-PEG gel expressed more β1 integrins than those cultured on the pure collagen gel (Figure 2b,d).

To further clarify that the zebrafish hepatoid ultrastructure was a direct result of the elastic modulus of the hydrogel, singularized hepatocytes were pre-exposed to PEG prior to plating (Figure S1). After 14 days of culture, the cells were immunostained for actin filaments as well as β1 integrin expression. The cells pre-exposed to the PEG, which is the same PEG used to assemble the collagen-PEG hydrogels, did not show significant changes in actin or integrin expression from cells plated on the collagen-PEG hydrogel without pre-exposure to the soluble PEG. In addition, cells pre-exposed to PEG formed canaliculi-like structures, like cells that were not pre-exposed to the soluble PEG. Thus, it is suggested that the PEG that is present in the collagen-PEG hydrogel acts as a neutral softener with minimal interactions with the zebrafish hepatocytes during hepatoid formation.

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Environ. Sci. Technol. 2020, 54, 13797−13806

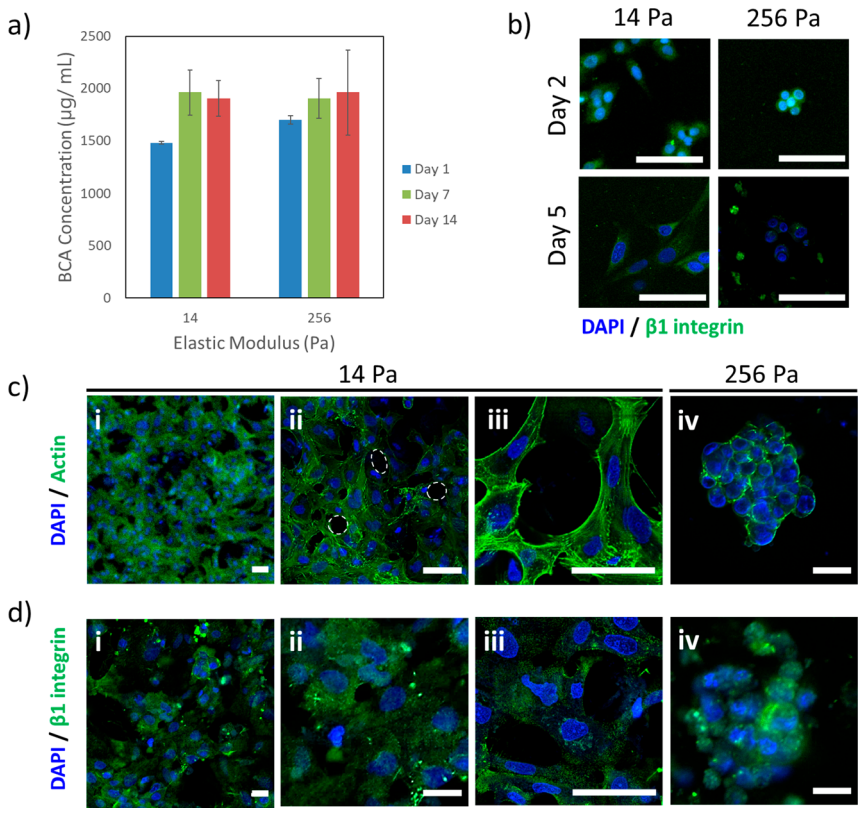

Figure 2. Zebrafish hepatocytes (ATCC CRL-2643) cultured on the collagen and collagen-PEG gels with different elastic moduli. (a) The total protein concentration assay after 1, 7, and 14 days of culture. The values and error bars represent the mean and standard deviation of three samples per condition, respectively. (b) Immunofluorescent staining of hepatocytes at days 2 and 5 for blue-colored nuclei and green-colored β1 integrin. (c and d) Immunofluorescent staining of hepatocytes after 14 days of culture on the gel. The images in part c show blue-colored nuclei and green-colored actin filament in cells cultured on the pure collagen gel with an elastic modulus of 14 Pa (i, ii) and collagen-PEG gel with an elastic modulus of 256 Pa (iii). White dashed circles mark hollow lumen formed by the self-organization of hepatocytes. The images in part d show blue-colored nuclei and green-colored β1 integrin in cells cultured on the pure collagen gel with an elastic modulus of 14 Pa (i, ii) and collagen-PEG gel with an elastic modulus of 256 Pa (iii). In each image, white scale bars represent 50 μm.

When observing the β1 integrin expression, which indicates cellular adhesion to the matrix, the softer collagen-PEG hydrogel served to increase the β1 integrin expression of cells than the stiffer, pure collagen hydrogel. Previous studies report that cell clusters formed or placed on a bioactive 2D substrate sense and respond to biophysical properties of the matrix via integrin-ligand bonds and cell−cell junctions.[25,26] As such, we suggest that hepatocytes bound to collagen molecules of the softer gel sense the mechanical signal from the substrate, increase cellular adhesion and growth, and finally form a cell sheet in which cells are interconnected to form the canaliculi-like structure. Cells homogeneously mixed into the hydrogel failed to associate with each other, thus resulting in few clusters with the physiologically relevant ultrastructure. These results indicate that the cells cultured on the 14 Pa collagen-PEG hydrogel were the most physiologically organized even though there was an independency of cell growth on the gel stiffness.

This study demonstrates that the softness of the collagen-PEG hydrogel plays a significant role in regulating the intercellular organization of engineered zebrafish hepatoids. *Ex vivo* cells frequently de-differentiate when cultured on a substrate which has a different modulus from the native tissue. By plating the hepatocytes onto a soft hydrogel with stiffness relatable to the yolk of the zebrafish egg, cells will experience biophysical cues which will result in hepatoids with higher physiological relevance. In particular, mouse hepatocytes cultured on stiffer collagen-polyacrylamide hydrogels exhibited larger cell areas and decreased expression of HNF4α, a transcriptional regulatory factor.[13] Because this factor controls many downstream factors and functions, the culturing of mouse hepatocytes on stiff substrates results in reduced hepatocyte function. Specifically, functional mRNA expression levels of *Baat*, *F7*, and *Gys2* are lowered in addition to that of HNF4α, according to the previous study made with mouse hepatocytes.

Compared with this study, the cells cultured on the softer gel, with an elastic modulus of 14 Pa, formed cohesive sheet-like structures with a canaliculi-like intercellular organization as seen in the actin staining images (Figure 2c). Canaliculi eventually fuse to form bile ducts in the adult zebrafish.[9,27] The bile ducts and blood vessels are jointly responsible for clearing metabolic waste from the hepatocytes.[8,28] In contrast, the hepatocytes on the stiffer collagen hydrogel, with an elastic modulus of 256 Pa, formed separated, small cell clusters. With the introduction of the hepatocytes into a softer microenvironment, the organotypic 3D organization resembled that of a zebrafish liver.

The physiological activity of hepatocytes cultured on the gels was analyzed by examining the ornithine-urea cycle, which is the primary pathway to detoxify ammonia and amino acids in the liver. Cells were analyzed at 27 °C and 37 °C, which represents the zebrafish and human body temperature, respectively (Figure 3). Cellular hepatic cycle activity increased with the cell culture period. After 2 weeks, cells cultured on the collagen-PEG gel

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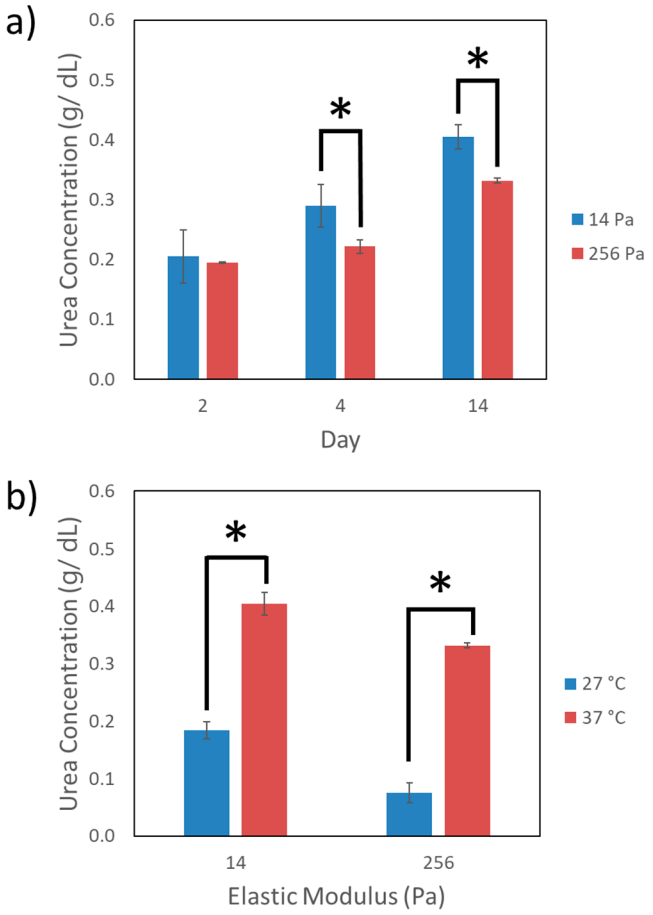

Figure 3. Analysis of urea cycle activity for zebrafish hepatocytes cultured on the gels with different elastic moduli. (a) The urea concentration was measured after 2, 4, and 14 days of culture. (b) Comparison of the urea cycle activity was made between 27 °C and 37 °C to evaluate the effects of environmental temperature. The values and error bars represent the mean and standard deviation of three samples per condition, respectively. The asterisk indicates statistical significance of the values between conditions ($*p < 0.05$).

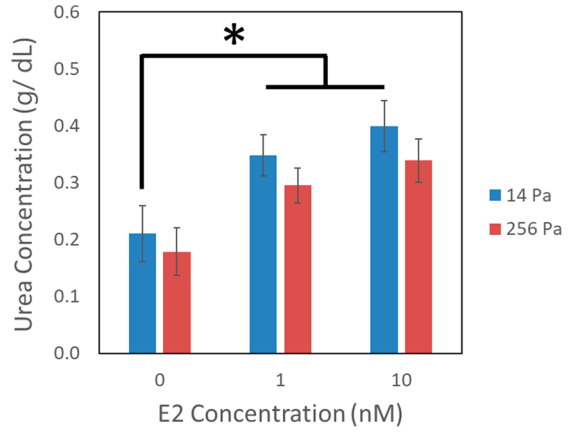

Figure 4. Analysis of hepatic urea cycle activity of zebrafish hepatocytes. Cells were cultured on the collagen gel with an elastic modulus of 14 Pa and the collagen-PEG hydrogel with an elastic modulus of 256 Pa for 2 weeks before exposure to 17β-estradiol (E2). Then, cells were exposed to 0, 1, and 10 nM of E2 for 24 h. The values and error bars represent the mean and standard deviation of three samples per condition, respectively. The asterisk indicates statistical significance of the values between conditions ($*p < 0.05$).

with an elastic modulus of 14 Pa displayed higher hepatic urea activity than those on the pure collagen gel with an elastic modulus of 256 Pa (Figure 3a). The inverse dependency of hepatic urea activity on the elastic modulus was more significant at 37 °C (Figure 3b).

We further assessed the extent to which the elastic modulus of the gel modulates hepatic urea cycle activities at varied concentrations of 17β-estradiol (E2). E2 binds with estrogen receptors to produce vitellogenin (VTG) in the female zebrafish, and its concentration is regulated in the liver through biotransformation.[29] E2 stimulated the hepatic urea cycle activity as displayed, with an increase of the urea concentration with increasing E2 concentration (Figure 4). Interestingly, the dependency of the urea cycle activity on the E2 concentration was more substantial with cells cultured on the collagen-PEG gel with an elastic modulus of 14 Pa than those cultured on the pure collagen gel with an elastic modulus of 256 Pa. Such an inverse dependency of the urea concentration on the elastic modulus of the gel is attributed to the cellular β1 integrin expression increased with the softer gel.

In addition, as shown in Figure 5, bisphenol A (BPA) and E2 were found to have effective VTG responses in the zebrafish hepatocyte clusters formed on collagen and collagen-PEG hydrogels. The hepatocytes cultured in a 2D monolayer on a polystyrene substrate did not markedly induce VTG production in the presence of E2 or BPA (Figure 5a). In contrast, zebrafish hepatocyte clusters formed on collagen-PEG hydrogels with an elastic modulus of 14 Pa resulted in significant increases of VTG activity at a concentration of 10 nM of E2 or 10 μM of BPA. Hepatocyte clusters formed on the collagen gel with an elastic modulus of 256 Pa showed minimal VTG activities after exposure to E2 or BPA, similar to cells cultured on a polystyrene substrate (Figure 5b).

VTG is the serum phospholipoglycoprotein precursor to egg yolk. It is potentially an ideal biomarker for environmental estrogens because estrogen receptors in the fish liver regulate vitellogenesis.[30] VTG is typically found in the blood of female fish, whereas in male fish, the level is very low. However, VTG synthesis can be induced in male fish if they are exposed to exogenous estrogens.[31] Therefore, the presence of VTG in a male fish can be considered a sensitive biomarker of estrogenic chemical exposure.[32] Therefore, E2 is considered to represent EDCs in the environmental system, and the synthesis of VTG in aquatic organisms is a very important marker for assessing endocrine disturbances by natural hormones and EDCs.

A previous study by the US Environmental Protection Agency (EPA) and the US National Toxicology Program (NTP) set 75 μM (17.2 mg/L) as the maximum BPA concentration detected in an environmental sample.[33] In addition, François Brio et al. showed that 10 μM of BPA revealed maximum estrogenic activity of the estrogen receptor and aromatase in zebrafish hepatocytes and larvae.[34] Therefore, zebrafish hepatocytes were exposed to either E2 or BPA. BPA stimulates estrogenic activity of hepatocyte clusters similar to E2. This result suggests that zebrafish hepatoids similarly metabolize both chemicals into VTG. However, the effect is significantly different depending on the substrate on which the zebrafish hepatocytes are cultured. The hepatoids cultured on the pure collagen hydrogel with an elastic modulus of 256 Pa did not respond to the EDCs as the hepatoids on the collagen-PEG hydrogels. From the earlier result, hepatoids on the stiffer hydrogel had disorganized tissue structure in comparison to the hepatoids cultured on the softer

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Environ. Sci. Technol. 2020, 54, 13797−13806

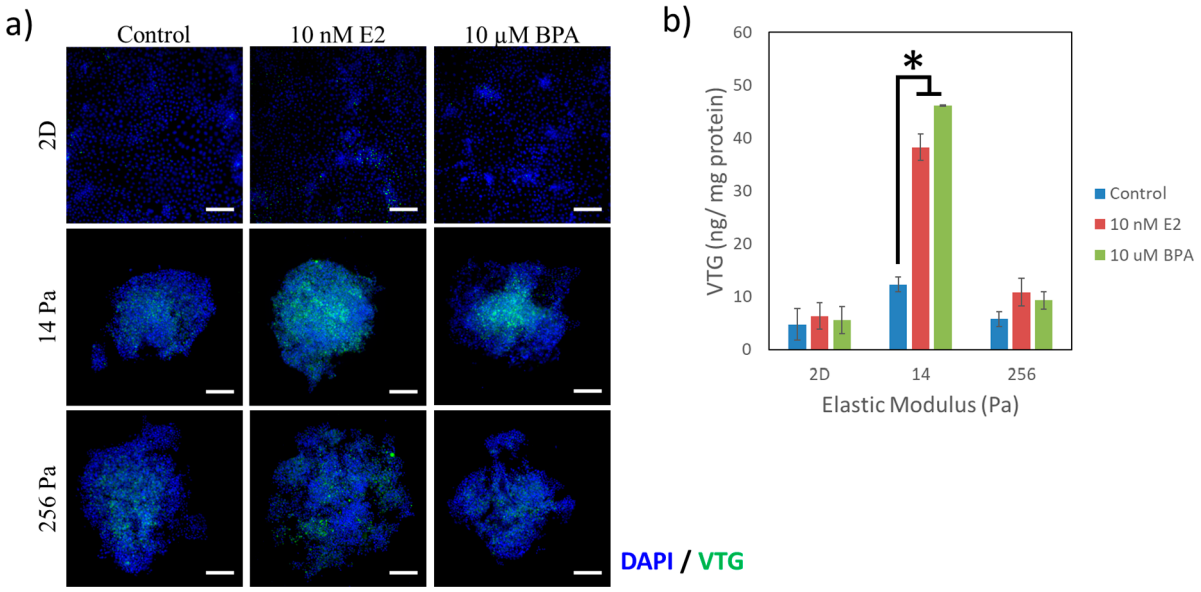

Figure 5. Analysis of the vitellogenin (VTG) in zebrafish hepatocytes after 14 days of culture on a 2D polystyrene substrate, collagen-PEG (elastic modulus ~ 14 Pa), and collagen (elastic modulus ~ 256 Pa) hydrogels. Immunofluorescent staining with DAPI (blue) and VTG (green) of zebrafish hepatocytes exposed to 10 nM of E2 or 10 μM of BPA for 24 h (a). The VTG concentrations as measured with ELISA (b). The white scale bars indicate 100 μm. The asterisk indicates statistical significance of the values between conditions (*$p < 0.01$).

hydrogels (Figure 2c). Therefore, the difference in VTG synthesis levels could be because physiologically similar tissue organization promotes high levels of hepatoid functionality. Thus, the disorganized cells on the 2D plastic substrate and the disorganized hepatoid formed on the pure collagen hydrogel had low responses to EDC exposure. It is likely that the rate of estrogenic activity is altered by the substrate stiffness. This possibility will be examined systematically in future studies.

The combined effects of estrogen concentration and gel softness on the cellular urea production are further related to the change in the ROS-mediated intracellular oxidative stress level. As the liver induces VTG synthesis using the excess E2, cells produce ROS which impacts cells negatively. In particular, we evaluated the general oxidative stress of the cells by monitoring the intracellular ROS level through the oxidation of the CellROX probe via live cell imaging for 16 h. This study discloses that hepatoid-like clusters formed on the softer collagen-PEG hydrogels are able to return the ROS level to a baseline at the elevated 17β-estradiol concentration. In contrast, cell clusters formed on the stiffer collagen hydrogel exhibited a higher ROS level for both elevated 17β-estradiol conditions. In addition, cell clusters showed a drop of ROS level below baseline when exposed to 10 nM 17β-estradiol, indicating that some of the cells likely lost viability and metabolic activities after exposure to excessive 17β-estradiol.

Because 17β-estradiol is localized in the liver for biotransformation into VTG, excessive accumulation from naturally produced and ingested 17β-estradiol will increase the generation of reactive oxygen species (ROS) through mitochondrial and genomic pathways. Live cell imaging was performed to visualize the ROS response of the zebrafish hepatocytes to 17β-estradiol using the positive signal from the CellROX reagent (Figure 6). The fluorescent intensity from the CellROX increases with increasing internal reactive oxygen species. The hepatocytes cultured on the gel with an elastic modulus of 14 Pa displayed consistent clustered cell morphology during the 16 h (Figure 6a). The fluorescent signal from the control conditions demonstrates the homeostatic level of ROS as a byproduct of metabolism and as a signaling molecule. As seen in all three concentrations of 17β-estradiol, the hepatocytes maintained their original morphology. The ROS signal marked by the oxidation of the fluorescent probe of the CellROX reagent was also consistent from 0 to 16 h. However, the morphology of the hepatocytes cultured on the stiffer gel with an elastic modulus of 256 Pa was changed rapidly during the 16 h after exposure to 17β-estradiol (Figure 6b). In particular, the change in morphology from spread to spheroidal was visible after 12 h of exposure to 1 nM 17β-estradiol (Figure 6b-v) and 6 h of exposure to 10 nM 17β-estradiol (Figure 6b-vi). Also, the hepatocytes exposed to 10 nM of 17β-estradiol lost most of the ROS intensity at 16 h.

The live images of ROS signal from the cells were then analyzed with the fluorescence signal intensity (Figure 6c,d). According to the quantitative analysis, even with a 0 nM concentration of 17β-estradiol, the zebrafish hepatocytes cultured on the hydrogel with an elastic modulus of 14 kPa exhibited lower ROS production than the cells cultured in the 256 Pa hydrogel (Figure 5c,d). When the concentration of 17β-estradiol was increased to 1 nM, the hepatocytes on the gel with an elastic modulus of 14 Pa showed an increase of the ROS signal up to 4 to 6 h, followed by the decrease of the intensity. In contrast, cells on the gel with an elastic modulus of 256 Pa displayed a steeper and continuous increase of the ROS signal up to 16 h.

Further increase of the 17β-estradiol concentration to 10 nM resulted in transient increases of ROS signal up to 2 h regardless of an elastic modulus of the gel. However, the hepatocytes cultured on the gel with an elastic modulus of 14 Pa showed a relatively slower increase in the ROS level than those on the gel with an elastic modulus of 256 Pa. In the stiffer hydrogel, ROS generation was decreased after 12 h, indicating that the cells were no longer functioning in metabolic degradation. Taken together, the cells cultured in the collagen-PEG hydrogel with a 14 Pa modulus were better able to metabolize the increased

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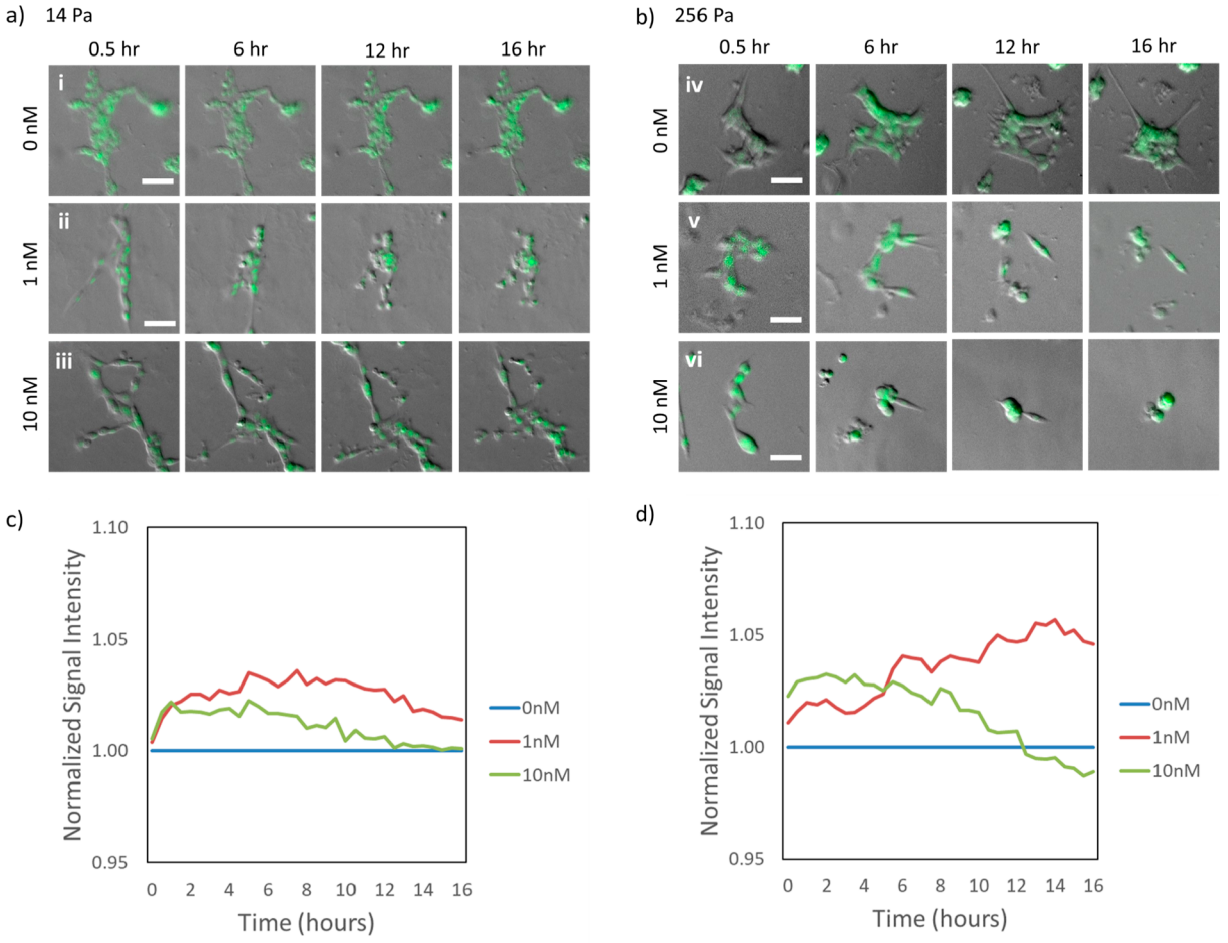

Figure 6. Live cell imaging of reactive oxygen species generation and morphology of zebrafish hepatocyte when exposed to 0 (i, iv), 1 (ii, v), and 10 (iii, vi) nM of 17β-estradiol. Images were taken every 30 min over 24 h after the addition of 17β-estradiol and CellROX reagent (green). The gray scale images are Gradient Light Interference Microscopy (GLIM) images showing the cellular morphology. (a) Zebrafish hepatocytes cultured on the collagen-PEG hydrogel with an elastic modulus of 14 Pa. (b) Zebrafish hepatocytes cultured in a pure collagen hydrogel with an elastic modulus of 256 Pa. The white scalebars indicate 40 μm. (c and d) The normalized signal intensity of CellROX for each hydrogel stiffness, 14 and 256 Pa, comparing their reactive oxygen species generation for increasing concentrations of 17β-estradiol. (c) The reactive oxygen species generation is increased for both 1 and 10 nM concentrations of 17β-estradiol in the 14 Pa hydrogel and the baseline ROS concentration is recovered after 5 h for the 1 nM concentration. (d) For the 256 Pa hydrogel, the ROS levels are increased in both concentrations of 17β-estradiol, but the ROS level drops below the control group after 12 h for the 10 nM concentration.

levels of 17β-estradiol without a significant impact on their morphology. In contrast, the hepatocytes cultured on the 256 Pa hydrogel demonstrated an early loss of functionality from elevated 17β-estradiol concentrations.

These experiments demonstrate an *in vitro* platform for the physiological impacts of endocrine-disrupting factors on zebrafish hepatoids that exhibited similar morphology and functionality as the live zebrafish. These hepatic tissues were engineered by the culturing of cells on the collagen-PEG gel with controlled stiffness while maintaining the pore area and fiber diameter of the hydrogels. The hepatocytes cultured on the collagen-PEG hydrogel with an elastic modulus of 14 Pa formed large sheets with intercellular canaliculi. In contrast, hepatocytes cultured on the pure collagen hydrogel with an elastic modulus of 256 Pa grew slowly without the formation of a cell sheet. Also, the hepatocytes cultured on the softer collagen-PEG hydrogel displayed elevated urea cycle activity and higher sensitivity to external 17β-estradiol than those cultured on the stiffer pure collagen gel. Overall, the cellular response to the gel is attributed to the difference of the gel softness.

Taken together, the results of this study report that the softness of the cell culture substrate plays a significant role in the assembly and physiological function of zebrafish hepatoids. The hepatoids engineered to present a canaliculi-like structure were active to restore intracellular ROS levels to normal by up-regulating detoxification activities upon exposure to 17β-estradiol. Since the zebrafish shares similar embryotic characteristics and homology as humans, the continued study of the zebrafish hepatoid would be beneficial to the understanding of human liver response to endocrine-disrupting chemicals such as 17β-estradiol. In addition, the results of this study will significantly impact current studies that are often plagued by the loss of morphology and functionality of live embryo or primary adult liver cells *in vitro*. Therefore, this study will be broadly useful in the development of an *in vitro* cell or organoid culture platform used for the rapid and precise screening of potentially toxic chemicals.

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Environ. Sci. Technol. 2020, 54, 13797−13806

ENVIRONMENTAL IMPLICATIONS

There are significant ethical and methodological concerns for using zebrafish as model organisms for testing EDCs as well as the aquatic environmental risks from EDC accumulation. The 3Rs, namely, Replacement, Reduction, and Refinement are essential values in legislation and guidelines governing the ethical use of animals in experiments. Hence, there is an ethical obligation to minimize the pain, stress, and suffering of fish, although there is still debate about whether fish can experience pain or have consciousness. Methodological challenges include the de-differentiation of primary zebrafish tissues for *ex vivo* cultures on conventional cell culture substrates. Therefore, the engineered *in vitro* zebrafish liver model not only mimics the natural zebrafish liver but also complements alternatives to animal testing.

Among many challenges faced by researchers in adapting *in vitro* tissue models one is that the cells do not form into physiologically similar units which maintain the structural and functional characteristics of their target organs. This study addresses this challenge by creating a hydrogel system which provides repeatable and consistent hepatoid formation from a singularized cell line. By creating a stable zebrafish liver model *in vitro*, researchers can test EDCs and other water-borne chemicals in a reliable manner.

The limitation of human health relatability is not covered in this study. Although some conclusions can be made about the effects of these EDCs on human health based on the zebrafish liver model, aspects of zebrafish liver analysis, such as the measurement of vitellogenin (VTG), are unrelatable to humans. However, from this study, one can use the hydrogel construct which can regulate phenotypes of human hepatocytes or other cell types and engineer human organoids. Therefore, this study is broadly applicable to toxicology testing where the use of live animal models is costly or ethically prohibitive.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c01988.

Real-time imaging of the ROS response to 17β-estradiol in zebrafish hepatocytes (MP4)

Scheme and figure of zebrafish hepatocytes coated with PEG, cultured on collagen-PEG hydrogel for 14 days (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Thorpe, K. L.; Cummings, R. I.; Hutchinson, T. H.; Scholze, M.; Brighty, G.; Sumpter, J. P.; Tyler, C. R. Relative Potencies and Combination Effects of Steroidal Estrogens in Fish. *Environ. Sci. Technol.* **2003**, *37* (6), 1142−1149.

(2) Pang, Q.; Li, Y.; Meng, L.; Li, G.; Luo, Z.; Fan, R. Neurotoxicity of BPA, BPS, and BPB for the Hippocampal Cell Line (HT-22): An Implication for the Replacement of BPA in Plastics. *Chemosphere* **2019**, *226*, 545−552.

(3) Nagorka, R.; Koschorreck, J. Trends for Plasticizers in German Freshwater Environments − Evidence for the Substitution of DEHP with Emerging Phthalate and Non-Phthalate Alternatives. *Environ. Pollut.* **2020**, *262*, 114237.

(4) Tian, H.; Gao, Z.; Wang, G.; Li, H.; Zheng, J. Estrogen Potentiates Reactive Oxygen Species (ROS) Tolerance to Initiate Carcinogenesis and Promote Cancer Malignant Transformation. *Tumor Biol.* **2016**, *37* (1), 141−150.

(5) Shao, Y.; Xiao, H.; Di Paolo, C.; Deutschmann, B.; Brack, W.; Hollert, H.; Seiler, T. B. Integrated Zebrafish-Based Tests as an Investigation Strategy for Water Quality Assessment. *Water Res.* **2019**, *150*, 252−260.

(6) Bergman, Å.; Heindel, J.; Jobling, S.; Kidd, K.; Zoeller, R. T. *State-of-the-Science of Endocrine Disrupting Chemicals* **2012**, *211*, 2012 DOI: 10.1016/j.toxlet.2012.03.020.

(7) Neale, P. A.; Ait-Aissa, S.; Brack, W.; Creusot, N.; Denison, M. S.; Deutschmann, B.; Hilscherová, K.; Hollert, H.; Krauss, M.; Novák, J.; Schulze, T.; Seiler, T.-B.; Serra, H.; Shao, Y.; Escher, B. I. Linking in Vitro Effects and Detected Organic Micropollutants in Surface Water

Using Mixture-Toxicity Modeling. *Environ. Sci. Technol.* **2015**, *49* (24), 14614−14624.
(8) Wilkins, B. J.; Pack, M. Zebrafish Models of Human Liver Development and Disease. *Compr. Physiol.* **2013**, *3* (3), 1213−1230.
(9) Menke, A. L.; Spitsbergen, J. M.; Wolterbeek, A. P. M.; Woutersen, R. A. Normal Anatomy and Histology of the Adult Zebrafish. *Toxicol. Pathol.* **2011**, *39* (5), 759−775.
(10) Kiamehr, M.; Heiskanen, L.; Laufer, T.; Düsterloh, A.; Kahraman, M.; Käkelä, R.; Laaksonen, R.; Aalto-Setälä, K. Dedifferentiation of Primary Hepatocytes Is Accompanied with Reorganization of Lipid Metabolism Indicated by Altered Molecular Lipid and MiRNA Profiles. *Int. J. Mol. Sci.* **2019**, *20* (12), 2910.
(11) Kim, M.; Lee, J. Y.; Jones, C. N.; Revzin, A.; Tae, G. Heparin-Based Hydrogel as a Matrix for Encapsulation and Cultivation of Primary Hepatocytes. *Biomaterials* **2010**, *31* (13), 3596−3603.
(12) You, J.; Park, S.-A.; Shin, D.-S.; Patel, D.; Raghunathan, V. K.; Kim, M.; Murphy, C. J.; Tae, G.; Revzin, A. Characterizing the Effects of Heparin Gel Stiffness on Function of Primary Hepatocytes. *Tissue Eng., Part A* **2013**, *19* (23−24), 2655−2663.
(13) Desai, S. S.; Tung, J. C.; Zhou, V. X.; Grenert, J. P.; Malato, Y.; Rezvani, M.; Español-Suñer, R.; Willenbring, H.; Weaver, V. M.; Chang, T. T. Physiological Ranges of Matrix Rigidity Modulate Primary Mouse Hepatocyte Function in Part through Hepatocyte Nuclear Factor 4 Alpha. *Hepatology* **2016**, *64* (1), 261−275.
(14) Simonetti, R. B.; Santos Marques, L.; Streit, D. P., Jr; Oberst, R. Zebrafish (Danio Rerio): Ethics in Animal Experimentation. *IOSR J. Agric. Vet. Sci. Ver. I* **2016**, *9* (7), 2319−2372.
(15) Couteau, C.; Coiffard, L. Regulation No 1223/2009 on Cosmetic Products. *Nouv. Dermatologiques* **2010**, *29* ().
(16) Uchea, C.; Owen, S. F.; Chipman, J. K. Functional Xenobiotic Metabolism and Efflux Transporters in Trout Hepatocyte Spheroid Cultures. *Toxicol. Res. (Cambridge, U. K.)* **2015**, *4* (2), 494−507.
(17) Baron, M.; Mintram, K.; Owen, S.; Hetheridge, M.; Moody, A.; Purcell, W.; Jackson, S.; Jha, A. Pharmaceutical Metabolism in Fish: Using a 3- D Hepatic In Vitro Model to Assess Clearance. *PLoS One* **2017**, *12*, No. e0168837.
(18) vom Saal, F. S.; Nagel, S. C.; Coe, B. L.; Angle, B. M.; Taylor, J. A. The Estrogenic Endocrine Disrupting Chemical Bisphenol A (BPA) and Obesity. *Mol. Cell. Endocrinol.* **2012**, *354* (1−2), 74−84.
(19) Nagel, S. C.; Bromfield, J. J. Bisphenol a: A Model Endocrine Disrupting Chemical with a New Potential Mechanism of Action. *Endocrinology* **2013**, *154* (6), 1962−1964.
(20) NCBI, N. C. for B. I. itgb1bp1 integrin beta 1 binding protein 1 [Danio rerio (zebrafish)] https://www.ncbi.nlm.nih.gov/gene/641420.
(21) Kandel, M. E.; Hu, C.; Naseri Kouzehgarani, G.; Min, E.; Sullivan, K. M.; Kong, H.; Li, J. M.; Robson, D. N.; Gillette, M. U.; Best-Popescu, C.; Popescu, G. Epi-Illumination Gradient Light Interference Microscopy for Imaging Opaque Structures. *Nat. Commun.* **2019**, *10* (1), 1−9.
(22) Clay, N. E.; Shin, K.; Ozcelikkale, A.; Lee, M. K.; Rich, M. H.; Kim, D. H.; Han, B.; Kong, H. Modulation of Matrix Softness and Interstitial Flow for 3D Cell Culture Using a Cell-Microenvironment-on-a-Chip System. *ACS Biomater. Sci. Eng.* **2016**, *2* (11), 1968−1975.
(23) Liang, Y.; Jeong, J.; DeVolder, R. J.; Cha, C.; Wang, F.; Tong, Y. W.; Kong, H. A Cell-Instructive Hydrogel to Regulate Malignancy of 3D Tumor Spheroids with Matrix Rigidity. *Biomaterials* **2011**, *32* (35), 9308−9315.
(24) Cha, C.; Kim, S. Y.; Cao, L.; Kong, H. Decoupled Control of Stiffness and Permeability with a Cell-Encapsulating Poly(Ethylene Glycol) Dimethacrylate Hydrogel. *Biomaterials* **2010**, *31* (18), 4864−4871.
(25) Yano, H.; Mazaki, Y.; Kurokawa, K.; Hanks, S. K.; Matsuda, M.; Sabe, H. Roles Played by a Subset of Integrin Signaling Molecules in Cadherin-Based Cell-Cell Adhesion. *J. Cell Biol.* **2004**, *166* (2), 283−295.
(26) Shkumatov, A.; Baek, K.; Kong, H. Matrix Rigidity-Modulated Cardiovascular Organoid Formation from Embryoid Bodies. *PLoS One* **2014**, *9* (4), 1−10.
(27) Yao, Y.; Lin, J.; Yang, P.; Chen, Q.; Chu, X.; Gao, C.; Hu, J. Fine Structure, Enzyme Histochemistry, and Immunohistochemistry of Liver in Zebrafish. *Anat. Rec.* **2012**, *295* (4), 567−576.
(28) Ip, Y. K.; Chew, S. F. Ammonia Production, Excretion, Toxicity, and Defense in Fish: A Review. *Front. Physiol.* **2010**, *1*, 1−20.
(29) Van Der Ven, L. T. M.; Holbech, H.; Fenske, M.; Van Den Brandhof, E. J.; Gielis-Proper, F. K.; Wester, P. W. Vitellogenin Expression in Zebrafish Danio Rerio: Evaluation by Histochemistry, Immunohistochemistry. *Aquat. Toxicol.* **2003**, *65* (1), 1−11.
(30) Cosnefroy, A.; Brion, F.; Maillot-Maréchal, E.; Porcher, J.-M.; Pakdel, F.; Balaguer, P.; Aït-Aïssa, S. Selective Activation of Zebrafish Estrogen Receptor Subtypes by Chemicals by Using Stable Reporter Gene Assay Developed in a Zebrafish Liver Cell Line. *Toxicol. Sci.* **2012**, *125* (2), 439−449.
(31) Mommsen, T. P.; Walsh, P. J. 5 Vitellogenesis and Oocyte Assembly. In *The Physiology of Developing Fish*; Hoar, W. S., Randall, D. J. B. T.-F. P., Eds.; Academic Press, 1988; Vol. *11*, pp 347−406. DOI: 10.1016/S1546-5098(08)60202-2.
(32) Sumpter, J. P.; Jobling, S. Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment. *Environ. Health Perspect.* **1995**, *103*, 173−178.
(33) Crain, D. A.; Eriksen, M.; Iguchi, T.; Jobling, S.; Laufer, H.; LeBlanc, G. A.; Guillette, L. J. An Ecological Assessment of Bisphenol-A: Evidence from Comparative Biology. *Reprod. Toxicol.* **2007**, *24* (2), 225−239.
(34) Le Fol, V.; Aït-Aïssa, S.; Sonavane, M.; Porcher, J.-M.; Balaguer, P.; Cravedi, J.-P.; Zalko, D.; Brion, F. In Vitro and in Vivo Estrogenic Activity of BPA, BPF and BPS in Zebrafish-Specific Assays. *Ecotoxicol. Environ. Saf.* **2017**, *142*, 150−156.