

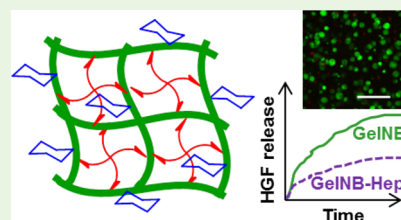
Modular Cross-Linking of Gelatin-Based Thiol–Norbornene Hydrogels for *in Vitro* 3D Culture of Hepatocellular Carcinoma Cells

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Supporting Information

ABSTRACT: Gelatin-based hydrogels are increasingly used to promote cell fate processes in 3D. Here, we report the use of orthogonal thiol–norbornene photochemistry to prepare modularly cross-linked gelatin-based hydrogels for studying the influence of independent matrix properties on hepatocellular carcinoma cell fate *in vitro*. In addition to demonstrating the ability to independently tune the mechanical and biological properties of modular gelatin–norbornene (GelNB) hydrogels, we also determined that network cross-linking density plays a key role in the mechanisms of proteolytic gel degradation. During *in vitro* degradation studies, GelNB hydrogels with lower cross-linking density degraded faster and followed a surface erosion mechanism, whereas dense GelNB hydrogels degraded in a bulk degradation mechanism. Hepatocellular carcinoma cells, Huh7, were encapsulated and grown in GelNB hydrogels with modularly tuned stiffness, bioactive motifs, and heparin content. We systematically evaluated the effect of matrix properties on cell viability and functions *in vitro*, including CYP3A4 activity and urea secretion. We found that encapsulated Huh7 cells exhibited higher cellular metabolic activity when encapsulated in modular GelNB hydrogels composed of higher gelatin contents or gels with lower stiffness. Interestingly, altering gelatin content and matrix stiffness did not significantly affect hepatocyte-specific cellular functions. To improve cellular function, we prepared norbornene and heparin dual-functionalized gelatin through a two-step synthesis protocol. Heparin-functionalized GelNB (i.e., GelNB-Hep) hydrogels were able to sequester and slowly release hepatocyte growth factor (HGF) *in vitro*. Finally, the conjugation of heparin on GelNB led to suppressed Huh7 cell metabolic activity and improved CYP3A4 activity and urea secretion.



KEYWORDS: thiol–ene, photopolymerization, gelatin, heparin, hepatocyte, hydrogel

INTRODUCTION

Hepatocytes are the major parenchymal cells of the liver, the largest organ in the body that plays crucial roles in detoxification, metabolism, and protein synthesis.^{1–3} Damage to hepatocytes can lead to hepatic diseases, including hepatitis, cirrhosis, and liver cancer such as hepatocellular carcinoma (HCC). An appropriate *in vitro* culture system is fundamental to the understanding of liver disease progression and repair mechanisms.¹ Conventionally, two-dimensional (2D) cell cultures are routinely employed to assess liver cell functions *in vitro*. However, when liver cells are cultured on 2D tissue culture plastics (TCP), they typically experience abnormal proliferation profiles and suffer from loss of hepatic functions.^{1–3} Additionally, 2D cell culture does not provide appropriate cell–extracellular matrix (ECM) interactions, which dictate natural cell polarity.² Three-dimensional (3D) scaffolds, on the other hand, can be designed to recapitulate aspects of the natural ECM microenvironment, including cell–ECM interactions crucial to cell survival and functions.⁴ As a result, there has been a paradigm shift in cell culture from 2D TCP to 3D biomaterials.⁵

A variety of synthetic and natural biomaterials have been developed for 3D cell culture, including poly(ethylene glycol) (PEG) and gelatin. Gelatin is a collagen-derived water-soluble protein that has lower immunogenicity compared to its

precursor.⁶ Similar to collagen, gelatin can undergo a thermo-reversible gelation process.⁷ Gelatin also contains peptide sequences as binding sites (e.g., Arg-Gly-Asp sequence) for integrins and protease-sensitive sites for matrix metalloproteinases (MMPs).^{7–9} Hence, gelatin has been routinely used to modify substrates/matrices that are otherwise inert to cells.¹⁰ Although gelatin can be formulated into 3D hydrogel through temperature-induced physical gelation, a high concentration of gelatin is often needed for the process. Furthermore, the resulting physical hydrogels are not stable and are not suitable for long-term *in vitro* cell culture. Alternatively, gelatin can be chemically modified with covalently cross-linkable motifs, such as methacrylamide. Chemically cross-linked gelatin hydrogels are more suitable for 3D cell culture because of their increased stability even at lower weight percentages.⁸ For example, Lau and colleagues used genipin to cross-link gelatin into microspheres to promote the formation of hepatocellular aggregates.⁴ Anseth et al. and Khademhosseini et al. prepared gelatin modified with methacrylamide (i.e., GelMA) to create covalently stabilized gelatin hydrogels for *in situ* cell encapsulation and 3D cell culture.^{11–13} In an effort to

Received: September 3, 2015

Accepted: October 20, 2015

Published: October 21, 2015

investigate hepatocyte viability and the restoration of hepatocellular functions, another group developed GelMA hydrogels with covalently immobilized galactose.¹⁴ Gelatin can also be functionalized with heparin, an important glycosaminoglycan (GAG) capable of sequestering growth factors and protecting them from proteolysis.^{15–18} In this regard, Nakamura et al. reported the conjugation of heparin to gelatin through a Borch reaction between aldehyde-modified heparin and the amino groups of gelatin.¹⁶ Kim et al. prolonged the cultivation of rat primary hepatocytes with the inclusion of thiolated heparin within PEG-diacrylate (PEGDA) hydrogels formed by Michael-type addition chemistry.¹⁵ The incorporation of heparin within gelatin-based hydrogels improved the viability and hepatocyte-specific functions (i.e., albumin and urea secretion) of the encapsulated cells. In the same study, the growth factor sequestering effect of heparin was examined through the sustained release of hepatocyte growth factor (HGF).¹⁵ You and associates created multilayered heparin/PEG microwells for enhancing hepatocyte function.¹⁸ Rat primary hepatocytes cultured in microwells coated with heparin presented cuboidal morphology indicative of a natural hepatocyte phenotype. It was also reported that cells cultured in heparin-containing microwells expressed more urea and albumin compared to those cultured in wells without heparin.

The development of GelMA hydrogels has allowed researchers to use stable chain-growth gelatin hydrogels for 3D cell culture and for soft and hard tissue regeneration.^{14,19–24} However, studies have shown that, compared with step-growth/orthogonal photoclick reactions (e.g., thiol-norbornene reaction), chain-growth photochemistries are less cytocompatible for radical-sensitive proteins and cells.^{8,25,26} In addition, it has been shown that step-growth thiol-ene photoclick hydrogels provide a facile method for immobilization of proteins/peptides to create a biomimetic microenvironment.^{27,28} Preparing a gelatin-based 3D cell culture system that utilizes step-growth thiol-ene photoclick chemistry will provide added benefits in the tunability of material properties. In this regard, we have synthesized norbornene-functionalized gelatin (GelNB) for formation of orthogonally cross-linked gelatin-based hydrogels.⁸ While GelNB hydrogels cross-linked by thiol-containing cross-linkers have proven cytocompatible, we have not reported the modification of GelNB with additional bioactive motifs (e.g., heparin), which may further impact cell fate processes, especially for hepatic cells.

Here, we describe the use of orthogonal thiol-norbornene photochemistry to form chemically modified gelatin hydrogels for 3D culture of hepatocellular carcinoma cells Huh7. Huh7 cells were used here because it is a common cell type used for drug testing and for modeling liver cancer. We are interested in developing a tunable 3D biomimetic matrix to study liver cancer cell fate, and this work is the first step toward establishing a therapeutically relevant biomimetic liver tumor niche. In this work, gelatin-based hydrogels were modularly cross-linked from reacting GelNB (either type A or type B gelatin), PEG4NB, and cross-linker PEG4SH. Hydrogel properties were tuned by changing compositions of the macromer and characterized via rheometry. The moduli of cell-laden hydrogels were measured over time to evaluate the stability of the gels in the presence of cells. Proteolytic degradation studies were performed to explore the effect of gelatin or cross-linking density on protease-mediated degradation of orthogonally cross-linked gelatin-based hydrogels. We also evaluated the influence of modular gelatin hydrogel cross-

linking conditions on the viability and function of hepatocellular carcinoma cells Huh7. Finally, we prepared heparin-modified GelNB to probe the viability, function, and morphology of 3D cultured Huh7 cells.

EXPERIMENTAL SECTION

Materials. Type A gelatin (Bloom 238–282) and type B gelatin (Bloom 225) were obtained from Amresco and Electron Microscopy Sciences, respectively. The 4-arm PEG-OH (MW 20 kDa) and 4-arm PEG-SH (MW 10 kDa) were purchased from JenKem Technology. AlamarBlue reagents were purchased from Thermo Scientific. Urea assay and CYP3A4 assay were purchased from BioAssay Systems and Promega, respectively. Live/dead staining kit for mammalian cells was acquired from Life Technologies Corp. DPBS, fetal bovine serum (FBS), 100× antibiotic-antimycotic, and DMEM were acquired from HyClone. Heparin sodium salt was obtained from Akron Biotech. 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Fisher and TCI chemicals, respectively. Recombinant murine HGF and ELISA kit were procured from Peprotech and R&D systems, respectively. Bromocresol green sodium salt was purchased from Alfa Aesar. 1,9-Dimethyl-methylene blue (DMMB), zinc chloride, and all other chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Synthesis of GelNB and PEG4NB. GelNB was synthesized by reacting gelatin with carbic anhydride as previously published.⁸ PEG4NB was synthesized through reacting 4-arm PEG-OH with 5-norbornene-2-carboxylic acid as reported elsewhere.^{29–32}

Preparation and Characterization of Hydrogels. Macromer components including GelNB, PEG4NB, and PEG4SH were mixed at different concentrations as indicated in Tables 1–5 for forming

Table 1. Hydrogel Formulations Used in Figure 1

	GelNB		PEG4SH _{10kDa}		$R_{[\text{thiol/ene}]}$
	wt %	[ene] (mM)	wt %	[ene] (mM)	
A	2	4.4	0.6	2.2	0.5
B	2	4.4	1.1	4.4	1.0
C	3	6.7	1.7	6.7	1.0

hydrogels with independently controlled properties. Gelation was initiated via long wavelength ultraviolet (UV) light exposure (365 nm and 5 mW/cm² for 5 min) using lithium acylphosphinate (LAP, 1 mM) as the photoinitiator.

A Bohlin CVO 100 digital rheometer with a light cure cell attachment was used to probe *in situ* gelation kinetics and to investigate rheological properties with settings as described in our previously published work.⁸ For *in situ* photorheometry, a macromer solution (100 μ L) was pipetted on a quartz plate in the light cure cell and irradiated with light through a flexible light guide using Omnicure S1000 system as the UV light source. Light was turned on 10 s after starting time-sweep measurement using 25 mm parallel plate geometry (10% strain, 1 Hz frequency, and a gap size of 90 μ m). Gel points were determined at the time when storage modulus (G') surpassed loss modulus (G''). To form gels for shear modulus measurement, aliquots of prepolymer solutions were injected into glass slides separated by 1 mm Teflon spacers. After gelation, disc-shaped hydrogels were punched out with 8 mm diameter biopsy punch. Moduli of the gels were measured using a parallel plate with gap size 800 μ m. Oscillatory rheometry was operated in strain sweep mode (0.1–5% strain) at 1 Hz frequency, and averaged G' values were obtained from the linear portion of the modulus-strain curves.

Proteolytic Degradation of GelNB Hydrogels. After photopolymerization of hydrogels, gels were first weighed and then placed in wells containing 1 mL of chymotrypsin (1 mg/mL) in PBS. Every 10, 15, or 30 min, gels were removed from protease solution and blotted dry, and their mass was recorded. This process was repeated until the

Table 2. Hydrogel Formulations Used in Figure 2

	GelNB		PEG4NB _{20kDa}		PEG4SH _{10kDa}		$R_{[\text{thiol/ene}]}$
	wt %	[ene] (mM)	wt %	[ene] (mM)	wt %	[thiol] (mM)	
B	2	4.4	0	0	1.1	4.4	1
D	2	4.4	0.20	0.4	1.2	4.8	1
E	2	4.4	0.45	0.9	1.3	5.3	1
F	2	4.4	0.58	1.2	1.4	5.6	1
G	2	4.4	1.13	2.3	1.7	6.7	1
H	2	4.4	1.68	3.3	1.9	7.7	1
I	7	15.4	0	0	1.9	7.7	0.5
J	7	15.4	0	0	2.9	11.5	0.75
K	7	15.4	0	0	3.8	15.4	1

Table 3. Hydrogel Formulations Used To Tune Gelatin Content without Significantly Affecting Hydrogels Stiffness

	GelNB		PEG4NB _{20kDa}		PEG4SH _{10kDa}		G'_0 (kPa)
	wt %	[ene] (mM)	wt %	[ene] (mM)	wt %	[thiol] (mM)	
L	1	2.2	1.0	2.0	1.05	4.2	2.0 ± 0.76
M	3	6.7	0	0	1.2	4.7	2.2 ± 0.13
N	5	11	0	0	0.9	3.7	2.1 ± 0.95
O	7	15.4	0	0	0.85	3.4	2.0 ± 0.42

Table 4. Formulations and Initial Moduli of Hydrogels Used in Figure 3

	GelNB		PEG4SH _{10kDa}		$R_{[\text{thiol/ene}]}$	G'_0 (kPa)
	wt %	[ene] (mM)	wt %	[ene] (mM)		
C	3	6.7	1.7	6.7	1	4.9 ± 0.12
P	5	11	2.7	11.0	1	10.6 ± 0.70
Q	7	15.4	3.8	15.4	1	22.0 ± 0.75
R	7	15.4	1.9	7.7	0.5	6.7 ± 0.34
S	7	15.4	2.9	11.5	0.75	14.2 ± 0.37

gel itself had completely degraded. Results were presented as percent mass loss to the original gel mass prior to protease treatment.

Cell Encapsulation. Huh7 cells were encapsulated at a final cell density of 5×10^6 cells/mL in all hydrogels. All macromers (i.e., GelNB, PEG4NB, PEG4SH) and photoinitiator (i.e., LAP) were dissolved in PBS and sterilized by passing the solutions through 0.22 μm syringe filters. Macromers, photoinitiator, and cells were mixed at desired concentrations with gentle pipetting. Twenty μL aliquots of precursor solutions were subjected to the same light irradiation conditions as described above. After which, the cell-laden hydrogels were cultured in growth media (high-glucose DMEM supplemented with 10% FBS and 1 \times antibiotic–antimycotic) maintained at 37 °C in 5% CO₂. Culture media were refreshed every 2–3 days.

Cell Viability, CYP3A4 Activity, and Urea Secretion. To investigate cell viability within hydrogels, encapsulated Huh7 cells were stained with live/dead staining kit (Life Technologies) and imaged with confocal microscope (Olympus Fluoview FV100 laser scanning microscope). Z-stack images (100 μm thick, 10 μm per slice) from at least three random fields were captured for all experimental conditions. On Day-0, live (green) and dead (red) cells were counted in each of the three captured images, and the viability was determined by normalizing the number of live cells to the total number of cells.

For each image, the total number of cells ranged from about 140 to 280 cells. Cell viability was also quantitatively evaluated using AlamarBlue reagent. Briefly, cell-laden hydrogels were incubated in the diluted reagent (1:10 dilution in cell culture media as recommended by the manufacturer) for 90 min, and the fluorescence generated from the cell-laden hydrogels was measured using a microplate reader (ex 560 nm, em 590 nm). Hepatocyte-specific functions in the encapsulated cells, including urea secretion and CYP3A4 enzymatic activity, were measured on day 14 postencapsulation. The cell culture media were refreshed 48 h prior to sampling, and the collected media samples were analyzed for urea content using a commercially available urea assay kit (QuantiChrom Urea assay kit, Bioassay systems). CYP3A4 enzyme activity assay was performed using P450-Glo CYP3A4 Luciferin-IPA kit (Promega) following a published protocol.³ Urea secretion and CYP3A4 activity levels were normalized by the cell metabolic activity from the respective cell-laden hydrogel.

Synthesis, Purification, and Characterization of Heparin-Conjugated GelNB (GelNB-Hep). GelNB-Hep was synthesized using standard EDC/NHS chemistry^{33,34} but with GelNB as the starting material. Briefly, heparin (1 wt/vol %) was dissolved in ddH₂O, and the concentration of carboxyl groups on heparin was determined by bromocresol green assay. To the heparin solution, 10 \times molar excess of EDC and NHS were added, and the reaction was stirred for 30 min at room temperature. In another vessel, GelNB (~2.5 wt/vol %) was dissolved in ddH₂O and added to the activated heparin solution. The reaction was allowed to proceed for another 18 h at room temperature with gentle stirring. After which, the GelNB-Hep solution was collected and dialyzed (MWCO of dialysis membrane: 6–8 kDa) against 0.2 M NaCl for 72 h, followed by another dialysis in ddH₂O for 72 h. The dialyzed samples were lyophilized, and the degree of heparin substitution (DS) was determined with DMMB assay using unmodified heparin at known concentrations as a standard (DS ~ 3 μg heparin per 1 mg of GelNB). DMMB was further used to qualitatively detect the immobilization of

Table 5. Hydrogel Formulations Used To Tune Moduli of Hydrogels without Affecting Gelatin Content

	GelNB		PEG4SH _{10kDa}		$R_{[\text{thiol/ene}]}$	G'_0 (kPa)	
	wt %	[ene] (mM)	wt %	[ene] (mM)		day 0	day 14
N	5	11	0.9	3.7	0.34	1.50 ± 0.09	0.52 ± 0.06
T	5	11	1.4	5.6	0.50	3.76 ± 0.30	2.42 ± 0.10
U	5	11	2.1	8.3	0.75	6.90 ± 0.12	5.40 ± 0.40

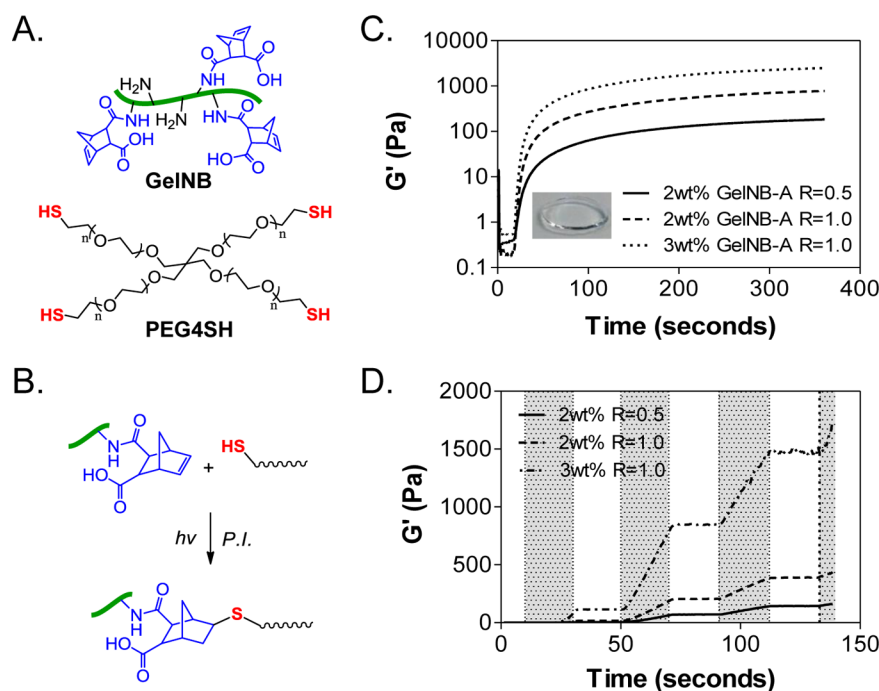


Figure 1. (A) Chemical structures of norbornene-functionalized gelatin (GelNB) and PEG-tetra-thiol (PEG4SH). (B) Light and radical-mediated thiol-norbornene photoclick reaction. (C) *In situ* photorheometry of GelNB-A using PEG4SH as cross-linker. Light was turned on 10 s after initiating the measurements. Inlet image shows a representative gelatin-based thiol-ene hydrogel. (D) Temporal control of thiol-ene gelation using intermittent light exposure. Shaded regions represent the presence of light.

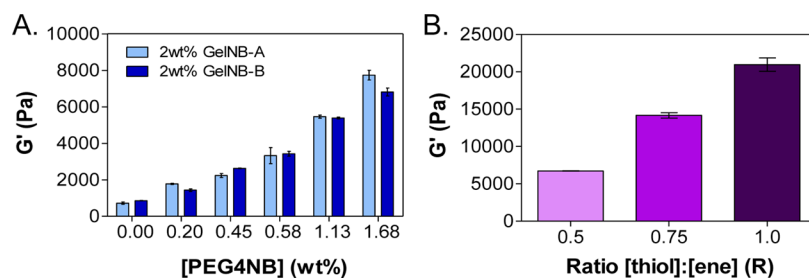


Figure 2. Fine-tuning the cross-linking density through altering concentrations of macromer (GelNB, PEG4NB, or PEG4SH). (A) At a constant gelatin content (2 wt %) hydrogel cross-linking density could be tuned by adjusting PEG4NB and PEG4SH (Table 2 compositions B, D–H) while maintaining a constant ratio of mM thiol per mM ene ($R = 1$). (B) At a fixed GelNB-B content (7 wt %) cross-linking density can be tuned by varying the ratio of mM thiol per mM ene via adjusting only the cross-linker PEG4SH (Table 2 compositions I–K).

heparin within GelNB hydrogels. Briefly, hydrogels (pure PEG, 1% GelNB or 1% GelNB-Hep, both type A and type B) were washed in PBS (pH 7.4) for 1 h to allow un-cross-linked elements to be leached from the gel. After 1 h, gels were moved to a 2 mL bath of DMMB solutions and incubated at room temperature for 24 h. After which, gels were transferred to PBS to remove residual DMMB solution, and the gels were washed in PBS for another 24 h, followed by imaging with a digital camera.

***In Vitro* HGF Release from Heparin-Conjugated GelNB Hydrogels.** The 7 wt % type A GelNB or GelNB-Hep hydrogels were prepared as described earlier, but the gels were loaded with 200 ng/gel of recombinant mouse HGF. After gelation, gels were submerged in 5 mL of release buffer composed of 0.1 wt % BSA and kept at 37 °C. At predetermined time periods, 400 μ L of solution samples were collected, and the buffer was reconstituted to 5 mL with fresh release buffer. Immediately following collection the samples were stored at -80 °C until analysis with HGF ELISA kit following manufacturer's protocol.

Statistics. All experiments were conducted independently for at least three times, and each experimental or control group contained at least three samples. Results were presented as mean \pm SD. Two-way ANOVA with Bonferroni post-test was performed to evaluate the

statistical significance between the indicated groups (* denote $p < 0.05$, ** denote $p < 0.001$, *** denote $p < 0.0001$).

RESULTS AND DISCUSSION

Light-Mediated Orthogonal Thiol–Norbornene Cross-Linking of GelNB Hydrogels. Previously, we have shown that macromer compositions affect the cross-linking efficiency of modularly cross-linked GelNB hydrogels.⁸ To further investigate the effect of GelNB macromer content and thiol-to-ene molar ratio (R) on light-mediated orthogonal thiol-norbornene gelation, we conducted gelation studies using *in situ* photorheometry. Macromers GelNB and PEG4SH (Figure 1A) were used to form gelatin hydrogels with orthogonal thiol-ene cross-links (Figure 1B). As shown in Figure 1C, precursor solutions begin to gel very rapidly upon exposure to 365 nm light at an intensity of 5 mW/cm². Increased [GelNB] from 2 to 3 wt % or increased R from 0.5 to 1 did not change gel point significantly (~ 6 –8 s). However, gels formed by higher [GelNB] or higher R yielded higher stiffness (Table 1, formulations A–C). The rapid gel points of GelNB hydrogels

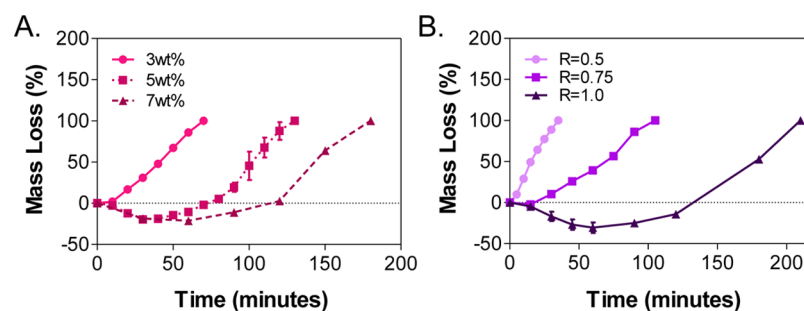


Figure 3. Chymotrypsin-mediated (2 mg/mL) proteolysis and mass loss of GelNB hydrogels cross-linked at (A) different GelNB content and (B) different thiol-to-ene ratios.

can be attributed to the rapid radical-mediated photopolymerization, the non-oxygen-inhibited thiol–norbornene reaction, as well as the presence of multiple norbornene functionalities on a single GelNB macromer.

Because gelatin can gel physically, it is crucial to verify that the formation of GelNB hydrogels is a result of light-mediated thiol–ene gelation. Here, prepolymer solutions were subjected to alternating rounds of light exposure (i.e., alternating light exposure and darkness for at least 3 cycles) during *in situ* rheometry (Figure 1D). One can see that gel modulus only increased in the presence of light and leveled off immediately upon switching off the light, indicating that hydrogel cross-linking was indeed a result of light-mediated thiol–ene photopolymerization and not physical gelation.

Effect of Macromer and Cross-Linker Concentration on Gel Modulus. In chain-growth polymerized GelMA hydrogels, elastic modulus of a hydrogel is often coupled to the network cross-linking density dictated by macromer concentration.^{11,12} The modular and orthogonal cross-linking of GelNB hydrogel allows easy decoupling of the dependency between mechanical and biological properties. Specifically, the mechanical property of the GelNB hydrogel was tuned by adjusting the concentration of bioinert macromers (i.e., PEG4NB or PEG4SH) in the precursor solution (Table 2, formulations B, D–H). Results shown in Figure 2A demonstrate that, at a fixed concentration of GelNB (i.e., 2 wt %), hydrogel shear moduli increased (~ 0.8 to ~ 8 kPa) with increasing concentrations of bioinert cross-linkers for both types of GelNB.

Another method to modularly tune the cross-linking density of orthogonal gelatin hydrogel is through adjusting R , the molar ratio of thiol to norbornene groups. At a fixed gelatin content (i.e., 7 wt % GelNB-B), gels could be easily prepared with a wide range of stiffness (i.e., ~ 6 to ~ 20 kPa) by simply adjusting R from 0.5 to 1 (Figure 2B, Table 2, formulations I–K). Additionally, Table 3 demonstrates the independent control of gelatin content (1–7 wt %) without significantly affecting gel stiffness. This allows the concentration of bioactive motifs to be adjusted while maintaining a similar cross-linking density in all groups. These experiments demonstrated that it is possible to modularly control the mechanical (e.g., cross-linking density and stiffness) and biological (e.g., cell adhesiveness and protease sensitivity) properties of the cell-laden GelNB hydrogels. It is worth noting that tuning the stiffness of gelatin hydrogel independent of its biological contents is rather challenging using gelatin hydrogels prepared from chain-growth photopolymerization. While it will be interesting to compare the differences in material properties of chain-growth and step-

growth gelatin hydrogels, such study is beyond the scope of the current work.

Proteolytic Degradation of GelNB-B Hydrogels.

Gelatin contains protease-sensitive sequences that can be cleaved by enzymes secreted by nearby cells. In an effort to understand the protease degradability of orthogonally cross-linked GelNB hydrogels, we conducted proteolytic degradation studies using chymotrypsin as a model enzyme. Instead of collagenase or gelatinase, chymotrypsin was used since it is a more economical option and can achieve similar proteolytic gel degradation.²⁶ Chymotrypsin cleaves C-terminal peptide bonds following large hydrophobic amino acids like tyrosine, proline, and tryptophan contained in gelatin.^{7,26,35} GelNB hydrogels were formed with different concentrations (3, 5, 7 wt %) at a unity of R or at the same GelNB content (i.e., 7 wt %) but different R -values ($R = 0.5, 0.75$, or 1.0). Clearly, gel moduli increase at higher weight contents of GelNB or higher R values (Table 4). Within 3 h of chymotrypsin treatment, all GelNB hydrogels eroded (Figure 3). Interestingly, the hydrogel degradation mode seemed to be influenced by the initial gel cross-linking density. Specifically, the weights of hydrogels formed with 5 and 7 wt % GelNB increased (i.e., negative % in mass loss) during the initial hours of chymotrypsin treatment (Figure 3A). These hydrogels also appeared to swell in size during the first 1–2 h of protease treatment, indicating that the degradation had happened throughout the hydrogel network, and in turn, the gels imbibed more protease solution, leading to a gain in gel mass. After a sufficient amount of gelatin molecules were cleaved, the gel mass started to decrease (positive in mass loss) and the gels eventually lost their structural integrity. On the other hand, gelatin hydrogels that formed at 3 wt % started to lose mass immediately upon placing gels in chymotrypsin solution. The gels also appeared thinner as the enzyme cleaved the gels from the exterior through a surface erosion mechanism. This process continued until the entire hydrogel disappeared.

Similar to controlling the weight percent of GelNB, altering the R -value also dictates how GelNB hydrogels are degraded proteolytically. As shown in Figure 3B, all GelNB hydrogels eroded within 4 h, but hydrogels formed at $R = 0.5$ and 0.75 degraded faster and with a surface-erosion mechanism. On the other hand, gels formed at $R = 1$ degraded slower and in a bulk degradation mechanism. These results suggest that when the cross-linking density of GelNB hydrogels is low, the cleavage of gelatin chains by protease leads to easy removal of gel fragments, and the degradation exhibits a typical surface erosion mechanism. On the other hand, when the cross-linking density of GelNB hydrogels is high, initially most of the protease-cleaved gelatin chains are still attached to the network because of the high amount of cross-links. This leads to the infiltration

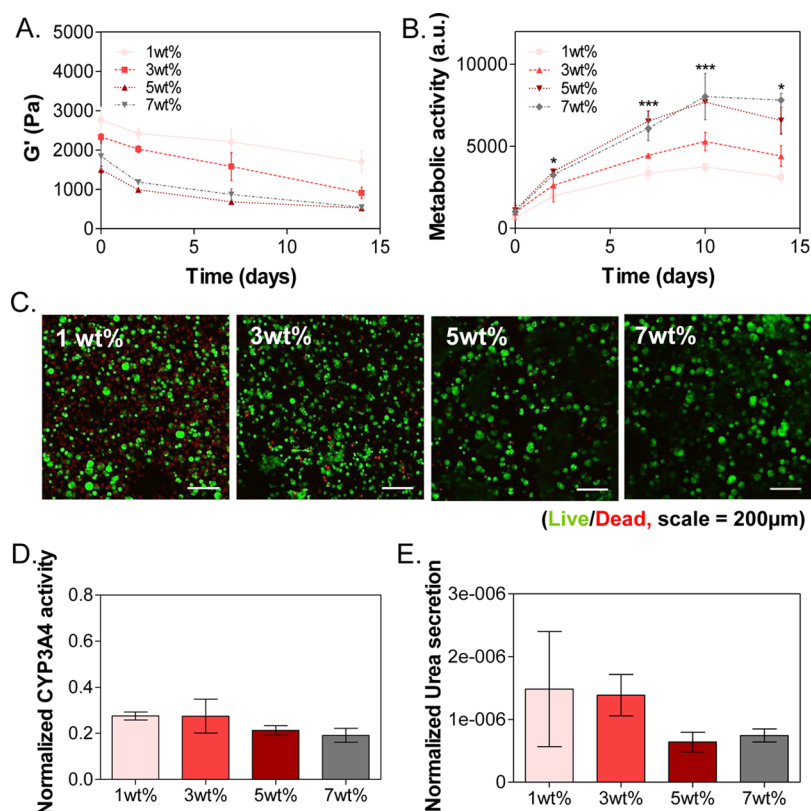


Figure 4. Effect of type B GelNB content on Huh7 cell viability. (A) Shear moduli of cell-laden hydrogels and (B) metabolic activity of Huh7 cells as a function of time. (* $p < 0.05$, *** $p < 0.0001$). (C) Representative live/dead staining and confocal Z-stack images of encapsulated Huh7 cells on day 14. (D) Normalized CYP3A4 enzymatic activity and (E) urea secretion from encapsulated Huh7 cells on day 14. CYP3A4 and urea levels were normalized to the metabolic activity on the same day of experiments.

of more protease into the gel network that causes increased network cleavage and water uptake (hence the increased in gel mass), and eventually the loss of gel integrity. The variation in degradation mechanisms may be explored for delivering growth factors in a dynamic fashion.

Influence of Gelatin Content on Viability of Encapsulated Huh7 Cell. To evaluate the effect of bioactive motif (i.e., gelatin content) on cell fate without the complication of different gel mechanical properties, we encapsulated Huh7 cells in modular GelNB hydrogels with increasing gelatin content (1–7 wt %) but with similar initial stiffness using formulations listed in Table 3. Live/dead images were used for counting the percentage of live cells following light-mediated cell encapsulation. We estimated that the percent of live cells was ~85% and 87% in 1 and 7 wt % of GelNB hydrogels, respectively. This level of cell viability is similar to the numbers reported in the literature (~80–90%).^{26,29,31} In Figure 4A it can be seen that the presence of cells affected, although not statistically significantly, the initial moduli of the GelNB gels. It is likely that the proteases secreted by Huh7 cells begin to degrade gelatin as soon as the cells were suspended in the prepolymer solution. While the gel moduli in all gels decreased significantly over the course of 14 days, all hydrogels remained intact throughout the experiment. To understand the effect of gelatin content on Huh7 viability, metabolic activity of the encapsulated cells was monitored over a two week period (Figure 4B). Huh7 cells encapsulated in gels with higher gelatin contents (5 and 7 wt %) showed higher metabolic activity from day-2 to day-14 when compared to gels at lower gelatin content (e.g., 1 wt %). Cells encapsulated in 7 wt % GelNB showed

significantly higher metabolic activity over all other groups by day-14 (* $p < 0.05$). Live/dead staining and confocal imaging results show that almost all cells remained alive and formed cell clusters in 5 and 7 wt % GelNB hydrogels whereas significantly more dead cells were found in hydrogels containing lower gelatin content (i.e., 1 and 3 wt % GelNB) (Figure 4C). These results demonstrate that higher gelatin content facilitates Huh7 cell survival.

One of the most important functions of the liver is the conversion of fat-soluble toxins into water-soluble waste, such as urea, by members of the CYP450 family. Among all the members of the CYP450 family, CYP3A4 is the most abundant enzyme. Therefore, CYP3A4 enzymatic activity (Figure 4D) and urea secretion (Figure 4E) were assessed to gain insight into the hepatocyte-specific function of the encapsulated cells. Interestingly, even though higher gelatin content improved cell survival (Figure 4B,C), it did not enhance CYP3A4 activity and urea secretion. This result suggests that, in addition to the biological motifs provided by gelatin, additional ECM cues may be required to enhance cellular functions.

Effect of Matrix Stiffness on Huh7 Viability. Mechanical properties of 2D substrates or 3D matrices have been shown to affect cell fate processes.^{36–38} While the mechanical properties of gelatin hydrogels formed by chain-growth polymerization can be tuned by adjusting functionalized gelatin content, this manipulation also simultaneously alters the concentrations of cell signaling motifs afforded by gelatin. We aimed to adjust the stiffness of our modularly cross-linked GelNB hydrogels for studying the effect of matrix mechanical cues on cell fate without the confounding effects of cell–ECM interactions.

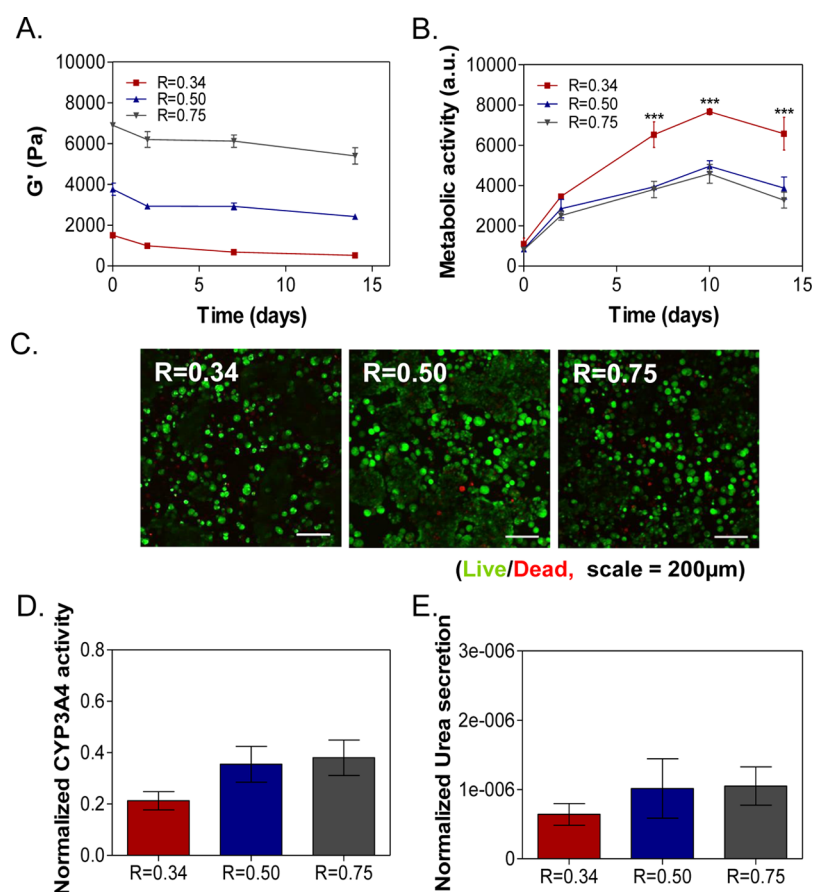


Figure 5. Effect of matrix stiffness on Huh7 viability (formulations listed in Table 5). (A) Stiffness of cell-laden gels and (B) metabolic activity of encapsulated Huh7 cells as a function of time ($***p < 0.0001$). (C) Representative live/dead staining and confocal Z-stack images of encapsulated Huh7 cells in GelNB hydrogels on day 14. (D) Normalized CYP3A4 enzymatic activity and (E) urea secretion from Huh7 cells cultured in gelatin-based hydrogels on day 14. CYP3A4 and urea levels were normalized to the metabolic activity on the same day of experiments.

Since results shown in Figure 4 reveal that higher concentration of GelNB facilitates Huh7 cell viability in 3D, we encapsulated Huh7 cells in gels containing 5 wt % GelNB while adjusting the R -value to yield the gels with a wide range of stiffness ($G' \sim 0.7$ to ~ 5.5 kPa). Day 0 viability results indicate a trend similar to that reported in the previous section; cell viability following encapsulation remained to be $\sim 87\%$. All cell-laden GelNB hydrogels degraded over time, but the moduli of the cell-laden hydrogels at day 14 were still significantly different from each other (Table 5). The metabolic activity of Huh7 cells encapsulated in stiffer gels ($R = 0.5$ and 0.75) appeared to be suppressed in a comparison with that in the softer gels ($R = 0.34$). A significant difference in metabolic activity was observed when cells were encapsulated in softer environments on day 7 and onward ($***p < 0.0001$). The results found here coincide with our previous findings that the metabolic activities of Huh7 cells were suppressed in stiffer hydrogels formed by PEG-based mixed-mode thiol–acrylate hydrogels.³⁹

One would speculate that the decreased metabolic activity from cells encapsulated in stiffer gels was a result of cell death in stiffer gels. However, this was not the case for Huh7 cells encapsulated in GelNB hydrogels with different stiffness as live/dead staining and confocal imaging performed at day 14 postencapsulation demonstrated that cells were mostly alive and formed clusters with no distinct difference (Figure 5C). It is likely that Huh7 cells encapsulated in stiffer GelNB hydrogels

received increased mechanical stimuli that suppressed their cellular metabolic activity.

Matrix stiffness (G') at 3–6 kPa might be relevant in hepatocyte functions as this stiffness range is similar to the stiffness found in healthy liver (< 6 kPa).⁴⁰ However, we found that CYP3A4 enzymatic activity was only slightly enhanced in cells grown in stiffer gels ($R = 0.50$ or 0.75 , $G' \sim 5$ kPa) at day 14 postencapsulation (Figure 5D). The same trend was also found in urea secretion (Figure 5E). This result was different from that of our previous work where Huh7 cells encapsulated in bioinert PEG-based thiol–acrylate hydrogels exhibited lower metabolic activity but higher urea secretion.³⁹ Future mechanistic studies are required to elucidate whether additional signaling crosstalks between integrins and mechanosensors exist in Huh7 cells that regulate protease activity and urea secretion.

Heparinization of GelNB Hydrogels. Since adjusting the concentration of bioactive gelatin and matrix stiffness did not yield statistically significant differences in Huh7 cell functions, we sought to further modify GelNB hydrogels with other relevant bioactive motifs, such as heparin, a highly sulfated and growth factor sequestering glycosaminoglycan that is found in abundance in the liver.⁴¹ To immobilize heparin within the modularly cross-linked gelatin hydrogels, we synthesized a heparin-modified form of GelNB (GelNB-Hep, Figure 6A) through standard EDC/NHS chemistry. Following purification, the degree of heparin substitution on GelNB was about 0.003

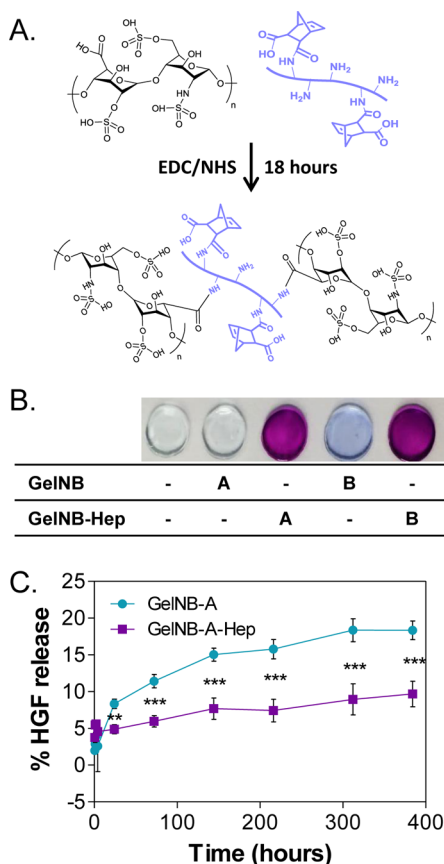


Figure 6. (A) Schematic of heparin conjugated gelatin–norbornene (GelNB-Hep) synthesis. (B) Dimethyl methylene blue (DMMB) qualitative assay to verify immobilization of heparin within hydrogels. All gels contained 1.7 wt % PEG4NB and 1.4 wt % PEG4SH except type A and B gelatin which include an additional 1 wt % of gelatin. (C) Release of hepatocyte growth factor (HGF) from 7 wt % type A GelNB or GelNB-Hep gels cross-linked by PEG4SH (** $p < 0.001$; *** $p < 0.0001$).

wt % of heparin per 1 wt % of GelNB (or 3 μg heparin/mg GelNB).

GelNB-Hep hydrogels were prepared with the same gelation method using PEG4SH as the cross-linker. The presence of heparin in the GelNB-Hep hydrogels was qualitatively assessed by a modified dimethyl methylene blue (DMMB) assay (Figure 6B). The binding of positively charged DMMB to negatively charged glycosaminoglycan (e.g., heparin) yields a shift in the absorption spectrum and the color of DMMB (i.e., from blue to pink upon binding to GAGs). Clearly, gels containing no gelatin appeared transparent since no DMMB was able to bind to the noncharged PEG hydrogel. Similarly, gels made with positively charged type A GelNB also appeared transparent whereas hydrogels containing negatively charged type B GelNB appeared slightly blue in color. The blue color obtained by type B gelatin hydrogel was a result of electrostatic interactions between the negative charge of type B GelNB and the positive charge of DMMB. Both types of GelNB-Hep, on the other hand, appeared dark pink in color after DMMB incubation, suggesting that the immobilized heparin was able to react with the DMMB dye as it infiltrated the hydrogels.

To exploit heparin's ability to sequester and sustain growth factor release, hepatocyte growth factor (HGF) was loaded into hydrogels made of either 7 wt % type A GelNB or GelNB-Hep ($R = 0.40$ and $R = 0.47$, respectively). To account for the potential influence of heparin on gel cross-linking, the two gel formulations were tuned to yield almost identical stiffness ($G' \sim 5.5$ kPa, data not shown). HGF-loaded GelNB or GelNB-Hep hydrogels were placed in PBS at 37 $^{\circ}\text{C}$, and buffer aliquots were sampled at various time points for analyzing HGF release. As shown in Figure 6C, no significant difference in HGF release was observed between the two groups during the initial few hours of release. However, at 24 h, significantly more HGF was released from the GelNB hydrogel than from GelNB-Hep gels. Nearly 400 h into the release study, only $\sim 17\%$ of HGF was released from GelNB hydrogels, and only about $\sim 7\%$ of HGF was released from GelNB-Hep hydrogels. While current study

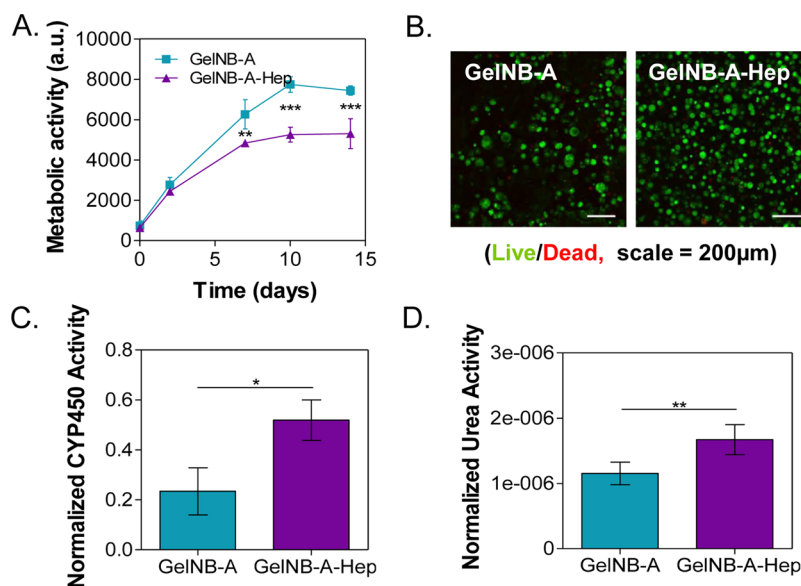


Figure 7. (A) Effect of heparin on metabolic activity of Huh7 cells (** $p < 0.001$; *** $p < 0.0001$). (B) Representative live/dead staining and confocal Z-stack images of encapsulated Huh7 cells in type A GelNB or GelNB-Hep hydrogels on day 14. (C) Normalized CYP450 enzymatic activity in Huh7 cells cultured in gelatin-based hydrogels on day 14 (* $p < 0.05$). (D) Normalized urea secretion of Huh7 cells cultured in gelatin-based hydrogels (** $p < 0.001$).

only demonstrates the ability of GelNB-Hep hydrogels to sequester and control the release of HGF, this gel format should have broader impact on supporting cell fate processes in 3D due to the broad affinity of heparin to various growth factors and cell-secreted proteins.

Effect of Heparin-Conjugated GelNB on Huh7 Cell Viability and Function. We sought to investigate the effect of heparin on Huh7 cells without the complications from gel stiffness and bioactive motifs. Here we prepared hydrogels with the same gelatin content (7 wt % type A GelNB and GelNB-Hep) and with similar stiffness (~ 5.5 kPa). Following the encapsulation of cells on day 0, Huh7 cells within GelNB gels indicate $\sim 87\%$ viability, whereas cells within heparin conjugated GelNB hydrogels are more than 95% viable. Huh7 cells encapsulated in GelNB and GelNB-Hep hydrogels remained viable, as demonstrated by the steady increase in metabolic activity (Figure 7A) and live/dead staining (Figure 7B). However, Huh7 cells encapsulated in GelNB-Hep hydrogels showed significantly lower metabolic activity at days 7, 10, and 14 as compared with cells in GelNB hydrogels ($**p < 0.001$; $***p < 0.0001$). Since the reduction of cell metabolic activity was not due to cell death (Figure 7B), it is logical to suggest that the immobilization of heparin altered intracellular signaling through sequestering growth factors contained in the supplemented serum (i.e., 10% FBS) or secreted by the encapsulated cells. In addition to suppressing metabolic activity, immobilized heparin also significantly enhanced CYP450 enzymatic activity (Figure 7C) and urea secretion (Figure 7D). The enhanced hepatocyte-specific functions may be attributed to local enrichment of growth factors from serum through binding to immobilized heparin. It has previously been reported that FBS contains a number of growth factors, many of which have the ability to influence hepatocyte functions (i.e., TGF β 1, IGF-2, IGFBP2).^{42,43} TGF β 1 has been known to inhibit the growth of Huh7 cells and induce partial differentiation.⁴⁴ It has also been documented that IGF-2 is a contributor in neoplastic hepatocyte proliferation (heterogeneous distribution of cells within a mass).⁴⁵ Therefore, it is possible that the presence of heparin locally enriched some growth factors that synergistically enhance hepatocyte-specific functions. It is also possible that the presence of heparin affected growth factor–receptor binding kinetics, which in turn resulted in different cell function.

CONCLUSION

In summary, we have prepared highly tunable gelatin-based hydrogels using orthogonal thiol–norbornene photochemistry, and the resulting gels were cytocompatible for *in vitro* 3D hepatocellular carcinoma cell culture. The biophysical and biochemical properties of GelNB hydrogels can be easily and independently tuned through adjusting the components in the precursor solution. As expected, these gelatin-based covalent hydrogels can be degraded by proteases. Through systemic variation of gelatin content and matrix stiffness, we found that higher gelatin content and lower matrix stiffness promote cellular metabolic activity. However, suppressed metabolic activity does not necessarily correlate well with cell viability and function. The modularly cross-linked GelNB hydrogels were also modified with heparin to sequester growth factors and to positively influence CYP450 activity and urea secretion from encapsulated Huh7 cells. The ability to fine-tune matrix biophysical properties (e.g., stiffness) independent of biochemical properties (e.g., cell-responsive motifs) in gelatin

hydrogels will allow researchers to study how independent local matrix properties affect cancer cell fate processes and drug responsiveness. Hence, the modularly cross-linked GelNB hydrogels should provide a highly favorable 3D cell culture platform for *in vitro* cancer cell research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsbomaterials.5b00436](https://doi.org/10.1021/acsbomaterials.5b00436).

Supporting figure showing viability of Huh7 cells postencapsulation (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This project was supported in part by the Indiana University Office of the Vice President for Research through an IU Collaborative Research Grant (IUCRG), IUPUI Office of the Vice Chancellor for Research through a Research Support Funds Grant (RSFG), and IUPUI Biomechanics and Biomaterials Research Center through a Pilot Grant. The authors thank Ms. Han Shih and Dr. Tsai-Yu Lin for their technical assistance and helpful discussion in data processing and presentation.

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