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COMMUNICATION

Viral infection of human progenitor and liver-derived cells encapsulated in three-dimensional PEG-based hydrogel

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Abstract
We have studied the encapsulation of human progenitor cells into 3D PEG hydrogels. Replication-incompetent lentivirus promoter reporter vectors were found to efficiently detect the *in vivo* expression of human hepatic genes in hydrogel-encapsulated liver progenitor cells. Similarly, hydrogel-encapsulated cells could be efficiently infected with hepatitis C virus, and progeny infectious virus could be recovered from the media supernatants of the hydrogels. Provocatively, the diameters of these virus particles range from ∼50 to 100 nm, while the calculated mesh size of the 8 k hydrogel is 44.6 ± 1.7 Å. To reconcile how viral particles can penetrate the hydrogels to infect the encapsulated cells, we propose that microfractures/defects of the hydrogel result in a functional pore size of up to 20 fold greater than predicted by theoretical mesh calculations. These results suggest a new model of hydrogel structure, and have exciting implications for tissue engineering and hepatitis virus studies.

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(Some figures in this article are in colour only in the electronic version)

Introduction

End-stage liver disease (ESLD) is a major cause of worldwide morbidity and mortality [1]. Liver transplantation is the only available treatment. Viral infections such as from hepatitis C virus (HCV) are important etiologies of ESLD. About 170 million people worldwide are infected with HCV, and it is the leading cause of liver cancer and liver transplantation in the United States [1]. Current interferon-based therapies are inadequate for most patients, and there is a great need to develop and evaluate new potential antiviral strategies [2]. Engineered artificial human liver tissues could be of great benefit to the above efforts. Soon after plating into standard two-dimensional (2D) tissue culture, however, primary hepatocytes lose many features of advanced differentiation, including the ability to support infection with hepatitis viruses. Because of this and the critical shortage of transplantable liver tissue, we have been exploring the potential to create

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three-dimensional (3D) liver tissues by encapsulating human progenitor cells in a 3D matrix that might better promote the maintenance of hepatic features and enable the study of the HCV replication cycle in a 3D microenvironment. An attractive biomaterial for such purpose is photopolymerizable poly(ethylene glycol) di-acrylate (PEG-DA)-based hydrogels [3]. PEG is a non-toxic, robust material with easily controlled mass transfer properties that can be cross-linked into 3D hydrogels by introducing terminal acrylate functional groups that are capable of polymerization in the presence of UV light and a photoinitiator [4]. Moreover, cells can be encapsulated during the polymerization process, and hydrogels can be patterned using advanced photolithographic or micro-fluidic technologies into shapes, and structures of defined micro-dimensions that offer structural support and high tissue density while maintaining an *in vivo* like environment for cells [5]. Furthermore, the chemical properties of PEG-based hydrogels allow for functionalization with biologically relevant molecules such as growth factors, degradable linkages or peptides promoting cell attachment [3].

In this communication, we studied the encapsulation of human progenitor cells into 3D PEG hydrogels and determined their ability to be infected by viruses. Provocatively, the diameters of the latter are larger than the calculated mesh size of the hydrogel, and lead us to propose a new model of hydrogel structure.

Material and methods

Materials

Poly(ethylene glycol) diacrylate (*MW* 575,700), poly(ethylene glycol) (PEG) (*MW* 1.5 k, 3.4 k and 8 k), acryloyl chloride and acrylic acid (AA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). PEG (*MW* 1.5 k, 3.4 k and 8 k) was converted into PEG-DA from a reaction between the hydroxyl moieties associated with the PEG end groups and acryloyl chloride according to the following procedure.

Synthesis of PEG diacrylate (PEG-DA)

PEG-DA was synthesized as described previously [6]. In a two-way reaction flask in a 50 °C water bath, 10 g of PEG powder (Aldrich) was added along with 100 mL of tetrahydrofuran (THF) (Aldrich) in order to dissolve the PEG powder. After the PEG powder was dissolved, acryloyl chloride (Aldrich) was added to the reaction flask (2 mL for 8 k and 4 mL for 3.4 k), and was left to react with the PEG powder for 5 h. After the mixture cooled to room temperature, the reaction flask was left in a refrigerator overnight, and the PEG crystallized. After the THF solution was disposed of, 100 mL of fresh THF was added and the crystallized PEG was dissolved in the reaction flask in a water bath (50 °C). This process of replacing the THF, adding new PEG powder, letting the mixture cool down and leaving it in the refrigerator overnight was repeated four times. The resulting PEG-DA is dried in an oven (30 °C), where residual THF evaporates and only the synthesized PEG-DA powder remains.

Cell culture

Huh-7.5 cell monolayers (a subline derived from Huh-7 hepatoma cells [8]) were propagated in Dulbecco's modified minimal essential medium (DMEM) (Cellgro) in the presence of 10% fetal bovine serum (Omega Scientific), 2 mM L–glutamine (Cellgro), 100 IU of penicillin mL^{-1}, 100 μg of streptomycin mL^{-1} (Cellgro) and 0.1 mM nonessential amino acids (GIBCO). Huh-7.5 cells were grown in plastic dishes at 37 °C with 5% CO_2.

Preparation of hydrogels for cell encapsulation and HCV infection

Huh-7.5 cells were resuspended (up to 2.5 × 10^6 cells mL^{-1}) in a 8 k PEG-DA pre-polymer solution in PBS (Gibco). The photoinitiator, Irgacure 2959 (I-2959, Ciba), was added until final concentrations of the photoinitiator reached 0.05% (w/v). Fifty microliters of uncured solution was loaded between glass slides (VWR) with a Teflon spacer and exposed to 320–390 nm UV light (10 mW cm^{-2}) for 60 s (DYMAX, Cure spot 50). The resulting hydrogel was submerged in 1 mL of growth media (DMEM, 10% FBS, non-essential amino acids). After 24 h post-encapsulation, hydrogel cultures were infected with tissue culture generated hepatitis C virus particles (HCVcc) [8], or were subjected to viability determination using Live–Dead™ assay, as previously described [7]. Infection was performed as follows: the hydrogel-encapsulated cells were submerged in 0.5 mL of growth media containing 1.35 × 10^3 TCID50 of HCVcc (MOI ∼ 0.1). The cells were incubated at 37 °C for 3 h under gentle rocking; 0.5 mL of media were added. At 24 h post-infection, the hydrogel was washed three times for 10 min with 2 mL of PBS at 37 °C with gentle rocking, followed by incubation in growth media. The cells were maintained in the hydrogel for 10 days, and the media were replaced every 4 days. After 10 days following infection, the growth media were pre-cleared and used to infect naïve Huh-7.5 cells in regular 2D culture as described [8]. The hydrogel-encapsulated cells were fixed with 4% formaldehyde for 1 h, and the resulting whole mount was stained for the HCV core antigen, as previously described [8]

Measuring cell viability by Live/Dead assay and Alamar Blue

Live and dead cell numbers in reference samples labeled with calcein AM (5 mg mL^{-1}) for live cell staining and ethidium homodimer (2.5 mg mL^{-1}) for dead cell staining fluorescent stains (Live-Dead™, Molecular Probes) were manually counted. Six regions on duplicate samples were examined, and the mean was determined. Images were acquired by a Nikon Ellipse TE300 fluorescence microscope and a Qimage RETIGA EXi camera.

Alamar Blue (Trek Diagnostic Systems), a fluorometric indicator of cell metabolic activity, was performed to determine relative live cell number to control samples. Samples were washed with PBS after aspirating the medium. They were then refed with 10% (v/v) Alamar Blue dye and

90% (v/v) cell culture medium, and allowed to incubate for 5 h. The resulting solution was removed from the sample, and the fluorescence was measured at room temperature on a plate reader, FLEXstation II 384 (Molecular Devices), using excitation and emission wavelengths of 520 and 590 nm, respectively. The relative live cell number was obtained by comparing the relative fluorescent intensity of experimental samples and references.

Lentivirus reporter constructs

Human hepatic-specific promoters of alpha-fetoprotein and albumin were cloned and then inserted into lentiviral vectors so as to drive the expression of EGFP or DsRed reporter genes, respectively, as described in [9]. VSV-G pseudotyped virus particles were prepared, and the viral titer determined, as described in [9].

Encapsulation of stem cells and liver progenitors

Human differentiating embryonic stem cell clusters (hDESC) derived from embryoid bodies were prepared as described in [10]. Human fetal livers (14–22 weeks) were purchased from Advanced Biosciences Research Inc. (Alameda, CA) in accordance with all institute, state and federal regulations. Human fetal liver progenitor cells (LPC), defined by EpCAM positive cell population [11, 12], were isolated using anti-EpCAM antibody-conjugated magnetic beads as described in [11, 12]. Around 20–40 hDESC clusters, or 2×10^5 LPC, were encapsulated in 100 μl of hydrogel. For LPC encapsulations, 5 mM GRGDS peptide (Peptide International Inc, Louisville, KY) was added to the hydrogel before polymerization [13]. Media were changed every 3 days. At day 4, LPC in hydrogel were transduced by pseudotyped lentivirus particles at an MOI of 50:1. After 6 days of culture, the expression of reporter genes was examined under fluorescence microscopy. EGFP and DsRed were examined under fluorescence microscopy. BrdU labeling for hDESC in hydrogel was performed as described in [9].

Mechanical properties of PEG-DA hydrogels

Uniaxial strip extensometry has been used to measure the stress and strain of different *MW* of poly(ethylene glycol) diacrylate [6]. In this study, strips of hydrogels were tested using an Instron 5844 materials testing apparatus equipped with a 10 N load cell (Instron Corp., Norwood, MA). After making gels and allowing for swelling in PBS buffer, the samples were cut into 3.0 mm wide and 12.0 mm long strips using a parallel blade device, and thickness was measured using a digital caliper (VWR International, Westchester, PA). The set grip distance was set at 5 mm, and the gels were clamped. The crosshead speed was controlled at 3.6 mm min^{-1} for all samples. During the tests, an ultrasonic humidifier was used to maintain the water content of the samples. Five strips of each type of material were tested, and an average value for Young's modulus (E), maximum strength ($\sigma_{\max}$) and elongation at break (ε_{b}) of water-saturated PEG-DA single networks were measured along with associated standard deviations.

Swelling studies

The water content of the hydrogels was evaluated in terms of the weight swelling ratio, and swelling studies were performed by immersing the weighted dry hydrogel in water. The swollen gels were lifted, patted dry, and weighed at regular intervals until equilibrium was attained. The percentage of water content (WC) is calculated from a swollen and dry weight of the hydrogel as described previously [14, 15]:

$$\text{Water content} = \frac{W_s - W_d}{W_s} \times 100, \tag{1}$$

where W_s and W_d are the weights of swollen state and dried state, respectively.

Number average molecular weight between cross-links and mesh size

Number average molecular weight between cross-links (M_c) and mesh size of hydrogels was calculated using a previously published method [3, 16, 17] based on swelling data. The swelling of a hydrogel depends on the number of effective chains per unit volume, V_e, which is given by the following equation:

$$V_e = -\frac{\frac{\nu}{V_1}\left[\ln(1 - V_{2,s}) + V_{2,s} + \chi V_{2,s}^2\right]}{V_{2,r}\left[\left(\frac{V_{2,s}}{V_{2,r}}\right)^{1/3} - \frac{1}{2}\left(\frac{V_{2,s}}{V_{2,r}}\right)\right]}, \tag{2}$$

where ν is the specific volume of the polymer, which is 0.893 cm^3 g^{-1} for PEG, V_1 is the molar volume of water (18 cm^3 mol^{-1}), and χ is the Flory–Huggins interaction parameter of polymer–water ($\chi_{\mathrm{PEG}} = 0.426$). $V_{2,r}$ and $V_{2,s}$ are the volume fractions of polymer in the relaxed gel and swollen gel, respectively. These are defined as

$$V_{2,r} = \frac{V_p}{V_r}, \qquad V_{2,s} = \frac{V_p}{V_s}, \tag{3}$$

where V_p is the volume of polymer, V_r is the volume of relaxed gel, and V_s is the volume of swollen gel. From V_e and the number average molecular weight of polymer before cross-linking ($M_{n(0)}$), the number average molecular weight between cross-links, M_c, can be determined using the following equation:

$$M_c = \left(V_e + \frac{2}{M_{n(0)}}\right)^{-1}. \tag{4}$$

The mesh size of the hydrogel network was then determined as described by Peppas *et al* [16]. Briefly, the average end-to-end distance of the polymer chain in the unperturbed (solvent-free) state was determined using the following equation:

$$\left(r_0^{-2}\right)^{1/2} = l\left(2\frac{M_c}{M_r}\right)^{1/2} C_n^{1/2}, \tag{5}$$

where l is the carbon–carbon bond length, typically 1.50 Å, M_r is the molecular weight of the repeating unit, and C_n is the Flory characteristic ratio ($C_{n,\mathrm{PEG}} = 4.0$). The mesh size in angstroms, ξ, was then calculated using the following equation:

$$\xi = \left(r_0^{-2}\right)^{1/2} V_{2,s}^{-1/3}. \tag{6}$$

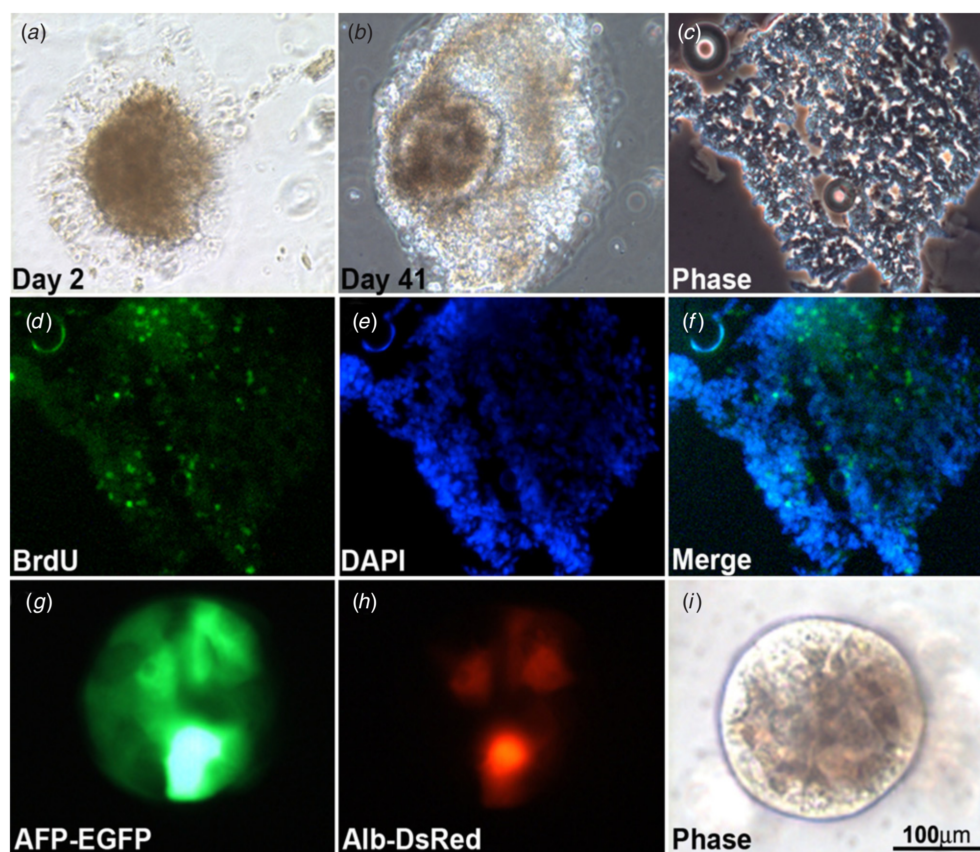

Figure 1. Growth and gene expression of human differentiating embryonic stem cells and liver progenitor cells in PEG hydrogels. (*a*), (*b*) Embryoid bodies derived cells survive for at least six weeks encapsulated in 8 k PEG hydrogel. (*c*)–(*f*) BrdU labeling (stained with FITC-conjugated anti-BrdU antibody) indicating active DNA replication within numerous nuclei (counterstained with DAPI) of the encapsulated hDESC. (*g*)–(*i*) Liver progenitor cells (LPC) encapsulated in PEG 8 k hydrogels containing RGD peptide and expression of hepatic genes monitored in live cells using lentiviral promoter reporter vectors, wherein human promoters for AFP (*g*) or albumin (*h*) are fused to the genes encoding the fluorescent proteins, EGFP and DsRed, respectively.

Results and discussion

To determine conditions for encapsulation of cells in the hydrogel, we first investigated the viability of cells in different molecular-weight-based PEG (*S*1) and optimized a variety of parameters including water content (*S*2), UV intensity and exposure time (*S*1–2, *S*1–3), and photoinitiator concentration (*S*1–1) (see Supporting Information (SI) at stacks.iop.org/BMM/4/011001). Optimal conditions and viability were obtained with PEG-DA 8 k hydrogels, and these were used throughout this study. Next, we encapsulated two types of cells that represent attractive potential starting sources for the fabrication of human liver tissues, a complex mixture of differentiating human embryonic stem cells (hDESC) cultured in conditions designed to promote early hepatic differentiation [10] and liver progenitor cells (LPC) isolated from human fetal liver [12].

As shown in figure 1, hDESC could be maintained for at least six weeks within the hydrogel scaffold (figures 1(*a*), (*b*)). BrdU labeling indicated that the encapsulated cells were undergoing DNA replication associated with active cell division (figures 1(*c*)–(*f*)). LPC isolated from human fetal liver could be similarly encapsulated in hydrogels (figures 1(*g*)–(*i*)). Moreover, not only could these cells be encapsulated and grown in hydrogel for extended periods of time, they also retained the ability to express genes characteristic of liver cells such as alpha-fetoprotein (AFP) and albumin. Indeed, we were able to monitor the *in vivo* expression of the latter using pseudovirus particles capable of infecting the target cells (figures 1(*g*), (*h*)). These particles pseudotyped with the VSV-G envelope protein contained replication-defective lentiviral vectors encoding fluorescent reporter proteins under the transcriptional control of the AFP or albumin promoters [9].

Infection of the encapsulated LPC with the pseudovirus particles of about 100 nm in size [18] was somewhat provocative, given the estimated mesh size ∼40 to 45 Å (PEG-DA 8 k) of the hydrogels calculated using a previously established method [17, 19] (see SI stacks.iop.org/BMM/4/011001). Given the above mesh size, virus particles (50–100 nm) should not be able to diffuse in or out of the hydrogel network and infect the cells encapsulated in a 3D microenvironment. Even allowing for the estimated increase in mesh size from the unperturbed to the theoretical maximally stretched state (see SI stacks.iop.org/BMM/4/011001 equation (4)) [16], this would still be predicted to prevent virus particles from diffusing through the mesh. Moreover, the hydrogel retained its full

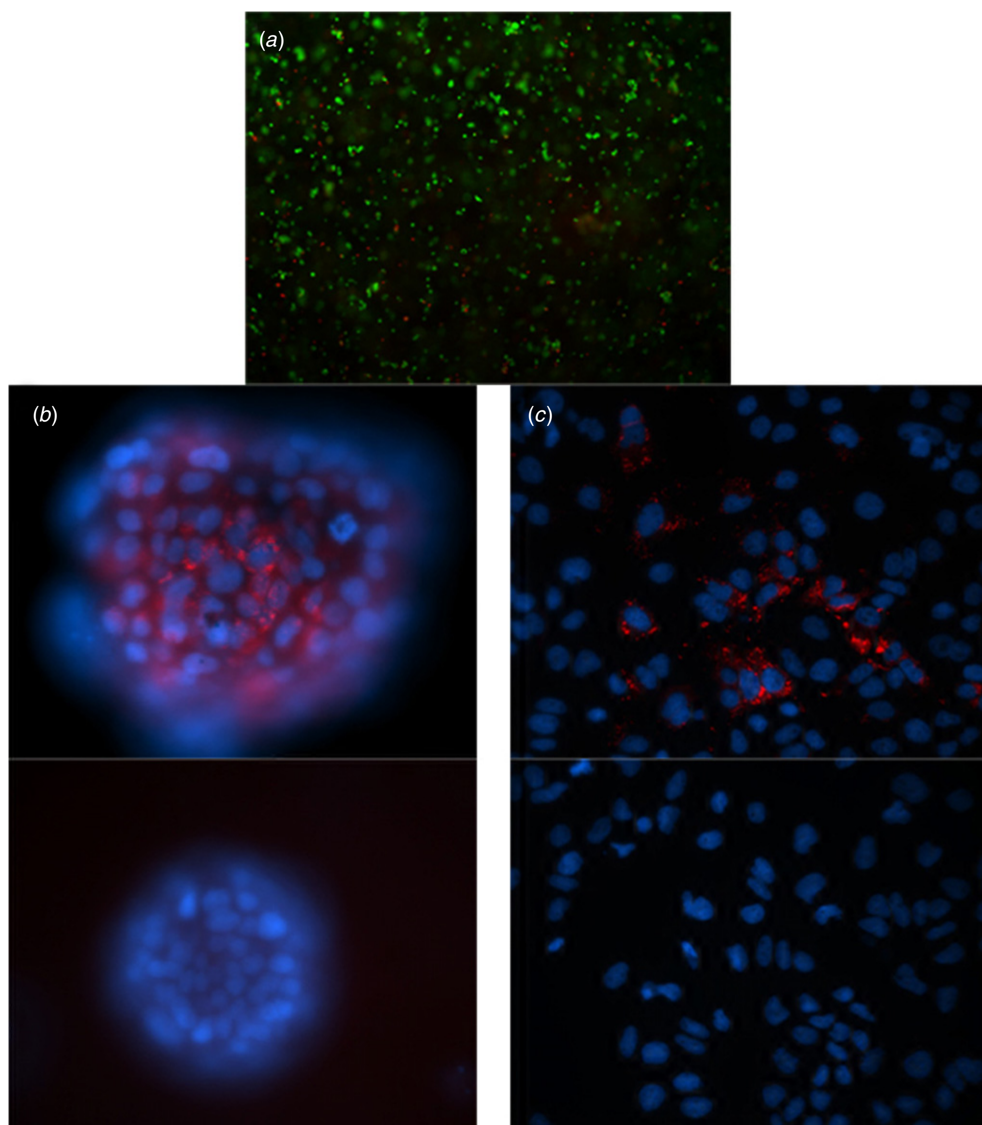

Figure 2. HCV infection of Huh-7.5 cells in hydrogels. (*a*) Viability of hydrogel-encapsulated Huh-7.5 cells performed at 24 h post cell encapsulation. Live cells are stained green and dead cells stained red. (*b*) Immunofluorescence staining for HCV core antigen (red) in hydrogel encapsulated cells 10 days after infection with HCV (top panel) or mock (lower panel) inoculums. Note the distinct foci within cells that are in the focal plane resembling the core antigen localization as seen in 2D culture infected cells. (*c*) Media supernatants from wells containing the hydrogels of (*b*) collected 10 days after initial infection were inoculated onto Huh-7.5 cells grown in standard 2D culture. Seventy-two hours post-infection the cells were stained for HCV core antigen. Note again the characteristic HCV core-staining pattern (top panel), demonstrating that infectious progeny virions could be obtained from the supernatants of HCV-infected, hydrogel-encapsulated cells.

tensile strength over the time course of the experiment (*S*4). We, therefore, hypothesize that the ability of virus particles to infect encapsulated cells is a result of the heterogeneity of hydrogels that persists throughout the swelling process. Typically, hydrogels are constructed with cross-links induced chemically or by irradiation, both of which result in structures with cross-links that are distributed throughout the hydrogel. In the PEG 8 k diacrylate system, cross-linking occurs at the termini of polymer chains. Each cross-linked site in the network consists of an oligoacrylate that serves as a node from which a number of PEG chains emerge, depending on the degree of polymerization of the network. Presumably, encapsulated materials (in our case, cells ∼10 μm), restrict local PEG chain network formation, leading to microfractures or defective formations. Indeed, the latter are the likely result of previous purposeful attempts to alter the hydrogel network structure or create pores using freeze thaw, porosigens, nanoparticles and gas bubbles [20–22].

Based on the lentiviral reporter infection results, we hypothesized that not only can defective pseudoviruses infect hydrogel-encapsulated cells, but fully infectious virus could also infect permissive cells leading to a complete viral replication cycle. To test this hypothesis, Huh-7.5 cells (the only cell line known to be capable of efficient HCV infection) were encapsulated in PEG-DA hydrogel followed by infection with an HCV inoculum (virus particle size ∼50 nm) produced *in vitro*, as described in [8]. As shown in figure 2(*a*), the human hepatocellular carcinoma-derived Huh-7.5 cells can

survive encapsulation in PEG hydrogel. As hypothesized, the encapsulated cells were efficiently infected within the PEG-DA hydrogel (figure 2(*b*)). Moreover, the infected encapsulated cells also produced fully infectious progeny virus capable of reinfecting naïve Huh-7.5 cells (figure 2(*c*)), demonstrating that a complete HCV replication cycle could be supported by the hydrogel-encapsulated cells.

Human embryonic stem cells have been cultured on the surface of PEG hydrogels [23]; cells derived from human embryonic stem cells have been differentiated toward a chondrogenic lineage in PEG hydrogels [24]; and mouse liver progenitor cells have been successfully encapsulated within PEG hydrogels [7]. However, to the best of our knowledge, this is the first comunication to demonstrate that progenitor cells isolated from human fetal liver tissue can be successfully encapsulated and maintained within PEG hydrogels. Moreover, this is the first report of *de novo* infection with virus (both replication-defective pseudovirus particles and fully infectious hepatitis C virus).

Conclusion

Human progenitor and liver-derived cells can be readily encapsulated in 3D PEG-based hydrogels. Reconciling the ability to infect the hydrogel-encapsulated cells with viruses of greater diameter than the calculated mesh size of the hydrogel leads us to propose that microfractures/defects of the hydrogel result in a functional pore size of up to 20 fold greater than predicted by theoretical mesh calculations. Moreover, this novel platform provides a unique opportunity to study HCV infection of cells cultured in a three-dimensional environment and the evaluation of novel antiviral therapies. In the long term, hydrogel-based, artificially engineered human liver tissues might form the basis for liver assist devices and cell replacement therapies [25].

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