Published in final edited form as:

Macromol Rapid Commun. 2014 September ; 35(18): 1578–1586. doi:10.1002/marc.201400278.

Cell-Friendly Inverse Opal-like Hydrogels for Spatially Separated Coculture System

Dr. Jaeyun Kim^[+],

School of Engineering and Applied Sciences and Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 01238. School of Chemical Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea

Dr. Sidi A. Bencherif^[+],

School of Engineering and Applied Sciences and Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 01238

Weiwei Aileen Li, and

School of Engineering and Applied Sciences and Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 01238

Dr. David J. Mooney

School of Engineering and Applied Sciences and Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 01238

David J. Mooney: mooneyd@seas.harvard.edu

Keywords

hydrogels; co-culture system; inverse opal structure; alginate; gelatin

Three-dimensional (3D) scaffolds are widely used for the modeling, regeneration, replacement, or improvement of the functions of tissues as they can mimic the microstructure as well as the chemical and biological characteristics of the target tissues.^[1] Porous and biodegradable polymer scaffolds have been extensively studied as a structural supporting matrix or cell adhesive substrate for cell based tissue engineering.^[2] Usually a highly porous structure with well-interconnected pores is required to achieve sufficient cell seeding and migration within the scaffold, and to facilitate mass transfer of nutrients and metabolites for proliferation and differentiation of cells. Various approaches have been taken to generate porous networks in polymer scaffolds, including gas foaming,^[3–5] salt leaching,^[6–8] and freeze drying.^[9–11] However, the use of these technologies to make macroporous scaffolds typically yields irregular pore sizes, shapes, and structures, as well as limited pore connectivity.

Recently, inverse opal structured polymer scaffolds have been utilized as 3D constructs for cell culture, as they can be designed with a uniform pore size and a high degree of pore interconnectivity.^[12–24] Solid beads, such as polystyrene (PS), poly (methyl methacrylate)

^[+]J.K. and S.A.B. contributed equally to this work.

(PMMA), or poly(caprolactone) (PCL), have been used as sacrificial templates, while silica, polyacrylamide (PAM), chitosan, poly(ethylene glycol) (PEG), or poly(lactic-co-glycolic acid) (PLGA) have been used as the polymer matrix (Scheme 1a). However, the use of organic solvents or acidic solutions to remove the template beads precluded the possibility to encapsulate cells in the inverse opal polymer matrix (Scheme 1a). Similarly, cells also cannot be encapsulated within the polymer matrix in other conventional macroporous scaffolds due to use of unfriendly organic solvents, freeze drying processes, or high pressure of gases in the fabrication step.^[3–11] This has led to the use of macroporous polymer scaffolds made from these techniques as a mechanical support for cell adhesion and growth on the outer surface of macropores. Ideally, one could encapsulate one cell type within the polymer matrix while attaching another cell type on the surface of pores. A more flexible synthetic system that mimics tissue microenvironments would be achieved with this approach, and could be useful to study many biological processes including stem cell niches and cancer microenvironments.

Here we describe the preparation of cell-friendly inverse opal-like hydrogels (IOHs) allowing cell encapsulation within the matrix and cell seeding on the surface of macropores. IOHs were fabricated using ionically crosslinked alginate microbeads as a sacrificial template and photocrosslinkable biocompatible polymers as the main component of the matrix (Scheme 1b). To achieve a cell-friendly generation of macropores in the inverse opal structure without using organic solvents, the alginate microbeads were removed using non-toxic chelating agents, allowing encapsulated cells to remain viable within the polymer matrix. In addition to encapsulating cells in a porous 3D matrix, additional cells can be seeded on the inner or outer surface of macropores of IOH. We demonstrate the potential use of the newly developed cell-friendly IOH technology as 3D macroporous scaffolds with tunable gel macropore size and physical properties. Further, we show that the IOH system could be used as a spatially separated coculture system, possibly mimicking a complex cellular microenvironment, by simultaneously culturing one cell type within the scaffold and another on the surface of the macropores.

Alginate hydrogel microbeads were used as a sacrificial template for the formation of cell-friendly IOHs. Alginate is a natural polysaccharide composed of α -L-guluronic and β -D-mannuronic acid sugar residues, and is ionically crosslinkable to form hydrogels in the presence of divalent cations, such as Ca^{2+} .^[25] Uniform and rigid alginate hydrogel microbeads prepared by dropping aqueous alginate droplets into a Ca^{2+} solution^[26, 27] were closely packed into a mold in order to be used as a sacrificial solid template for the infiltration of polymer precursors in the interstitial space (Scheme 1b). Alginate gels are known to dissolve quickly in the presence of chelating agents for divalent metal ions, such as ethylenediaminetetraacetic acid (EDTA). To confirm EDTA can dissolve alginate microbeads, three different sized microbeads (500, 800, 1500 μm) were prepared and analyzed (Figure S1a). Rhodamine-labeled bovine serum albumin (BSA) was mixed in the alginate solution and physically encapsulated during hydrogel formation to better visualize and monitor the fate of alginate beads. After 20 min of incubation in 50 mM EDTA, the spherical alginate beads fully dissolved, resulting in a pink solution due to the release of rhodamine-labeled BSA (Figure S1b). The use of EDTA circumvents the need for toxic organic solvents in the sacrifice of templates.

As EDTA is commonly used in the detachment of cells from solid substrates,^[28] its ability to provide a cell friendly-process for the removal of alginate microbeads was next analyzed. Mouse MSCs cultured in flasks were incubated in a 50 mM EDTA solution for 10, 30, 60, 120, or 180 min, and the viability of cells was measured using calcein AM and ethidium homodimer-1. Representative fluorescent images showed that although the average cell morphology changed from an elongated to a spherical shape, there was no significant toxicity of the EDTA solution to the cells (Figure S2a). Quantitative analysis confirmed that approximately 98% of cells were viable even after 3 h incubation in the presence of EDTA (Figure S2b). These observations suggest that incubation of the gels in a 50 mM EDTA solution for up to 3 h would be safe and nontoxic for the encapsulated cells within IOHs.

Next gelatin-based IOHs were prepared using alginate microbeads as a sacrificial template (Figure 1a–c). Gelatin is a derivative of collagen, one of the most common natural extracellular matrix proteins. To enable polymerization, photocrosslinkable methacrylate groups were coupled to the amine groups of gelatin, resulting in methacrylated gelatin (MA-gelatin).^[29, 30] Alginate microbeads were closely packed in a Teflon mold with a preselected shape and volume, and a 10 wt% solution of MA-gelatin was added and allowed to infiltrate the interstitial spaces of the closely packed alginate microbeads (Figure 1a). MA-gelatin was subsequently polymerized under long-wave UV (365 nm) irradiation for 20 min (Figure 1b). The complete crosslinking conversion of MA-gelatin was confirmed by comparing ¹H-NMR of MA-gelatin before and after photocrosslinking (Figure S3). The disappearance of methylene protons from MA-gelatin after 10 min of UV irradiation in the presence of photoinitiator suggests that nearly full vinyl conversion was achieved in the crosslinked gelatin hydrogels. Alginate microbeads were completely removed from the IOHs by incubation in a 50 mM EDTA solution for 1 h at 37 °C, generating interconnected macropores within the photocrosslinked gelatin matrix while retaining the original overall shape and size (Figure 1c). The mechanical strength of outer phase was important to maintain the macroporous structure in IOHs. For example, we used 10 wt% gelatin-MA solutions as a precursor, resulting in a well-preserved pore structure, while 5 wt% gelatin-MA resulted in poor structural integrity after dissolution of alginate microbeads. The pore size was uniform throughout the matrix and 3D interconnected pores were clearly observed in the IOH. The size of macropores in IOH could be easily controlled by using different sized alginate microbeads as templates (Figure 1d). The shape of the IOH could be controlled using molds with various geometric shapes (e.g. discs, cylinders). For example, a cylindrical shaped gelatin IOH was prepared by using a centrifugal tube as a mold (Figure 1e).

The swelling characteristics of a network are important in various applications as it affects solute diffusion, surface properties, mechanical properties, and surface mobility.^[31] The mass swelling ratio of gelatin-IOHs prepared from 10 wt% MA-gelatin solutions was investigated as a function of the size of alginate beads (Figure 2a). The mass swelling ratio was independent of the size of alginate microbeads and was similar to the plain MA-gelatin hydrogel prepared without alginate microbeads, suggesting that the gel microstructure of gelatin was not compromised during encapsulation and dissolution of alginate beads. Additionally, increasing the polymer concentration in gelatin hydrogels led to a significant

decrease in the mass swelling ratio (Figure 2b), demonstrating that the crosslinking density had a significant effect on the material's ability to attract and store water.

The elasticity of the gel composites (alginate beads encapsulated within the gelatin matrix) and gelatin IOH (alginate beads-free gelatin matrix) were measured. The elastic modulus of pure 10% gelatin hydrogel without alginate microbeads was around 19.0 kPa, while the elastic moduli of alginate/10% gelatin composites were 24.1, 27.0, and 27.4 kPa for 300, 800, and 1500 μm alginate bead template, respectively (Figure 2c). The higher elastic modulus in composite gels in comparison to pure gelatin hydrogels is likely due to higher mechanical strength of alginate beads than gelatin. The removal of alginate microbeads led to a dramatic reduction in the initial modulus, to 1~10 kPa. Therefore a possible application of these inverse opal hydrogels would be as an *in vitro* co-culture system rather than *in vivo* application where strong compressive and shear forces exist. The mechanical strength of inverse-opal hydrogels may be enhanced by combining with other hydrogels or nanomaterials, which may allow their *in vivo* application via implantation; this approach also could be used to modulate the degradation rate of the gels and extend their lifetime. Changing the concentration of MA-gelatin led to alterations in the initial modulus of bulk gels (Figure 2d), which could be important as the elastic modulus of polymer supports can modulate the behaviors of adherent cells.^[32, 33]

Gelatin-based IOHs encapsulating MSCs were next fabricated to examine cell viability (Figure 3a). Mouse MSCs were dispersed in MA-gelatin solution prior to infiltrating around the alginate microbeads, and the prepolymer MA-gelatin solution was then photocrosslinked. Later, the alginate beads were removed using the EDTA solution. MSCs were uniformly distributed throughout the polymeric construct and the IOHs maintained a 3D interconnected pore structure after cell encapsulation (Figure S4). The cells exhibited a spherical morphology at day 1 likely due to the physical constraints of the gel (Figure 3b). At day 4, presumably due to matrix remodeling, a fraction of cells were able to adopt an elongated morphology (Figure 3c), and by day 7 a substantial fraction of cells were able to spread over the IOH matrix (Figure 3d). Cells can spontaneously adhere to the gelatin-IOH matrix as it contains cell-binding motifs.^[29] Further analysis demonstrated that MSCs proliferated over time (Figure 3e). These results suggest that gelatin polymerization and EDTA treatment did not affect cell survival, and this process can be used for the fabrication of cell-containing IOHs. Although there was no significant effect of EDTA treatment on the viability of the cells we tested, there could be a risk for cell death for other cell types. Further accelerating the dissolution of alginate may reduce this risk. In addition, to reduce the negative effect of UV irradiation to the cells, the current UV exposure time (20 min) could be further decreased by decreasing the thickness of target hydrogels, or other chemical crosslinking, such as redox-crosslinking method, may be applied instead of photocrosslinking.

IOH can also be used as a mechanical support for attachment of cells to the surface of the pores subsequent to construct fabrication (Figure 3f). Gelatin-based IOHs were prepared and MSCs seeded into the IOH. Cells successfully attached on the inner pore surfaces and covered the polymer construct (Figure 3g). The cells proliferated over time, and cell proliferation on the IOH surface was higher (170%, Figure 3h) compared to cells

encapsulated in the gels (120%, Figure 3e). This is likely due to the fact that cells on the pore surfaces have more available space to spread and proliferate and greater nutrient availability than cells encapsulated within the gel matrix.

The versatility of the IOH system was demonstrated by instead utilizing PEG as the matrix component. PEG-based IOH were prepared by using dimethacrylated-PEG (DM-PEG, 20 wt %) in a protocol similar to the one used to prepare gelatin-based IOHs. As PEG is non-adherent to cells,^[34, 35] the behavior of cells seeded on the IOH is expected to be distinct from the behavior on gelatin-based IOH. MSCs expressing mCherry fluorescent protein were seeded on the pores of IOH (Figure S5). As the cells could not attach they formed spheroids over time in the macropores. Overall, the size of cell aggregates was around 150 μm . Interestingly, the cells in aggregates maintained their capability of spreading and proliferating after being re-seeded on a 2D culture plate (Figure S6). Cell attachment to the PEG-based IOHs could be promoted by modifying the PEG matrix with arginine-glycine-aspartic acid (RGD) peptides; RGD-modified PEG was used as a co-monomer in the fabrication of IOH (Figure S7). Cells seeded on RGD-modified PEG-based IOH were adherent and covered the entire surface of macropores. These results indicate that one can readily change the gel composition to alter the cellular response to the IOH.

Finally we examined the capability of IOHs to provide heterogeneous cell-cell compartments, possibly mimicking a complex cellular microenvironment, by simultaneously culturing one cell type within the scaffold and another on the surface of the macropores. Human umbilical vein endothelial cells (HUVECs) expressing green fluorescent proteins (GFPs) were physically encapsulated within the gelatin-based IOH during the polymerization process, while MSCs expressing mCherry protein were seeded on the surface of IOHs after polymerization and removal of alginate microbeads. After culturing 3 days, the IOHs were fixed and imaged under confocal microscope (Figure 4a). Only GFP-expressing cells encapsulated in IOH matrix were imaged in the bottom-most focal plane (Figure 4b), as expected. When the focal plane was moved to a higher position, mCherry-expressing cells appeared in the middle of the GFP-expressing cells (Figure 4c). The top focal plane imaged demonstrated a concentric circular pattern of outer GFP-expressing cells, and inner mCherry-expressing cells (Figure 4d). The side view of the intermediate position clearly showed the layered structure of mCherry-expressing cells over the encapsulated GFP-expressing cells. This analysis demonstrates the ability of this system to co-culture two distinct cell populations in different spatial positions. Recently there have been significant efforts to create biomaterial culture systems that allow one to study paracrine signaling between different types of cells.^[36–40] Most current 3D co-culture methods use co-encapsulation of different types of cells in a single type of hydrogel or simple separated encapsulation of different cell types in core-shell hydrogels. Although these approaches provide co-culture systems and spatial separation of each cell types, it is still challenging to prepare a 3D co-culture system with macroporous structure that can provide spatially controlled cellular microenvironments with high mass transfer property to mimic the natural cellular microenvironments. Our results suggest that IOH could be a useful biomaterial-based system to culture different types of cells in physically separated compartments with interconnected macropores, and could be used as a model scaffold to study paracrine signaling or other cell-cell interactions.

Taken together, these data indicate that cell friendly inverse opal-like hydrogels can be easily prepared using ionically crosslinked alginate beads as templates and photocrosslinked hydrogels as a matrix. The template beads can be removed quickly through EDTA treatment with minimal loss of viability for encapsulated cells. Inverse opal-like hydrogels prepared in this method allow both cell encapsulation within the hydrogel matrix and cell seeding on the surface of gel macropores. These IOHs may provide a useful 3D cell culture system to investigate biological processes such as paracrine signaling in stem cell niches or cancer-stroma interactions.

Experimental Section

Fabrication of alginate microbeads

Alginate microbeads were formed by dropping a 2 wt% MVG alginate (NovaMatrix) solution in PBS into a bath of 100 mM calcium chloride (Sigma-Aldrich). The alginate mixture was forced through the nebulizer using nitrogen gas, and resulting alginate droplets were ionically crosslinked as spheres in the calcium chloride bath. Alginate microbeads were rinsed three times in deionized water and stored in deionized water until use. The size of alginate microbeads was controlled by changing the pressure of nitrogen gas flowing to the nebulizer.

Synthesis of methacrylated-gelatin (MA-gelatin)

MA-gelatin was prepared by treating an 8 wt% solution of type A gelatin (Aldrich) in deionized water ($\text{d-H}_2\text{O}$), and dimethyl sulfoxide (DMSO) with a 60-fold molar excess of methacrylic anhydride (Aldrich) in the presence of excess triethylamine. Briefly, gelatin (8.0 g) was first dissolved in 100 mL of $\text{d-H}_2\text{O}$ and 50 mL of DMSO, and subsequently mixed with 40 g of MA and 3 g of TEA. After 3 d reaction, the solution was precipitated twice in a large excess of ethanol (20 times the volume of the reaction solution), filtered, dried in vacuum oven overnight at room temperature, and dialyzed for 3 d against $\text{d-H}_2\text{O}$. The solution was lyophilized for 2 d to generate a white powder and stored at -20°C until further use.

Characterization of MA-gelatin by ^1H NMR

High-resolution, 300 MHz proton NMR spectra were taken on a Varian Unity-300 (300 MHz) spectrometer. Deuterium oxide (D_2O) was used as solvent, and the polymer concentration was 0.5% by mass fraction. All spectra were run at room temperature, 15 Hz sample spinning, 45 tip angle for the observation pulse, and a 10 s recycle delay, for 128 scans. The standard relative uncertainty for calculation of reaction conversion via ^1H NMR arises from the choice of the baseline and is estimated to be 8%. ^1H NMR spectroscopy was used to determine the functionalization conversion on modified gelatin. The degree of methacrylation, which is defined as the ratio of the number of amino groups functionalized with methacrylamide groups to the total number of amino groups present in gelatin prior to the reaction, can be determined by comparing the integrated intensity of the aromatic region, representing the concentration of gelatin, with the intensity of the double bond region. NMR spectrometry was also used to characterize vinyl conversion of MA-gelatin macromonomers after photocrosslinking. Macromonomer solutions were prepared by mixing 10 wt% MA-

gelatin solution with D₂O in the presence of photoinitiator (I2959, 0.1 wt%). One milliliter of the solution was transferred and sealed in an NMR tube before being cured under a UV source (365 nm, 300 $\mu\text{W cm}^{-2}$) to cross-link hydrogels. ¹H NMR was used to characterize the efficiency of vinyl group reactivity during photocrosslinking. The conversion was evaluated by comparing the relative peaks of uncross-linked and cross-linked methylene protons.

Synthesis of RGD-modified ACLT-PEG

GRGDS peptide was dissolved in anhydrous DMF containing 4 M excess of TEA. ACLT-PEG3500-NHS (Jenkem, USA) was also dissolved in anhydrous DMF and immediately mixed with 1.1 M excess of peptide in DMF/TEA^[41]. The reaction occurs via substitution of the NHS moiety, which is replaced by a peptide GRDGS. After incubating for 1 d at room temperature, RDG-modified ACLT-PEG was precipitated twice in cold anhydrous ether and dried in a vacuum oven overnight at room temperature.

Fabrication of IOHs

Alginate microbeads were closely packed in a Teflon mold with designated shape. Then 10 wt% MA-gelatin aqueous solution with 0.1 wt% photoinitiator (Irgacure2959, BASF, USA) was infiltrated by adding on the top of the packed alginate microbeads and subsequently polymerized under UV (365 nm) irradiation for 20 min. The resulting alginate beads/hydrogel composites were incubated in 50 mM EDTA solution for 1 h at 37 °C under shaking to remove alginate microbeads. The resulting gelatin-IOH were washed in excess PBS 5 times and stored in PBS until use. PEG-IOH was prepared by using 20 wt% diacrylated-PEG (DA-PEG) with the same protocol described above. RGD-modified PEG-IOH was prepared using a mixture of 1 wt% of RGD-modified ACLT-PEG solution with 18 wt% DA-PEG solution in the synthesis.

IOHs swelling analysis

Immediately following hydrogel formation, an 8 mm radius disc of each composition was punched from a flat thin sheet and placed in PBS at 37 °C for 1 d. Discs were removed from PBS and blotted with a KimWipe to remove the residual liquid and the swollen weight was recorded. Samples were then lyophilized and weighed once more to determine the dry weight of polymer. The mass swelling ratio was then calculated as the ratio of swollen hydrogel mass to the mass of dry polymer.

Cell encapsulation in IOHs

Mouse MSCs (D1) were mixed in MA-gelatin solution (5×10^6 cells/mL). The mixture solution was added on the packed alginate microbeads and polymerized under UV (365 nm) irradiation for 20 min. The composite gel encapsulating cells was put in complete cell culture media for 10 min and the media was replaced 3 times. Then the composite gel was immersed in 50 mM EDTA solution for 1 h under shaking. After washing 3 times with complete media for 30 min, the IOH encapsulating cells were cultured at 37 °C.

Cell seeding on IOHs

1.4×10^6 cells of mouse MSCs (D1) or human breast cancer cells (MDA-MB-231) in 1 mL of complete media were added on the top of IOHs and incubated for 1 h at 37 °C. Then 5 mL of complete media was additionally added and incubated overnight. After exchanging with complete media, the IOH seeded with cells were cultured at 37 °C.

Cell proliferation study

Cell proliferation in IOHs was analyzed using alamarBlue assay according to the manufacture's instruction (Molecular Probes). Typically, after incubation with alamarBlue for 1 h at 37 °C, the fluorescence of culture media was measured with a plate reader using 560 nm of excitation and 590 nm emission filter settings.

Co-culture of GFP-expressing HUVECs and mCherry-expressing MSCs in IOHs

First, 1.4×10^6 GFP-expressing HUVECs were encapsulated in the gelatin-IOHs prepared using alginate beads with 800 μ m diameter according to above procedure. After culturing the resulting gelatin-IOHs for 3 d, 1.4×10^6 mCherry-expressing MSCs were seeded on the IOH gels and the cells were cultured for 3 d. The IOHs were fixed in 4% paraformaldehyde and imaged on the confocal microscope (Leica SP5 XMP).

Statistical Analysis

All values in the present study were expressed as mean $\pm$ S.D. The significance of differences between the groups was analyzed by a, two-tailed, student's t test and a P value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (1R01EB015498, F30DK088518-03, T32-GM008152), the Wyss Institute for Biologically Inspired Engineering at Harvard University, and NRF grants (2010-0027955, 2012R1A1A1042735) funded by the National Research Foundation under the Ministry of Science, ICT & Future Planning, Korea.

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Figure 1.

(a) Alginate microbeads packed in a disc-shaped Teflon mold and infiltrated with a 10 wt% MA-gelatin solution. (b) Alginate/gelatin composite retrieved from the mold after photopolymerization of MA-gelatin. (c) Gelatin-based IOH obtained after EDTA treatment to dissolve alginate microbeads. (d) Gelatin-based IOHs prepared with various sizes of alginate microbeads (left: 1500 μm , center: 800 μm , right: 500 μm). (e) Cylindrical shaped gelatin-based IOH using a centrifugal tube as a mold and fluorescent BSA-loaded alginate microbeads as templates (left: alginate/gelatin composite, right: the corresponding IOH after removal of alginate microbeads). All scale bars represent 5 mm.

Figure 2.

Mass swelling ratio of (a) 10 wt% gelatin-based IOHs prepared from various alginate microbead sizes and (b) non-macroporous gelatin hydrogels prepared at various concentrations. Compressive elastic moduli of (c) alginate/gelatin composites prepared using different sized alginate microbeads and gelatin-based IOH before and after removal of alginate microbeads and (d) gelatin hydrogels prepared at various concentrations. Small, medium, and large in (a, c) represent 500, 800, and 1500 μm sized alginate microbeads, respectively. Values represent mean and s.d. *represents $p < 0.05$.

Figure 3.

(a) A schematic representation of cell-encapsulating gelatin-based IOH. Fluorescent images of gelatin-based IOH encapsulating MSCs at (b) 1, (c) 4, and (d) 7 days after encapsulation. Image on right of (d) is enlarged image of yellow boxed region. (e) The number of encapsulated MSCs as a function of time (normalized to the number at day 1); an increase in cell number is used as an indication of cell proliferation. (f) A schematic representation of gelatin-based IOH seeded with cells. (g) Fluorescent image of MSCs seeded on gelatin-based IOHs at 4 days after seeding. (h) The number of encapsulated MSCs as a function of time (normalized to the number at day 1); an increase in cell number is used as an indication of cell proliferation. Values represent mean and s.d. *represents $p < 0.05$.

Figure 4.

(a) Schematic depicting gelatin-IOH encapsulating GFP-expressing HUVECs within the matrix, and mCherry-expressing MSCs seeded on the surface of macropores in IOH. The dotted lines represent the focal planes imaged on confocal microscope. (b, c, d) The confocal fluorescent microscopic images (top view) corresponding to the focal planes shown in (a). (e) Fluorescent image (side view) of the yellow dashed line in (c). All scale bars: 100 μm .

Scheme 1.

Illustration of the fabrication process of (a) conventional inverse opal scaffold and (b) cell-friendly inverse opal-like hydrogels (IOHs).