Manipulating hepatocellular carcinoma cell fate in orthogonally cross-linked hydrogels

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ABSTRACT

De-differentiation and loss of function in hepatocytes during two-dimensional (2D) tissue culture significantly hinders the progress of liver research. An ideal three-dimensional (3D) in vitro liver parenchymal cell culture platform should restore cell–cell and cell–matrix interactions, as well as normal hepatocyte polarity. Here, we report an orthogonal thiol-ene hydrogel system for culturing liver cell lines (e.g., Huh7 and HepG2). The hydrogels were prepared by a radical-mediated orthogonal thiol-norbornene photo-click chemistry using poly(ethylene glycol)-tetra-norbornene (PEG4NB) macromer and di-thiol containing linker (e.g., dithiothreitol (DTT) or bis-cysteine matrix metalloproteinase (MMP)-sensitive peptide). This system also allows facile incorporation of bioactive peptides (e.g., fibronectin-derived RGDS) to improve cell–matrix interactions. Encapsulated Huh7 and HepG2 cells showed elevated urea secretion and CYP3A4 enzymatic activities, as well as up-regulated mRNA levels of multiple hepatocyte genes (e.g., CYP3A4, BESP, and NTCP). Importantly, this is the first 3D hydrogel system that up-regulates the expression of NTCP in encapsulated Huh7 and HepG2 cell lines without any genetic modification or the addition of growth factors and chemical additives. Furthermore, the encapsulated cells displayed hepatocyte-like polarity distinctively different from the polarity displayed in 2D culture. These characteristics not only allow the study of hepatology in 3D using inexpensive cell lines, but also permit large-scale small-molecule screening. The up-regulation of NTCP expression and restoration of hepatocyte-like polarity in our hydrogels also shed light on future study of hepatitis B virus infection in vitro.

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1. Introduction

The liver is an important organ responsible for the metabolism of proteins, lipids, and steroids, the production and secretion of bile components, as well as the storage of sugar, vitamins and iron. It is also essential in xenobiotic transport, biotransformation, and detoxification. Studies in liver diseases and regeneration often utilize primary human hepatocytes (PHHs), the parenchymal cells in the liver [1–4]. However, in vitro culture of PHHs has been challenging as these primary cells lost their liver-specific characters and functions rapidly when plated in conventional tissue culture plastics (TCP). The difficulty in maintaining viability and function of PHHs in vitro using two-dimensional (2D) culture also hampers large-scale drug screening [1,3,5]. Alternatively, liver cells isolated from hepatoblastoma or hepatocellular carcinoma (e.g., HepG2 [6] and Huh7 [7]) have been used to study liver function. These immortalized cells are inexpensive and easy to maintain. Unfortunately, some of the critical liver functions and hepatocyte phenotypes were deprived off of these cells [3,4], making the use of these cells sub-optimal in liver tissue engineering research.

Many biomaterial platforms have been developed for studying liver parenchymal cells in vitro. For example, Pishko and his co-workers studied the viability and function of SV-40 transformed murine hepatocytes in chain-growth photopolymerized poly(ethylene glycol)-diacyrylate (PEGDA) hydrogels co-polymerized with fibronectin-derived RGDS peptide [8,9]. Bhatia and colleagues have exploited PEGDA hydrogels for the study of hepatocellular functions [10] and cell–cell interactions [11] in three-dimensional (3D) microenvironment, as well as for constructing micro-scale hepatic tissues for large-scale biological analysis, such as drug screening and vaccine development [12,13]. Glenn, Frank, and co-workers utilized 3D PEGDA hydrogels for encapsulating human progenitor or liver-derived cells to study hepatitis C viral infection [14]. Prestwich and colleagues prepared synthetic
extracellular matrix (sECM) hydrogels through nucleophilic reactions between PEG-based macromers (e.g., PEGDA, PEG-dimaleimide, etc.) and thiol-modified glycosaminoglycan and polypeptides for encapsulating HepG2-C3A cells [15]. Various PEG-based hydrogels modified with glycosaminoglycans (e.g., heparin) [16,17] or polysaccharides (e.g., galactose) [18] are also increasingly used to promote viability and function of primary hepatocytes in 3D.

While significant efforts have been devoted to the development of 3D in vitro hepatocyte culture [3,4,19–22], the utility of these platforms are still limited, in large part due to the difficulty in re-establishing normal cell polarity and cell–matrix interactions [3]. We hypothesize that an appropriate 3D microenvironment will restore normal hepatocyte functions and polarity in these immortalized cells. To test this hypothesis, we prepared highly cytocompatible PEG-based hydrogels formed by orthogonal thiol-norbornene photo-click chemistry [23]. The orthogonally and modularly cross-linked hydrogel network was composed of PEG-tetra-norbornene (PEG4NB) and di-thiol bearing cross-linker (e.g., dithiothreitol (DTT) or bis-cysteine peptide linker). Gelation and cell encapsulation were achieved simultaneously by a light-mediated (365 nm, under 2 min) orthogonal thiol-ene reaction, which has been shown to exhibit exceptional cytocompatibility compared with other PEG-based hydrogels formed by random chain polymerization [24,25]. This gelation method also permits easy incorporation of pendant peptides and proteins derived from ECM, which are critical for maintaining survival and function of the encapsulated cells and for preserving critical characteristics in primary cells [26–30]. In this contribution, we report the use of thiol-ene hydrogel system for encapsulation of hepatocellular carcinoma cells Huh7 and HepG2. We evaluated the influence of matrix compositions and culture medium conditions on viability, expression of liver-specific genes and functionality, as well as hepatocyte polarity in the encapsulated cells.

2. Materials and methods

2.1. Materials

4-arm PEG-OH (M.W. 20 kDa) was procured from JenKem Technology USA. Fmoc-amino acids, Fmoc-Rink-amide MBHA resin, and peptide synthesis reagents (HOBt, HBTU) were acquired from Anaspec or Chempep Inc. CellTiter Glo® and AlamarBlue® reagents were purchased from Promega and AbD Serotec, respectively. Live/Dead staining kit for mammalian cells was acquired from Life Technologies Corp. HPIC grade acetonitrile was purchased from Fisher Scientific. All other chemicals were purchased from Sigma–Aldrich unless otherwise noted.

2.2. Synthesis of PEG-tetra-norbornene (PEG4NB), photoinitiator, and peptides

PEG4NB was synthesized by reacting 4-arm PEG-OH with 5-norbornene-2-carboxylic acid as described in our previous publications [28,31–33]. The photoinitiator lithium arylyphosphinate (LAP) was synthesized following a published protocol without modification [34]. All peptides (KCGPQGWQCK, CRGDS, and CRDGS; terminal cysteines were added for radical-mediated thiol-ene reactions) were prepared following standard F-moc solid phase peptide synthesis (SPPS) protocol in a microwave peptide synthesizer (CEM Discover SPS). Crude peptides were cleaved in cleavage cocktail solution (95% trifluoroacetic acid (TFA), 2.5% water, 2.5% trisopropylisilane (TIS), and 5 wt/v% phenol), precipitated in cold ethyl ether, dried overnight, and purified using preparative scale RP-HPLC (PerkinElmer Flexar System). All peptides were purified to at least 90% purity and characterized by analytical RP-HPLC and mass spectrometry (Agilent Technologies). Purified peptides were stored at −20 °C prior to usage. The concentration of thiol groups on purified cysteine-containing peptides was quantified using Ellman’s reagent (PIERCE) following manufacturer’s protocol.

2.3. Hydrogel fabrication

Thiol-ene hydrogels were prepared by step-growth photopolymerization using PEG4NB (M.W. 20 kDa) and di-thiol crosslinkers, such as dithiothreitol (DTT) or KCNQGWWQCK (denoted as MMP-sensitive peptide linker). Thiol-ene

![Fig. 1. Orthogonally cross-linked thiol-ene hydrogel for 3D hepatocyte culture. (A) Chemical structure of PEG-tetra-norbornene (PEG4NB). (B) Light initiated thiol-ene reaction between norbornene and thiol-containing motifs. (R1: PEG, R2: cross-linkers, hv: light, PI: photo-initiator). (C) Schematic of a hepatocyte encapsulated in a biomimetic hydrogel. (D) Images of a cell-laden hydrogel disc (top: 1x photograph, bottom: 40x phase-contrast image). (E) Representative confocal z-stack images of Huh7 cells encapsulated in PEG4NB/DTT hydrogels after live/dead staining. Green: live cells; red: dead cells. (F) Viability of Huh7 cells encapsulated in PEG4NB/DTT hydrogels. Viability was defined as the ratio of live (green) cell counts to total (live and dead) cells (n = 3, mean ± SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
photopolymerization was initiated using 1 mM of LAP (dissolved in PBS) as the photoinitiator. Pre-polymer solution containing PEG4NB, cross-linker, and LAP was exposed to 365 nm light (5 mW/cm²) for 2 min (Fig. 1A–C). For all hydrogels, a stoichiometric ratio between thiol and ene groups was maintained.

2.4. Cell culture and encapsulation

Huh7 and HepG2 cells were gifts from Dr. Guoli Dai (Biology, IUPUI) and maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM, HyClone) containing 10% fetal bovine serum (FBS, Gibco) and 1 × Antibiotic-Antimycotic (Invitrogen, 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL Fungizone). Media used for differentiation experiments were 10% FBS and 1 × Antibiotic-Antimycotic in DMEM with additives noted in the text. Cells were routinely maintained in tissue culture plastic at 37 °C and 5% CO₂. Cell encapsulation was performed using a procedure similar to the gel fabrication method described above. Briefly, Huh7 or HepG2 cells (at a cell density of 5 × 10⁶ cells/mL or otherwise indicated) were suspended in pre-polymer solutions containing PEG macromer, cross-linker, integrin ligand CRGDS (or CRDGS, a non-functional control peptide), and photoinitiator. The precursor solutions were exposed to 365 nm light (5 mW/cm²) for 2 min. Cell-laden hydrogels (20 μL) were maintained in identical cell culture conditions as previously described.

2.5. Cell viability, immunostaining, and imaging

Cell viability was evaluated by live/dead staining and confocal microscopy as described previously [32]. Hepatocyte metabolic activity was assessed with AlamarBlue assay following previously developed protocol [32] with a modified incubation time of 90 min. For comparison, cell metabolic activity in hydrogel post-encapsulation was normalized to the value obtained at day 0.

Table 1

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<th>Name</th>
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<tr>
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<td>ABCB11 (BSEP)</td>
<td>TGATCCTCTGCAAGAAGGAAAGG</td>
<td>[69]</td>
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<tr>
<td>Sodium/taurocholate cotransporting polypeptide</td>
<td>SLC10A1 (NTCP)</td>
<td>TGTTTCGTCGGAAGAACATTCR</td>
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Fig. 2. Evaluation of Huh7 cell proliferation and CYP3A4 mRNA expression. Huh7 cells were encapsulated in PEG4NB/DTT hydrogels with pendent peptides CRDGS (RGD) or CRGDS (RGD). (A) Live/dead staining and confocal z-stack images (scale = 200 μm) and (B) metabolic activities of Huh7 cells on 0, 4, and 14 days post-encapsulation. (C) CYP3A4 mRNA levels from Huh7 cells cultured on 2D TCP and 3D hydrogels. (n = 3, mean ± SEM). *: p < 0.05.
ZO-1 protein was stained to visualize intercellular tight junction formation. In 2D TCP culture, Huh7 cells were seeded on an 8-well chamber glass slide (Nalgene Nunc) at $1 \times 10^5$ cells per well and incubated at 37°C with 5% CO₂. When cells reached ~80% confluency, culture media were removed, and cells were rinsed with 1× PBS before fixed by 4% paraformaldehyde (PFA) at room temperature for 15 min. After 3 washes with 1× PBS, PFA-fixed Huh7 cells were permeabilized by 1 mg/mL of saponin (Chem-impex international) at room temperature for 15 min. Then, cells were washed with 1× PBS for 3 times, and blocked with 1% BSA at room temperature for 2 h. The cells were then labeled with rabbit anti-human ZO-1 primary antibodies (1:100, Cell signaling, Cat. #: 8193) at room temperature for 2 h, washed with 1× PBS (1× PBS containing 0.05% Tween 20) for 5 min for 3 times, and immunostained by rhodamine-conjugated anti-rabbit IgG secondary antibodies (1:100, Santa Cruz, Cat. #: SC-2095) at room temperature for 2 h. After washing with 1× PBS for 5 min for 3 times, chamber walls were removed, and a cover slip was mounted onto Huh7 cells by mounting reagent pre-mixed with DAPI (Life technologies, Cat. #: P36931) for nuclei counter-stain. For 3D immuno-staining, cell-laden hydrogels were washed with 1× PBS at room temperature for 10 min before fixed by PFA, and the incubation time of gels with antibodies (both primary and secondary) were overnight at 4°C. Cell-laden hydrogels were not mounted but counter-stained with DAPI (Life technologies) at room temperature for 1 h, followed immediately by confocal imaging.

Confoal microscopy (Olympus Fluoview FV100 Laser Scanning Biological Microscope, RUPUI Nanoscope Imaging Center) was used to visualize immune-stained cells. Z-stack images (30–50 mm thick, 0.5 mm per slice) from two-three sample gels and at least four random views total were acquired for analysis by Olympus Fluoview software.

2.6. RNA extraction, reverse transcription, and quantitative real time PCR

On the indicated days of experiments, cells encapsulated in hydrogels were collected in DNase/RNase-free microtubes and flash frozen by liquid nitrogen and stored at -80°C until use. To extract RNA, frozen gels were homogenized in 500 μL of QIAzol (Qiagen, Cat. #: 79306) as the samples thawed. Samples were then incubated at room temperature for 20 min. Lysates were cleared by NucleoSpin Filters (NucleoSpin RNA II kit, Clontech, Cat. #: 740955.50), followed by adding 180 μL of 1-bromo-3-chloropropene (BCP, Sigma, Cat. #: B9673), vortexing for 15 s, incubating at room temperature for 3 min, and centrifuging at 12000 × g for 15 min at 4°C for aqueous/orGANic phase separation [35]. The aqueous layers (~600 μL) were transferred to clean microtubes and mixed in equal volume of RNase-free. Consequently, the mixtures were transferred to NucleoSpin RNA columns (NucleoSpin RNA II kit, Clontech, Cat. #: 740955.50). RNA extraction was performed following the manufacturer’s instruction. The isolated RNAs were eluted in 30 μL of DNase/RNase-free water and quantified by UV spectrometry. Aliquots of RNA samples were stored at –80°C until use.

Reverse transcription reactions were performed to convert 100–500 ng of the isolated total RNA into single-stranded cDNA using the FirstScript RT reagent kit (Clontech, Cat. #: BR017A). To assess gene expression level, quantitative real-time PCR was performed. Briefly, 2 μL of the reverse-transcribed cDNA was mixed with SYBR Premix Ex Taq II kit (Clontech, Cat. #: RR820Q) following manufacturer’s protocol. Quantitative real-time PCR was performed on an Applied Biosystems 7500 fast real-time PCR machine and run at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. Within the same experimental group, the detected levels of each gene were normalized to the level of GAPDH internal control. The normalized gene levels in day-14 2D culture were set as 1-fold for the comparison of gene expression (fold changes) under different culture conditions (2ΔΔC_t method [36]). Forward and reverse primers were listed in Table 1.

2.7. Cell function

CYP3A4 enzymatic activity was evaluated using P450 Glo CYP3A4 assays (Luciferin-IPA, Promega, Cat. #:V9001). On the day of the experiments, 2D TCP cultured Huh7 or HepG2 cells or cell-laden hydrogels were incubated with 300 μM of 100 nM Dex (images taken on day-14 post-encapsulation).

3. Results

3.1. In situ encapsulation of immortalized hepatocytes in thiol-ene hydrogels

PEG4NB (Fig. 1A) is a multifunctional macromer that reacts with thiol-bearing molecules through a radical-mediated orthogonal ‘photo-click’ reaction (Fig. 1B). The latter is not susceptible to oxygen inhibition and produces covalent thiol–ether–ester bonds (Fig. 1B). The identity of dithiol-bearing linker or mono-thiol bioactive motif can be altered to yield diverse biomimetic micro-environments for a variety of 3D cell culture applications (Fig. 1C). The rapid thiol-ene gelation is commonly performed under physiological relevant conditions and in aqueous solution; therefore it permits in situ cell encapsulation (Fig 1D). This thiol-ene hydrogel system is highly cytocompatible for in situ encapsulation of Huh7 cells as revealed by live/dead staining and confocal microscopic images (Fig. 1E). We found that at least 80% of the cells encapsulated at low cell density (5 × 10^5 cells/mL) remained viable post-

Fig. 1. Effect of Dex and DMSO on Huh7 cells encapsulated in hydrogels. (A) CYP3A4 mRNA levels from Huh7 cells cultured on 2D TCP or 3D PEG4NB/DTT hydrogels in the presence or absence of Dex or DMSO. (n = 3, mean ± SEM). *: p < 0.05. 1×: Gene level of day-14 2D TCP culture without Dex and DMSO additives. (B) Representative live/dead staining and confocal images of encapsulated Huh7 cells treated with different concentrations of DMSO in the absence or presence of 100 μM Dex (images taken on day-14 post-encapsulation).
encapsulation (Fig. 1F), while viability increased to over 95% when Huh7 cells were encapsulated at a higher cell density \((2 \times 10^7 \text{ cells/mL})\). Compared with a control non-active peptide CRDGS (denoted as RDG), the incorporation of a pendent integrin-binding peptide CRGDS (denoted as RGD) did not further increase Huh7 cell viability (Fig. 1E, F). Although the encapsulated cells proliferated to form large multi-cell clusters after two weeks of 3D hydrogel culture (Fig. 2A), the conjugation of integrin-binding ligand CRGDS did not alter cell morphology or growth kinetics (Fig. 2B).

3.2. CYP3A4 expression and cell viability in PEG4NB/DTT hydrogels

To understand the influence of culture context (i.e., 2D versus 3D) and matrix properties on hepatocyte phenotype, we evaluated the expression of hepatocyte-specific genes (e.g. CYP3A4, a member in the cytochrome P450 (CYP450) monoxygenases family) in bio-inert PEG4NB/DTT hydrogels. The experimental design was shown in Fig. 2C. We first examined CYP3A4 mRNA levels in Huh7 cells grown in 2D TCP (Fig. 2C, upper/right panel) and found that the expression level increased about 20-fold when the cells were cultured 10 additional days after reaching confluent coverage (Fig. 2C, upper/right panel), suggesting that CYP3A4 gene expression were affected by the degree of cell–cell interactions [37,38]. For comparison, we defined the gene expression levels from Huh7 cells cultured in 2D TCP for 14 days as 1-fold.

When Huh7 cells were encapsulated in 3D PEG4NB/DTT hydrogels and maintained in culture media containing 10% FBS without other additives, CYP3A4 mRNA expression was not affected (0.8x, RDG control). The incorporation of bioactive RGD motif did not facilitate the expression level (0.4x, RGD, lower/right panel of Fig. 2C). This phenotype might be a result of cell dedifferentiation. To enhance CYP3A4 expression in 3D, dexamethasone (Dex) and dimethyl sulfoxide (DMSO) were included in the culture media since these two components have been shown to facilitate hepatocyte differentiation [39–41]. Huh7 cells were encapsulated in PEG4NB/DTT hydrogels and cultured in regular media for 4 days before the addition of Dex and DMSO. On day 14, cells were harvested for evaluation (Fig. 3A, upper diagram). Interestingly, Dex and DMSO did not affect CYP3A4 mRNA expression in Huh7 cells cultured on 2D TCP (Fig. 3A, lower panel). In 3D PEG4NB/DTT hydrogels, however, Dex alone (100 nM) was sufficient to significantly up-regulate CYP3A4 mRNA expression (14-fold, \(p < 0.05\), +Dex, 0% DMSO, Fig. 3A, lower panel). Low concentration of DMSO alone (−Dex, 0.5% or 1% DMSO) also significantly elevated CYP3A4 mRNA level in Huh7 cells (both at ~17-fold, \(p < 0.05\), Fig. 3A, lower panel). We also found synergistic effect of Dex and DMSO on CYP3A4 mRNA levels (91-fold increase for +Dex, 0.5% DMSO and 164-fold increase for +Dex, 1% DMSO groups, Fig. 3A, lower panel).  

Surprisingly, DMSO at 2% alone did not further enhance CYP3A4 mRNA expression, and the addition of Dex only increased it to 15-fold (+Dex, 2% DMSO, Fig. 3A, lower panel). Live/dead staining and confocal microscopic image results showed low cell viability when DMSO was present in the culture media (Fig. 3B), suggesting...
that the addition of DMSO was cytotoxic to Huh7 cells even at low concentration (0.5% DMSO ± Dex).

### 3.3. Effects of biomimetic linkers on encapsulated hepatocyte fate

We have reported that pancreatic epithelial carcinoma cells encapsulated in orthogonally cross-linked PEG-based hydrogels behaved differently when matrix metalloproteinase (MMP)-sensitive peptide cross-linker was used [32]. To allow the liver cells to remodel their local matrix, which might facilitate cell viability, morphology, and hepatocyte-specific function, we encapsulated Huh7 cells in protease-sensitive hydrogels cross-linked by PEG4NB and MMP-sensitive peptide (Fig. 4). Live/dead staining and confocal microscopic images (Fig. 4A) and metabolic activity assays (Fig. 4B) showed that Huh7 cells were viable, metabolically active, and formed multi-cell clusters in the MMP-sensitive PEG hydrogels. Importantly, Huh7 cells encapsulated in MMP-sensitive hydrogels with RGD motif were significantly more viable, metabolically active, and separated from basolateral membranes by tight junctions (Fig. 4C). Strikingly, the incorporation of a functional RGD motif in the MMP-sensitive hydrogels further up-regulated the expression of CYP3A4 gene to 19-fold as compared with 2D culture (Fig. 4C). CYP3A4 enzymatic activities in both Huh7 and HepG2 cells encapsulated in MMP-sensitive hydrogels with RGD motif were significantly up-regulated (13- and 18-fold, respectively) when compared with 2D culture (Fig. 4D). Moreover, Huh7 cells encapsulated in MMP-sensitive hydrogels secreted significantly more urea (normalized to metabolic activity) on day 14 (compared with day 4 post-encapsulation, Fig. 4E), whereas no enhancement on urea secretion was observed in 2D culture (Fig. 4E).

### 3.4. Hepatocyte-specific gene expression in cells encapsulated in MMP-sensitive hydrogels

In addition to CYP3A4 mRNA expression, we found that other members in the CYP450 family monooxygenases family were up-regulated in a cell-type dependent manner. For example, CYP1A2 mRNA level in Huh7 cells was similar between 3D MMP-sensitive hydrogel and 2D TCP groups (Fig. 5A), whereas in HepG2 cells, CYP1A2 mRNA was 10-fold (RGD group) and 20-fold (RGD group) higher when cells were cultured in 3D hydrogels as compared with that in 2D culture (p < 0.05, Fig 5A). On the other hand, CYP2D6 mRNA level showed no statistical differences in HepG2 cells between 2D and 3D cultures, while it was down-regulated in Huh7 cells when cultured in 3D MMP-sensitive hydrogels (0.3–0.5 fold, Fig. 5A).

We also evaluated the expression of liver phase II metabolizing enzymes, such as UGT1A9 and UGT2B4, in Huh7 and HepG2 cells (Fig. 5B). UGT1A9 mRNA levels were within 2-fold differences between 2D TCP and 3D hydrogel culture, between RDG and RGD peptides, or between Huh7 and HepG2 cells. While UGT2B4 mRNA remained at similar levels between 2D and 3D hydrogel cultures and between RDG and RGD peptides in Huh7 cells, it was up-regulated in HepG2 cells encapsulated in 3D hydrogels, regardless of pendent peptide identity (13-fold, p < 0.05, Fig. 5B).

HNF4α, an important hepatocyte nuclear transcription factor, was elevated in HepG2, but not Huh7, cells encapsulated in MMP-sensitive hydrogels (both RDG and RGD: ~3-fold, Fig. 5C). Transporters on hepatocyte plasma membrane (e.g. BSEP and NTCP) are important for xenobiotic transport and are part of the detoxification pathways. Both BSEP and NTCP mRNA levels were up-regulated in Huh7 and HepG2 cells encapsulated in MMP-sensitive hydrogels (Fig. 5D). Notably, the incorporation of bioactive RGD motif in hydrogels facilitated BSEP and NTCP mRNA expression in Huh7 cells (i.e., up-regulated from 3-fold (RGD) to 5-fold (RGD)) and from 6-fold (RDG) to 15-fold (RGD) for BSEP and NTCP expression, respectively, p < 0.05, Fig. 5D). On the other hand, while 3D culture increased BSEP and NTCP expression in HepG2 cells (2-fold and 6-fold (p < 0.05), respectively), the presence of RGD motif did not change their expression levels (Fig. 5D).

### 3.5. Evaluation of hepatocyte polarity in PEG4NB/MMP-peptide hydrogels

Most epithelial cells form a monolayer in 2D culture and display a simple polarity with the apical membrane facing culture media and separated from basolateral membranes by tight junctions (Fig. 6A, left panel). Hepatocytes, however, display a complex hepatocyte polarity in which the apical and basolateral surfaces are defined by tight junctions and the apical membrane formed internally around the cell surface [42] (Fig. 6A, right panel). To examine...
the influence of culture platforms on the polarization of Huh7 cells, we first cultured these cells in 2D TCP or in DTT cross-linked hydrogels for 14 days, followed by immuno-staining to reveal the organization of a tight junction protein, ZO-1 (Fig. 6B). As expected, we found that ZO-1 localized on plasma membrane (2D, x–y axes, Fig. 6B) and between cells (2D, x–z axes, Fig. 6B), suggesting that Huh7 cells displayed a simple epithelial cell polarity when cultured in 2D TCP. When Huh7 cells were cultured in 3D hydrogels, ZO-1 was only visible within the multi-cell clusters (3D, Fig. 6B), suggesting that Huh7 cells restored the complex hepatocyte polarity when cultured in 3D. The types of cross-linkers or pendent peptides (RGD or RDG) did not affect the formation of hepatocyte clusters, and both Huh7 and HepG2 cells displayed the complex hepatocyte polarity when cultured in 3D (Fig. 6B). Interestingly, several ZO-1 staining were observed in HepG2 cell clusters, suggesting the formation of multiple apical surfaces, and possibly bile canaliculi-like structures, within a cluster.

4. Discussion

We have developed an orthogonally cross-linked hydrogel system for enhancing hepatocyte-specific functions in inexpensive hepatocellular carcinoma and hepatoblastoma cell lines Huh7 and HepG2 (Fig. 1). When encapsulated in hydrogels cross-linked by bioactive MMP-sensitive peptide, several hepatocyte-specific genes were up-regulated (Figs. 4 and 5). In addition, CYP3A4 enzymatic activity was improved (Fig. 4) while cells grew into multi-cell clusters (Figs. 2–4) with complex hepatocyte polarity (Fig. 6). One of the most crucial functions of the liver is biotransformation for metabolism and detoxification, in which the monoxygenase CYP450 family plays an important role. CYP3A4 is the most abundant CYP450 in the liver. While PEG4NB cross-linked by DTT was cytocompatible, CYP3A4 expression was not enhanced in Huh7 cells (Fig. 2C). Similar to CYP3A4, the expression of other hepatocyte genes (e.g. NTCP and HNF4a) was not improved when Huh-7 cells were encapsulated in bio-inert PEG4NB-DTT hydrogels (data not shown). This suggests the lost-of-function of Huh7 cells as hepatocytes. Dex and DMSO have both been used to promote hepatocyte differentiation [39–41]. Indeed, adding Dex and/or DMSO in culture media promoted CYP3A4 mRNA expression (Fig. 3). However, despite the wide use of DMSO as a solvent for hydrophobic molecules in tissue culture, even at a low concentration (0.5%) DMSO was cytotoxic to Huh7 cells cultured in 3D hydrogels. The most parsimonious explanation is that DMSO is cytotoxic to hepatocytes regardless of 2D TCP or 3D hydrogel culture; while dead cells were entrapped in 3D gels and become noticeable during analysis, they were removed when refreshing media in 2D TCP. Therefore, care must be taken when DMSO was used as a solvent for small hydrophobic molecules or as a reagent for promoting hepatocyte differentiation.

Although a few 3D cell culture systems have been developed for hepatitis C virus (HCV) and malaria infections [14,43–47], no 3D culture model has proven effective for in vitro hepatitis B virus (HBV) infection. We believe that our hydrogel culture platform may be highly useful in in vitro HBV infection because of the expression of NTCP from cells cultured in 3D hydrogels. The use of MMP-sensitive peptide as the hydrogel cross-linker was particularly useful as it enhanced the expression of many hepatocyte-specific genes, such as NTCP. While in this hydrogel platform the exact mechanisms by which hepatocytes adopt to improve (or restore) their function are unknown, this study represents, to the best of our knowledge, the first hepatocyte culture system capable of up-regulating NTCP mRNA level in immortalized hepatocyte cell lines without any genetic modification or culture media additives (Fig. 5D). This finding is especially important because NTCP not only is a transporter for bile products but also was recently identified as a receptor for HBV [48]. HBV infects around 2 billion people globally and poses huge public health burden worldwide [49–51]. Despite

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**Fig. 6.** Polarity of Huh7 and HepG2 cells. Huh7 or HepG2 cells were cultured on 2D TCP or 3D hydrogels for 14 days and stained for ZO-1 (red) and counter-stained by DAPI for DNA (blue). (A) Diagram of simple 2D epithelial polarity and complex 3D hepatocyte polarity. (B) Representative confocal images of Huh7 cells cultured on 2D TCP (left) and PEG4NB/DTT hydrogels. (C) Representative confocal images of Huh7 and HepG2 cells cultured on MMP-sensitive hydrogels. Images are representative of 4–8 fields examined (2–3 gels per condition). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the availability of HBV vaccines, the sero-conversion rate was estimated to be only around 57% of the vaccine recipients [52,53].

The study of HBV virology in vitro has been hindered because HBV infects only PHHs and a bi-potent cell line HepaRG [54–56]. The hopes of using PHHs to study HBV infection are dampened by the aforementioned difficulties, whereas the use of HepaRG cells is not ideal because these cells are highly heterogeneous with batch-dependent phenotypes after differentiation (10–93% biliary cells and 7–90% hepatocytes) [55,56]. Other inexpensive liver cell lines, such as Huh7 and HepG2 cells, are not susceptible to HBV infection due to the undetectable level of HBV receptor – NTCP [3]. Since its discovery as an HBV receptor [48], NTCP has been over-expressed in Huh7 and HepG2 cells by transduction of foreign plasmids [48,57,58]. This approach has allowed limited HBV infection in the transduced cells, albeit with a very low infectivity (~10%) [48,57,58]. Dex and DMSO have been used as additives in in vitro culture for differentiating hepatocytes, including bi-potent HepaRG cell line [39–41,54]. Pre-treating NTCP-overexpressing HepG2 cells with 2.5–3% of DMSO can enhance HBV infectivity to ~50–70% [57,58]; however, this method is suboptimal for HBV study because high DMSO concentration (~2%) is toxic to hepatocytes and may alter intercellular signaling events [40,41,59,60]. In the current study, even low DMSO concentration (0.5%) is cytotoxic to hepatocytes (Fig. 3B). Therefore, the ability of our orthogonally cross-linked hydrogels to promote NTCP expression (Fig. 5D) without genetic modification, or the addition of cytotoxic chemicals might provide a powerful culture platform to study HBV in vitro.

In addition to the improved hepatocyte-specific gene expression and cell function, we found that the encapsulated cells re-established complex hepatocyte polarity in 3D culture. This is critical for HBV infection as HBV infects hepatocytes only via the basolateral membranes on which NTCP localizes [56,61–63]. Hepatocytes grown on 2D TCP culture display simple epithelial polarity with limited receptor accessibility for HBV. The re-establishment of complex hepatocyte polarity in encapsulated Huh7 and HepG2 cells in our hydrogels (Fig. 6) overcomes this limitation and can potentially be a more appealing and appropriate method to study HBV virology in the future.

5. Conclusion

Our orthogonally cross-linked thiol-ene hydrogel system provides a cytocompatible niche for culturing Huh7 and HepG2 cells in 3D. When Huh7 cells were encapsulated in hydrogels cross-linked by bio-inert DTT, additives such as Dex and DMSO were required to promote hepatocyte phenotype (e.g. CYP3A4 expression). However, DMSO exhibited cytotoxicity to the encapsulated cells. Replacing DTT with bio-active MMP-sensitive peptide linkers could up-regulate the expression of several hepatocyte-specific genes and enhance hepatocyte functions in the encapsulated cells. In particular, the expression of NTCP, which is undetectable in 2D cultured hepatocytes, could be significantly up-regulated in 3D using MMP-sensitive thiol-ene hydrogels. The immobilization of RGDS motifs further enhanced NTCP expression. The cells encapsulated in the modularly cross-linked thiol-ene hydrogel also re-established complex hepatocyte polarity. Notably, these enhancements were achieved using non-genetically modified cells and without the addition of soluble additives.

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