

Preparation of Microporous Hydrogel Sponges for 3D Perfusion Culture of Mammalian Cells

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Abstract Three-dimensional (3D) perfusable organ models, primarily composed of liver cells, are expected as an efficient tool for *in vitro* cell-based drug screening and development. Various types of hydrogel-based 3D cell culture systems have been developed, but the lack in proper techniques to form vasculature networks in the hydrogel matrices results in inefficient supply of oxygen and nutrition to the cells. Here we propose a facile strategy to creating a perfusable hydrogel-based liver cell culture system. We utilized a bicontinuous dispersion of an aqueous two-phase system, which was composed of polyethylene glycol (PEG)-rich and gelatin methacrylate (GelMA)-rich phases, to produce cell-encapsulating microporous GelMA-based hydrogels. We successfully encapsulated HepG2 cells in the hydrogel matrix with a high cell viability, and confirmed that the spongy hydrogel was superior to homogeneous hydrogels for 3D cell culture. We performed perfusion culture for the cells encapsulated in the hydrogel sponge, to verify the usability and versatility of the presented hydrogel material for perfusion culture. The presented approach would be useful as a unique tool for developing organs-on-a-chip systems.

1 Introduction

The liver plays crucial roles in drug metabolism in our body, and hence, reconstruction of liver tissue models *in vitro* is of significant importance in developing cell-based drug screening systems. Recently, organs-on-a-chip devices or microphysiological systems (MPS) gain much attention that can reconstruct *in vivo* cellular environments and functions. These technologies could reproduce the interactions between multiple organs and/or control partial oxygen tension with the help of the circulating medium flow. These systems have been applied to liver cell culture experiments (Ho *et al.*, 2013), which are expected as alternative tools to animal models for liver-based drug testing.

Until now, many types of liver cell culture systems have been implemented into perfusion platforms; however, hepatocytes were mostly cultured in planar, 2D environments. Hepatocytes easily lose their differentiated characteristics in 2D culture systems, because cell-cell and cell-matrix interactions were limited. Consequently, expressions of hepatocyte-specific functions could not be maintained in conventional culture systems. In order to reproduce 3D cell-cell and cell-matrix interactions, 3D cell culture systems have also been reported. The representative examples are the formation of hepatocyte spheroids (Lee *et al.*, 2013) and the encapsulation of hepatocytes into biocompatible hydrogels (Kim *et al.*, 2010; Yamada *et al.*, 2012), mimicking the characteristics of *in vivo* cellular environments. These systems are

potentially useful for drug development, but the lack in proper technologies to create functional micro vasculature networks inside the prepared 3D tissue models hampers the wide applicability of these systems. Recently 3D bioprinting techniques are being developed, but complicated operations or machines are necessitated (Kang *et al.*, 2016).

Here we proposed a facile strategy to overcome these limitations by utilizing cell-encapsulating hydrogel sponges (Hori *et al.*, 2019). A newly found phenomenon of an aqueous two-phase “bicontinuous” dispersion system was used to create gelatin methacrylate (GelMA)-based hydrogel sponges with embedded continuous micropores. In this study, as a new application of the hydrogel sponges, we prepared a perfusion culture system for the liver cells encapsulated in hydrogel sponges, and evaluated the liver cell-specific functions. Then, we developed a perfusion cultivation system for the hydrogel sponge to demonstrate the usefulness of the presented system that can be potentially incorporated into organs-on-a-chip systems.

2 Materials and Methods

2.1 Cell culture

HepG2 cells (human hepatoma cell line) were provided by Riken BRC. Cells were maintained in a cell culture medium (Dulbecco’s modified Eagle’s medium (DMEM)

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with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) at 37°C in a CO₂ incubator. Cells were collected from cell culture dishes at 70% confluency by trypsin-EDTA treatment, and then, they were suspended in the culture medium and washed with saline twice. A cell pellet was formed via gentle centrifugation.

2.2 Preparation of gelatin methacrylate (GelMA)

The protocol for preparing gelatin methacrylate (GelMA) was previously described (Hori *et al.*, 2019). Briefly, gelatin from porcine skin was dissolved in PBS at 50°C. Methacrylic anhydride was added to this solution and kept at 50°C for 2 h. The obtained mixture was dialyzed in distilled water for 7 days at 50°C. During the dialysis, distilled water was replaced every 24 h. Finally, the dialyzed solution was dried to obtain GelMA powder. The dried powder was stored at -20°C until use.

2.3 Fabrication of porous hydrogel sponge

Fabrication process of microporous hydrogel sponges is shown in Figure 1. At first, aqueous solutions of GelMA and PEG were mixed thoroughly and then kept at 60°C for 12 h to form separated two phases; one was the PEG-rich phase and the other was the GelMA-rich phase. Next, the upper PEG-rich phase and the lower GelMA-rich phase were independently collected. The collected GelMA-rich phase and the PEG-rich phase were remixed by changing the volumetric re-mixing ratio. After adding a photoinitiator (Irgacure 2959) and bovine serum albumin into the mixture, the mixture was irradiated with UV light to selectively crosslink the GelMA-rich phase and to form the hydrogel. The obtained hydrogel was washed with PBS to remove the non-crosslinked PEG-rich phase.

For preparing cell-encapsulating hydrogels, bicontinuous solution was cooled to 37°C and poured onto the cell pellet, and it was mixed gently. The final cell concentration in this mixture was 5×10^7 cells/mL. Immediately after suspending cells in the mixture, the solution was irradiated by UV light for 1 min while the UV light shorter than 350 nm was cut using a longpass filter.

2.4 Observation of the cross section

To observe the cross-sectional morphologies of the obtained hydrogels, the hydrogel tablet (Φ of 10 mm, thickness of 2 mm) was embedded in O.C.T. compound and frozen at -80°C. Thin frozen sections with a thickness of ~10 μ m were prepared. The sections were fixed in 10% formalin neutral buffer solution, and the GelMA hydrogel matrices were stained red with Picrosirius Red Stain Kit and cells were stained green with Fast green.

2.5 3D cell culture and Cell viability test

To culture the encapsulated cells, the obtained hydrogel tablet was placed in the culture medium at 37°C at 5%

CO₂ condition in a CO₂ incubator for up to 7 days. The culture medium was replaced every two days. The viability of the encapsulated cells in the hydrogel sponge was analysed via Live/Dead assay. Briefly, after washing the cell-encapsulating hydrogel with PBS, the hydrogel was put in the working solution of Live/Dead assay for 30 min. Cells in the hydrogel were observed using a fluorescence microscope.

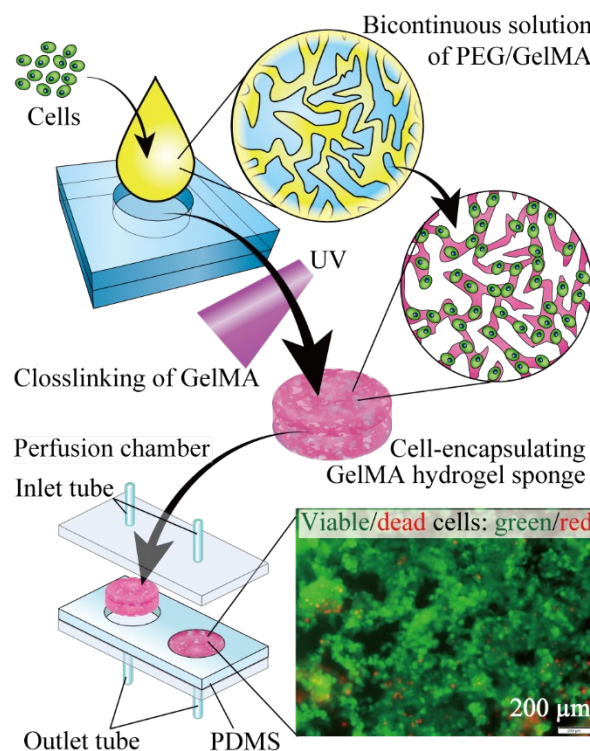


Figure 1. Fabrication process of the microporous hydrogelsponges and the perfusion system

2.6 Functional characterization of encapsulated cells

We examined the functions of the encapsulated HepG2 cells using quantitative reverse transcription PCR (RT-qPCR). We evaluated the gene expressions of HepG2 cells encapsulated in the hydrogel sponges at Day 1, 4, and 7. The expression levels of albumin (*ALB*) and cytochrome P450 3A4 (*CYP3A4*) were evaluated. The relative gene expression levels of these genes were normalized to the expression levels of *HPRT1* as the housekeeping control. The expression for the plate culture was normalized to be 1 for each gene.

2.7 Fabrication of perfusion culture system

We applied the obtained hydrogel sponges to perfusion cell culture system. We fabricated a perfusion chamber made of PDMS. The chamber was cylindrical in shape (Φ of 10 mm, depth of 2 mm) and had the inlet and outlet tubes (Φ of 1 mm) on the top and bottom sides, respectively, to smoothly introduce the cell culture medium. An obtained cell-encapsulating hydrogel sponge tablet was packed in the perfusion chamber and the cell culture medium was introduced. The flow rate was

constantly at 10 $\mu\text{L}/\text{min}$. The viability of the encapsulated HepG2 cells were also evaluated following the protocols described above.

3 Results and Discussion

3.1 Preparation of hydrogel sponge

We first prepared an aqueous two-phase system composed of the GelMA-rich and PEG-rich phases (Figure 2). We optimized the re-mixing ratio of these two phases. Immediately after mixing these two phases, GelMA hydrogels were obtained by irradiating UV light. Thin frozen sections of the obtained hydrogels are shown in Figure 3. When the ratio of the GelMA-rich and PEG-rich phases was 75:25, the PEG-rich phase was dispersed in the GelMA-rich phase, and the pores formed in the hydrogel were not continuous. In contrast, when the ratio was 60:40, the GelMA-rich phase was dispersed in the PEG-rich phase, and the obtained GelMA hydrogels were not continuous. When the ratio was 70:30, the cross-sectional shapes of the pores were not circular. This result indicates that these pores were continuous in a 3D space. This study provided a new means to form vasculature networks into gelatin-based hydrogels, with the help of the bicontinuous dispersion of aqueous two-phase system without harmful process to encapsulated cells.

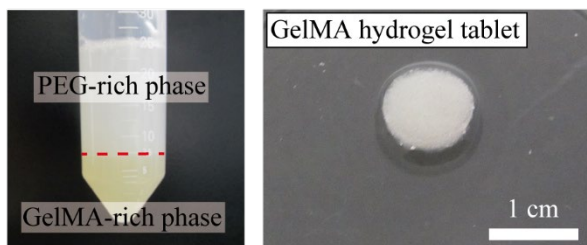


Figure 2. (Left) An aqueous two-phase solution composed of the GelMA-rich and PEG-rich (lower/ upper) phases. (Right) An obtained hydrogel tablet when the ratio of the GelMA-rich and PEG-rich phases was 70:30

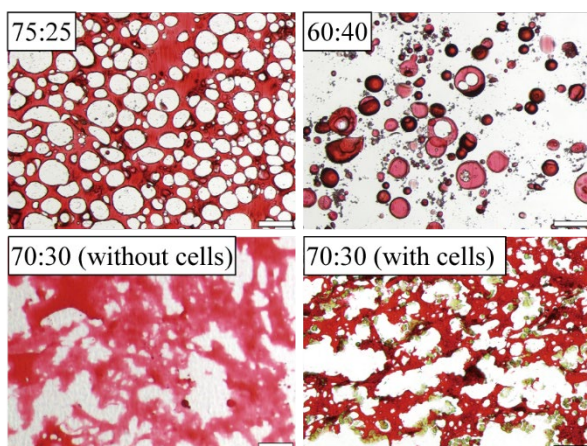


Figure 3. Micrographs of the frozen sections of the hydrogels when the re-mixing ratio of the GelMA-rich: PEG-rich phase was varied as indicated. The GelMA

hydrogels were stained red and cells were stained green. Scale bars are 100 μm

We next prepared cell-encapsulating hydrogels by suspending HepG2 cells in the bicontinuous solution before UV crosslinking. As a result, HepG2 cells were successfully encapsulated in the hydrogel sponge.

3.2 Cell viability analysis

We then examined the effect of vasculature networks inside the hydrogel on the viability of the encapsulated cells. The results of Live/Dead assay are shown in Figure 4. In the case of the uniform hydrogels, most of the cells were not viable after 3 days of cultivation, because of the lack in nutrition and oxygen. On the other hand, high cell viability (more than 85%) was maintained after 3 days of cultivation when HepG2 cells were encapsulated in the microporous hydrogel sponges. The encapsulated HepG2 cells proliferated and formed aggregating during 7 days of cultivation. This result indicated that vasculature networks in the hydrogel matrix were essential to supply nutrition and oxygen with the encapsulated cells and to maintain the viability.

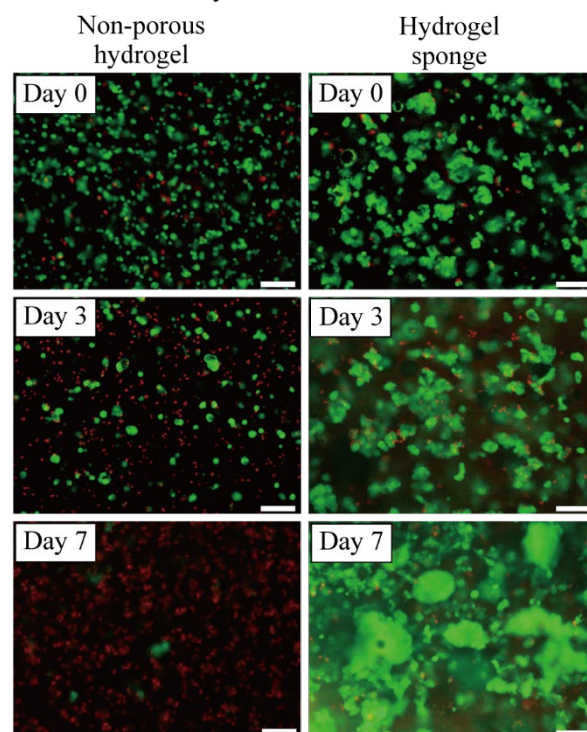


Figure 4. Live/Dead assay for the encapsulated HepG2 cells at the center regions of the hydrogel sponge and the nonporous hydrogel. Viable and dead cells were stained green and red, respectively. Scale bars are 100 μm

3.3 Evaluation of gene expression

The presented hydrogel sponges, in which vasculature networks were formed, maintained the viability of the encapsulated HepG2 cells. We investigated if the liver cell-specific functions of HepG2 cells were upregulated.

We examined albumin (*ALB*) and cytochrome P450 3A4 (*CYP3A4*) expressions via RT-PCR. Expressions of these genes in the hydrogel sponges were compared to those in the conventional plate culture (2D cell culture). The results are shown in Figure 5. At Day 1, the expressions of these genes were comparable to those in the 2D culture. This result was probably because the encapsulated cells were dispersed in the hydrogel matrix and proliferating. At Day 7, the expression levels of these genes were enhanced for the hydrogel sponges, because of the 3D configuration and the formation of proper cell-cell or cell-ECM interactions. From this result, we confirmed that the 3D culture in the microporous hydrogel sponge enhanced liver cell-specific functions.

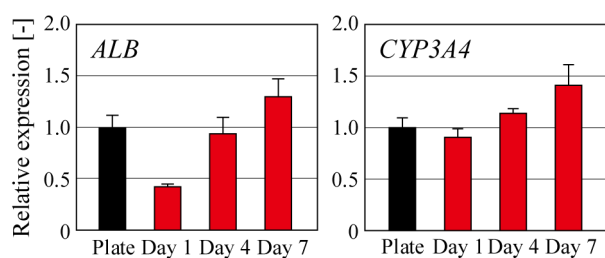


Figure 5. Gene expressions of liver-specific genes of the encapsulated HepG2 cells via RT-qPCR.

3.4 3D perfusion culture system

Finally, we attempted to perform perfusion culture for the cells in the hydrogel sponges. We prepared the 3D perfusion culture system as shown in Figure 6, and performed perfusion culture. After 4 days of perfusion culture, the shape of the packed hydrogel sponge was maintained and we were able to continuously introduce the culture medium into the perfusion chamber without clogging. The result of the Live/Dead assay for the encapsulated cells under perfusion culture is shown in Figure 6. We confirmed that most of the cells were viable. This method has advantages to previous 2D perfusion culture, in terms of the existence of 3D cell-cell or cell-matrix interactions. The presented system would be useful as one of the culture modules for 3D multiple organs-on-a-chip devices.

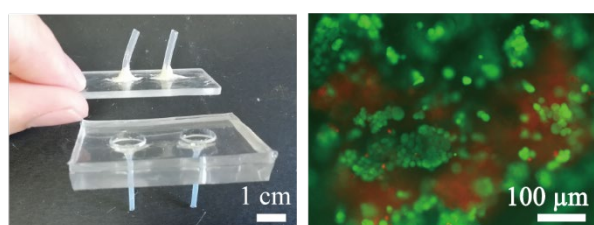


Figure 6. (Left) A perfusion chamber. (Right) The result of Live/Dead assay for the encapsulated cells under a perfusion culture at Day 4

4 Conclusions

We proposed a facile strategy to fabricate microporous hydrogel sponges, which can encapsulate living cells in

the matrix, using bicontinuous dispersion of an aqueous two-phase system. The obtained cell-encapsulating hydrogel sponges maintained high cell viability and enhanced the liver-specific functions. Encapsulated HepG2 cells were proliferating and formed aggregates during cultivation. Additionally, the presented hydrogel was applied to the perfusion culture and the encapsulated HepG2 cells were also viable. From these results, the presented hydrogel sponge would be a useful 3D cell culture platform for a variety of cell culture experiments with precisely controlled cellular microenvironments.

Acknowledgements

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