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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 NCTP 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*.

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)-diacrylate (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

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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 pendent 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. HPLC 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 arylphosphinate (LAP) was synthesized following a published protocol without modification [34]. All peptides (KCGPQGIWGQCK, 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% triisopropylsilane (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 dithiolthreitol (DTT) or KCGPQGIWGQCK (denoted as MMP-sensitive peptide linker). Thiol-ene

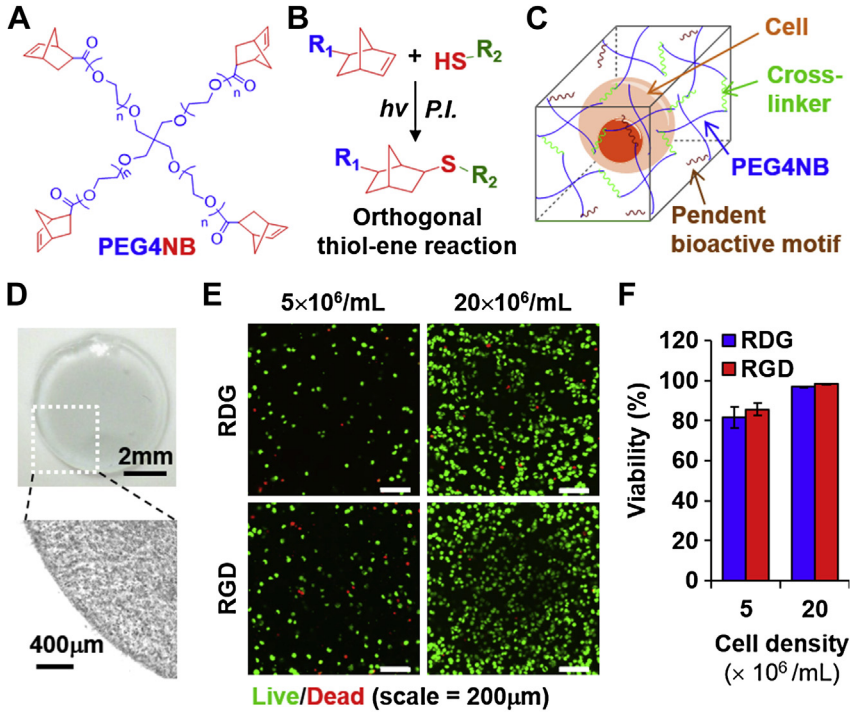

Fig. 1. Orthogonally cross-linked thiol-ene hydrogel for 3D hepatocyte culture. (A) Chemical structure of PEG-tetra-norbornene (PEG4NB). (B) Light initiated thiol-ene reaction between norbornene and thiol-containing motifs. (R1: PEG, R2: cross-linkers, *hv*: light, P.I.: photo-initiator). (C) Schematic of a hepatocyte encapsulated in a biomimetic hydrogel. (D) Images of a cell-laden hydrogel disc (top: 1× photograph, bottom: 40× 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.)