Fabrication of micrometer-scale porous gelatin scaffolds for 3D cell culture

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\textbf{A B S T R A C T}

In hydrogel scaffolds for 3D cell culture, porous structure plays a central role in maintaining cell survival by delivery of biochemical factors through the scaffolds. Thus, adequate pore structures should be a significant design factor for preparing cell culture scaffolds. Here, we present a non-cytotoxic approach to control porosity of a gelatin scaffold based on porogen leaching. Specifically, we fabricated photocrosslinkable hydrogel (gelatin methacrylate) containing porogens in a microfluidic channel, followed by dissolving out the porogens. Our approach allowed generation of micrometer-scale pores, increasing cell viability. This study could serve as a guide for tuning porosity of cell-seeded 3D scaffolds.

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\textbf{Introduction}

Three-dimensional (3D) cell culture has increasingly attracted attention as an alternative method to overcome limitations of traditional two-dimensional (2D) cell culture, moving toward recapitulating in vivo microenvironment in which cells interact with both surrounding cells and extracellular matrices (ECMs). Advances in techniques for the 3D cell culture have yielded several in vivo mimics; for example, gut models with 3D microvilli structures \cite{1,2,3}, liver models mimicking interactions with hepatocytes and hepatic stellate cells \cite{4}, and a skin model composed of dermal and epidermal multilayers \cite{5,6}.

In the fields of biomedical engineering and regenerative medicine, hydrogels are widely used biomaterials as scaffolds to support 3D cell cultures \cite{7,8,9}, owing to their 3D network structure in various scales ranging from cm to nm and high water content (typically \textgtr90\%). Hydrogels can be categorized into two groups: natural and synthetic polymers. Among natural polymers, alginate, collagen, and gelatin have been broadly used in tissue engineering due to their availability and simple solidification process after seeding cells in solution forms. Alginate, which is polysaccharide extracted from algae, is crosslinked ionically by divalent cations such as Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Fe\textsuperscript{3+}, and Ba\textsuperscript{2+} \cite{10,11,12,13,14}. Collagen, which is the most abundant ECM protein in our body, forms a fibrous matrix via self-assembly by neutralization at physiological temperature (e.g., 37°C) \cite{15,16,17,18,19}. Gelatin, which is composed mainly of denatured collagen, is gelled by cooling after heat-melting \cite{20}. Among synthetic polymers, polyethylene glycol (PEG) \cite{21,22,23} and poly (lactic-co-glycolic acid) (PLGA) \cite{24,25} have been prevalently used for drug delivery as well as tissue engineering owing to their biocompatibility, biodegradability and readily available molecular modifications. For instance, porosity, hydrophobic and hydrophilic properties of PEG can be controlled by varying molecular weight of PEG chains. In addition, PEG scaffolds can be modified by chemically conjugating various functional groups or biomolecules \cite{9}. PLGA is a copolymer composed of two monomers: glycolic acid and lactic acid. Degradability and diffusivity of PLGA hydrogel can be controlled by tuning molar ratio of the two monomers \cite{3}.

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For both categories of hydrogel scaffolds, microporous structure plays a central role not only in maintaining cell survival but also in enhancing cellular growth and functions by delivering soluble biochemical factors as well as oxygen through the scaffolds. The density and size of pores are associated with transport and mechanical properties of hydrogel scaffolds. In addition, implantation of scaffolds with appropriate pore sizes reportedly affects tissue regeneration and development [27]. Thus, adequate porosity and pore structures should be significant design factors for 3D cell culture scaffolds. Current techniques to control porosity of hydrogel scaffolds include gas foaming [28], fiber boiling [29], freeze drying [30], solvent casting, and particle leaching [1,31]. However, most of these techniques accompany a harsh process with likely damage to cells, such as mechanical stress in gas forming, excess heat in fiber boiling, and high pressure and low temperature in free-drying, all of which cannot be applied to cell-encapsulated hydrogel scaffolds. Therefore, when using aforementioned methods, hydrogel scaffolds could be seeded with cells only after complete fabrication. While the solvent casting and particle leaching were applicable to cell-encapsulated scaffolds, leaching out salts leads to high osmolality (>330 mOsm) and consequently affects cell survival. Although recent studies have reported non-cytotoxic porogens such as pre-fabricated microspheres with gelatin or PEG derivatives [32–35], procedure to both fabricate and leach out the microsphere porogens can be quite time-consuming until desired porosity and porous structures are acquired.

Here, we present a simple non-cytotoxic approach to control the porosity of a gelatin scaffold based on solvent casting and porogen leaching. Specifically, we lithographically fabricated photocrosslinkable hydrogel (gelatin methacrylate; gel-MA) containing a porogen (either gelatin or PEG) in a microfluidic channel, followed by simply dissolving the porogen out. We verified that we can control the porosity and molecular diffusion inside hydrogel by varying the concentration of porogens dissolved in gel-MA. Our approach led to micrometer-scale pore structure within a short period of time by utilizing a liquid phase porogen rather than solid phase porogens. We confirmed that the increase of the porosity enhanced not only diffusivity but also the viability of cell cultured within our gel-MA scaffolds.

Materials and methods

Synthesis of methacrylated gelatin

15% (w/w) gelatin solution was prepared by dissolving gelatin form porcine skin (Sigma) in Dulbecco’s phosphate buffered saline (DPBS; Gibco) at 50 °C. For methacrylation of gelatin, 2 ml of methacrylic anhydride (Sigma) was added to 20 ml of a gelatin solution at a rate of 0.5 ml/min using a syringe pump (NE-300; Just Infusion™). This mixture was allowed to react for 3 h under continuous stirring at 50 °C. The reaction was stopped by 3X dilution of the reaction mixture with warm (50 °C) DPBS. The diluted mixture was dialyzed in distilled water for 7 days using a dialysis cassette with 10 k molecular weight cut off (Thermo Fisher Scientific). During the dialysis, distilled water was replaced every day. After a dialyzed solution in the cassette was transferred to a 50 ml conical tube and lyophilized for 1 week. The lyophilized methacrylated gelatin (gel-MA) was stored at −80 °C prior to use.

Preparation of hydrogel pre-polymer solution

A hydrogel pre-polymer solution was prepared by dissolving lyophilized gel-MA (15% (w/w)) as a photopolymerizable precursor, 2-hydroxy-1-(4-hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) as a photoinitiator, and gelatin or polyethylene glycol (PEG; MW 600 Da; Sigma) as a porogen in DPBS at 80 °C. According to experimental conditions, various concentrations of Irgacure 2959 (0.5, 1, or 2% (w/v)), gelatin (0, 5, 10 or 15% (w/v)), and PEG (0, 2, 5, 10% (w/v)) were used. For cell-encapsulated scaffolds, a fully dissolved pre-polymer solution was mixed with a suspension of HeLa cells (5 × 10^6 cells/ml) in Dulbecco’s modified eagle’s medium (DMEM) at 37 °C.

Fabrication of a microfluidic channel

A layer of a polydimethylsiloxane (PDMS; Sylgard® 184; Dow Corning) channel was fabricated by creating a rectangular hole (35 mm (length) × 0.5 mm (width) × 0.5 mm (thickness)) on a flat PDMS slab (50 mm (length) × 20 mm (width) × 0.5 mm (thickness)). A microfluidic channel was constructed by plasma-bonding the channel layer between a bottom glass slide (76 mm (length) × 26 mm (width); Marienfeld-superior) and a top glass slide (20 mm (length) × 20 mm (width); Marienfeld-superior).

Fabrication of porous hydrogel scaffolds

The PDMS microfluidic channel was filled with a mixed solution of gel-MA and porogen (either gelatin or PEG; Fig. 1A) and exposed to ultraviolet (UV) light (13 mW/cm²; 320–390 nm; Omnicure s1000) for 30 s through a photomask of a transparent circle (diameter: 1 mm) (Fig. 1B). Then, the microchannel was flushed with warm DPBS or culture media for 5 min using a pipette in order to leach the porogen and to remove uncrosslinked gel-MA out of a gel-MA construct (Fig. 1C). For 3D culture, gel-MA scaffolds were seeded with HeLa cells at a density of 5 × 10^6 cells/ml.

Assessment of cell viability

To determine the appropriate concentration of a photoinitiator and UV irradiation time, the viabilities of cells after various crosslinking conditions were compared. Cytotoxic effect of the photoinitiator was assessed with HeLa cell-encapsulated scaffolds which were fabricated with 0.5, 1, or 2% (w/v) Irgacure 2959. Cells were cultured for 24 h and stained with a live/dead cell-staining solution prepared by mixing 1 μM calcein-AM (Life technologies) and 1 μM ethidium homodimer (Life technologies) in DPBS. In addition, effect of a porogen on cell viability was assessed with HeLa cell-encapsulated scaffolds which were fabricated with 15% (w/v) or without gelatin porogen. Cell viability was examined by staining live/dead cells after 72 h of culture. After staining the cells with live/dead staining solution, fluorescent microscope images of cells were taken at multiple locations. All images were imported to a freely available image processing software, ImageJ (NIH), and the number of live and dead cells in each image was quantified. The viability of cells was calculated as the ratio of the number of live cells to the number of total cells (live and dead).

Characterization of diffusion through porous hydrogel scaffolds

100 μl of fluorescein isothiocyanate-dextran (FITC-dextran; 20 kDa; Sigma) was injected to the microfluidic channel in which a gel-MA scaffold was immobilized. To analyze diffusion through the gel-MA scaffold, fluorescence images were acquired temporally and fluorescence intensity was analyzed using ImageJ software (National Institutes of Health). Diffusion coefficient was calculated from Eq. (1), which represents transient radial diffusion in a cylinder.

\[
\frac{C}{C_{\text{bulk}}} = 1 - \frac{4}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{\alpha_n^2} \exp\left(-\alpha_n^2 t\right)
\]  

(1)
where \( \frac{C_{\text{bulk}}}{C_{\text{0}}} \) is translated as fluorescence intensity of a cylindrical scaffold relative to that of a bulk solution surrounding the scaffold, \( a \) is radius of the scaffold, \( D \) is diffusion coefficient, \( t \) is time when fluorescence intensity was measured, and \( \alpha \) is a value calculated from Bessel function of 1st kind and order 0 using Eqs. (2) and (3).

\[
J_0(\alpha R) = 0
\]

(2)

\[
J_n(x) = \sum_{m=0}^{\infty} \frac{(-1)^m}{m!(n+m)!} \left( \frac{x}{2} \right)^{2m+n}
\]

(3)

To calculate effective diffusion coefficient \( (D_{\text{eff}}) \), a solver for nonlinear least-squares curve fitting was used in MATLAB (Mathworks).

**Scanning electron microscopy (SEM)**

Gel-MA scaffolds (diameter: 6 mm, thickness: 1 mm) with 15% (w/w) or without gelatin porogen were fabricated by photocrosslinking under UV light (13 mW/cm², 320–390 nm, Omniracle s1000) for 60 s. These constructs were mounted onto a cold stage immediately after cooling rapidly in liquid nitrogen, and observed on a cold-field-emission SEM (Hitachi S5000H) with electron beam energy of 10 kV. Because porous hydrogel scaffolds inherently contain water, we used Cryo-SEM that allowed for minimal deformation of the gel structure by freezing rapidly [36].

**Mercury intrusion porosimetry**

For examining the porosity of hydrogel scaffolds, a mercury intrusion porosimeter (Micromeritics AutoPore IV9500, Oak Ridge, TN, USA) was used. Briefly, the dried samples were subjected to a pressure cycle starting at approximately 0.2 psia and then increasing to 482 psia. Based on the amount of intrusion of mercury into samples via their internal pore structures, the analysis was performed using AutoPore IV9500 v1.07 software. A total of 0.1–0.3 g of each sample was used for the measurement.

**Cell culture**

HeLa cells (immortalized human cervical cancer cells; Korean Cell Line Bank) were maintained in DMEM (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 100 μg/ml streptomycin (Gibco), and 100 U/ml penicillin (Gibco) in 5% CO₂ at 37°C. Media was exchanged every 2 days and cells were passaged approximately 2 times per week. To culture cell-encapsulated gel-MA scaffolds, a microfluidic channel, in which a scaffold was immobilized, was filled with culture media. During the culture, a Petri dish containing the microfluidic channel was placed on a custom-made swing plate to generate gravity driven flows through the microchannel periodically. For initial 24 h, the entire cell culture dish was filled with culture media while the microchannel was immersed in the media in order to allow for complete leaching of a porogen. After 24 h, the media was removed
from the culture dish and media inside the microchannel was replenished.

Statistical analysis

A one-way ANOVA with Tukey’s post-hoc test was used to compare viability of cells with varying the concentration of the photoinitiator (Fig. 1B) in Prism (GraphPad Software, Inc.). *** and **** denote \( p < 0.001 \) and \( p < 0.0001 \), respectively. 2-way ANOVA with Sidak correction was used to compare the viability of cells in the hydrogel scaffolds (Fig. 3E). ** and *** denote \( p < 0.05 \) and \( p < 0.001 \), respectively.

Results

Determination of non-cytotoxic concentration of a photoinitiator

To develop a non-cytotoxic process for the fabrication of gel-MA hydrogel scaffolds, we first determined a non-cytotoxic concentration of Irgacure 2959 that generated radicals to initiate photocrosslinking of gel-MA precursors upon UV exposure without affecting cell viability. This was necessary as we wanted to develop methods for gel fabrication with cells encapsulated. The photocrosslinking process is likely to affect cells during the UV exposure. After 24h of HeLa cell culture within gel-MA scaffolds fabricated with 0.5, 1, and 2% (w/v) Irgacure 2959, we assessed viability of the cells. As shown in Fig. 1B, cell viability was ~95% for 0.5% (w/v) Irgacure 2959 while the viability declined dramatically below 60% for both 1 and 2% Irgacure 2959. Therefore, we used 0.5% (w/v) Irgacure 2959 for all the following experiments.

Porosity tuning of gel-MA hydrogel scaffolds

We first characterized diffusion of 20 kDa FITC-dextran (Stoke’s radius of 3.3 nm) into gel-MA scaffolds fabricated without porogen by varying the UV-exposure time from 10 to 30 s. Fig. 1C displays representative temporal evolution of fluorescence in the gel-MA scaffolds. As the exposure time increased, fluorescence intensity inside the scaffolds reached a plateau value (i.e., bulk concentration of the FITC-dextran outside the scaffolds) more slowly, which indicates that higher crosslinking density hindered diffusion. After we performed non-regression fitting with relative fluorescence intensity of 20 kDa FITC-dextran over time, while assuming that

![Figure 2](image-url)
the fluorescence intensity was linearly proportional to low enough concentration (e.g., 10 μM), we were able to estimate effective diffusivity ($D_{\text{eff}}$) of FITC-dextran in gel-MA scaffolds. Estimated effective diffusivities were 1.74 × 10⁻⁶, 0.53 × 10⁻⁶, and 0.19 × 10⁻⁶ cm²/s for UV-crosslinking time of 10, 20, and 30 s, respectively (Fig. 1D).

To control porosity of hydrogel scaffolds, we then cast gel-MA hydrogel with porogens which were eventually leached out. We chose gelatin or PEG as a porogen because these materials were highly miscible with gel-MA solution, and known to be biologically inert. We fabricated gel-MA constructs with varying concentrations of the gelatin porogen (i.e., 5, 10, and 15% (w/v)) and assessed porosity by estimating diffusivity of 20 kDa FITC-dextran in each scaffold. As shown in Fig. 2A, diffusion of the FITC-dextran into the gel-MA scaffolds became faster with increasing concentration of the gelatin porogen. Our time-lapse quantitative analysis of fluorescence images (i.e., fluorescence intensity within the gel-MA scaffolds relative to that in the bulk solution outside the scaffolds) up to 40 min showed a similar trend (Fig. 2B). Specifically, the relative fluorescence intensity was approximately 40% higher in a scaffold fabricated with 15% (w/v) gelatin compared with one without the porogen at 40 min. We also estimated effective diffusivity with various concentrations of porogen (Fig. 2C). $D_{\text{eff}}$ in the presence of 15% porogen (0.098 × 10⁻⁶ cm²/s) was about 2.5 times greater than $D_{\text{eff}}$ without the porogen (0.039 × 10⁻⁶ cm²/s).

We observed similar results from gel-MA scaffolds cast with the PEG (600 Da) porogen. Non-linear regression of relative concentration of the FITC-dextran that was measured experimentally (Fig. 2D) showed increasing fluorescence intensity with higher PEG porogen concentration. The effective diffusivity in scaffolds with the PEG porogen increased by ~3 folds as the concentration of the PEG porogen increased from 2 to 10% (w/v) (Fig. 2E). Interestingly, the effective diffusivity with the PEG porogen appeared to be within a range of 0.1–0.7 × 10⁻⁶ cm²/s, which is higher than that with 15% (w/v) gelatin porogen. These results indicate that the porosity of gel-MA scaffolds can be controlled by both the concentration and types of porogens. By varying the concentration of gelatin and PEG porogens, we were able to achieve the $D_{\text{eff}}$ values ranging from 0.04 to 0.6 × 10⁻⁶ cm²/s.

**Porous structures of gel-MA hydrogel scaffolds**

To confirm porogen-mediated alteration in porous structures, we observed microstructures of gel-MA surfaces using a scanning electron microscopy (SEM). As shown in Fig. 3A, the size of pores generated by 15% (w/v) gelatin porogen was considerably larger

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**Fig. 3.** 3D culture in porous structures of gel-MA hydrogel scaffolds. (A) Representative scanning electron micrographs showing porous structures of gel-MA hydrogel scaffolds fabricated with no porogen (left) and 15% (w/v) of gelatin porogen (right). (B) Bar graph presenting porosity measured in the gel-MA scaffolds shown in (A). Error bars represent standard deviation. (C) Optical micrograph showing a HeLa cell-seeded gel-MA scaffold at seeding density of 5 × 10⁵ cell/ml. White dotted squares (center and border) indicate regions of interest for estimation of cell viability as presented in (D) and (E). (D) Fluorescence micrographs showing live (green) and dead (red) cells at the center (top) and border (bottom) in gel-MA scaffolds fabricated with no porogen (left) and 15% (w/v) of gelatin porogen (right). (E) Bar graph displaying cell viability at the center (white) and border (gray) in gel-MA scaffolds fabricated with no porogen and 15% (w/v) of gelatin porogen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
than the pores in the scaffold without the porogen; the pore size was 4–6 μm with 15% (w/v) gelatin porogen. Although the calculated diameter of gelatin that we used in this study is ~6.5 nm [37], assuming molecular weight of 87.5 kDa for Bloom number of 300, the pore size appeared to span a micrometer scale. This result could be attributed to both concentration of the porogen and polymerization-induced phase separation, as similarly reported for PEG porogens [38,39]. We also measured porosity of gel-MA scaffolds with and without the gelatin porogen using a mercury intrusion porosimeter. Fig. 3B indicates that the porosity with 15% (w/v) gelatin porogen was 2.95%, which was 1.8 times higher than the no porogen case.

Cell culture in porous gel-MA hydrogel scaffolds

To investigate the effect of porosity on the viability of cells during 3D culture, we encapsulated and cultured HeLa cells in gel-MA scaffolds without and with 15% (w/v) gelatin porogen. After 72 h of the 3D culture, we assessed cell viability at the center as well as at the borders of scaffolds, as indicated in Fig. 3C. Viability of HeLa cells were significantly higher by ~20% when the cells were cultured in the scaffold with the gelatin porogen; average viability at the center and border was ~75 and 95%, respectively, whereas in case of scaffold without porogen, average viability at the center and border was ~50 and ~75%, respectively (Fig. 3D and E). We note that the viability at the center appeared to be significantly lower than that at the border in general (Fig. 3E). Especially at the center, the viability decreased down to ~50% when the porogen was not introduced. These results clearly suggest that the porosity of gel-MA scaffolds influences viability of cells and that pore size over ~3 μm (i.e., pore size of gel-MA scaffolds without the porogens) was critical for cell survival for 3 days.

Discussion

In tissue engineering and regenerative medicine, it is crucial to construct porous scaffolds not only to culture cell in three-dimension but also to allow for dynamic cellular phenotypes such as proliferation and migration, all of which recapitulate the in vivo tissue microenvironment. Among several approaches to fabricate the porous scaffolds are available, the porogen leaching method is relatively amiable for controlling both pore sizes and porosity while retaining cell viability. Our approach aimed to utilize the porogen leaching method with a photocrosslinkable hydrogel, where a base monomer, a porogen, a photoinitiator were required. Although the gel-MA as the base monomer and gelatin or PEG as the porogen have been known to affect cell viability minimally [40], Iracure 2959 as the radically reactive photoinitiator through photocrosslinking could serve as a major factor in influencing the viability [41]. Therefore, we first characterized phototoxicity by varying the concentration of the photoinitiator. We found that 0.5% (w/v) of Iracure 2959 led to nearly no phototoxicity while maintaining structural fidelity via complete photocrosslinking. Jung and Oh reported similar results that cell viability diminished dramatically when the concentration of the photoinitiator was greater than 0.5% [42]. In addition to the concentration, photocrosslinking time affects the viability. Specifically, a recent study showed that the viability of primary porcine aortic valvular interstitial cells (VICs) seeded in gel-MA, followed by UV-curing for 1 and 2 min, reduced to 70 and 60%, respectively, after 48 h culture; at 24 h after photocrosslinking, viability remained over 90% [43]. Because the exposure to UV for 30 s still led to >90% of viability, we chose this as our photocrosslinking time.

To validate our hypothesis that an increase in the concentration of the porogen consequently expanded porosity, we performed diffusion experiments by delivering fluorescent FITC-dextran into a microfluidic channel in which the porous gel-MA scaffold was immobilized. Although NaCl crystals were used in conventional porogen leaching, we chose gelatin as the porogen to minimize persistent side effects of residual porogen to cells [44]. We confirmed that 20 kDa FITC-dextran diffused more rapidly as the concentration of the porogen increased, similarly as reported elsewhere [39]. Furthermore, we found that the choice of the porogen was another way to control desired porosity. Specifically, gelatin was desirable for obtaining diffusivity of less than 0.1 × 10⁻⁶ cm²/s and PEG (600 Da) was suitable for obtaining diffusivity ranging 0.1–0.7 × 10⁻⁶ cm²/s. Lower effective diffusivity for the gelatin porogen could be attributed to a difference in average molecular weight and relative viscosity of the porogen. In other words, 50–100 kDa of gelatin might be entrapped physically to a higher extent within gel-MA networks, compared with 600 Da of PEG during leaching. We note that Lee et al. also reported a similar phenomenon called semi-interpenetrating network (SIPN) where an increase of PEG porogen within polyacrylamide scaffolds resulted in locally higher density of hydrogel and thus hindrance of diffusion [39]. Observation of relatively high diffusivity (1.7 × 10⁻⁶ cm²/s) despite no porogen was probably a result of incomplete photocrosslinking of gel-MA, due to a short UV-crosslinking time of 10 s. This result indicates that UV-crosslinking time indeed influences porosity. An additional rationale behind our choice of 30 s is that a similar diffusivity of 0.18 × 10⁻⁶ cm²/s was obtained for no gelatin porogen and 2% (w/v) PEG porogen conditions. Therefore, UV-crosslinking time should be chosen by considering both desirable porosity and cell viability.

Viability of HeLa cells seeded in gel-MA scaffolds showed that increased porosity due to the gelatin porogen permitted more efficient delivery of oxygen and nutrients such as glucose and oxygen toward the center region of a hydrogel scaffold. In addition, higher viability at the border region can be correlated with an interplay between the concentration of oxygen and glucose [45]; higher levels of oxygen caused lower viability in the presence of glucose, whereas higher concentrations of glucose increased viability with constant oxygen level. Conceivably, oxygen was delivered to a level sufficient for cell survival in the whole region of scaffold, whereas transport of larger molecules inside the scaffold were limited, causing depletion of nutrients primarily in the center region.

Conclusion

In this study, we presented a simple process not only to fabricate photocrosslinkable gel-MA scaffolds seeded with cells, but also to control porosity readily by leaching a solution phase of gelatin or PEG as a porogen. Our approach allowed for generation of relatively large micrometer-scale pores within a short period of time, compared to a typical method where sequential dissolution of solid phase particulates as porogens is accompanied, which increased cell viability during 3D culture. This study could serve as a practical guide in choosing a scaffold material, a porogen, and the concentration of the porogen for tuning porosity of cell-seeded 3D scaffolds.

Conflict of interest

The authors declare no financial or commercial conflict of interest.
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