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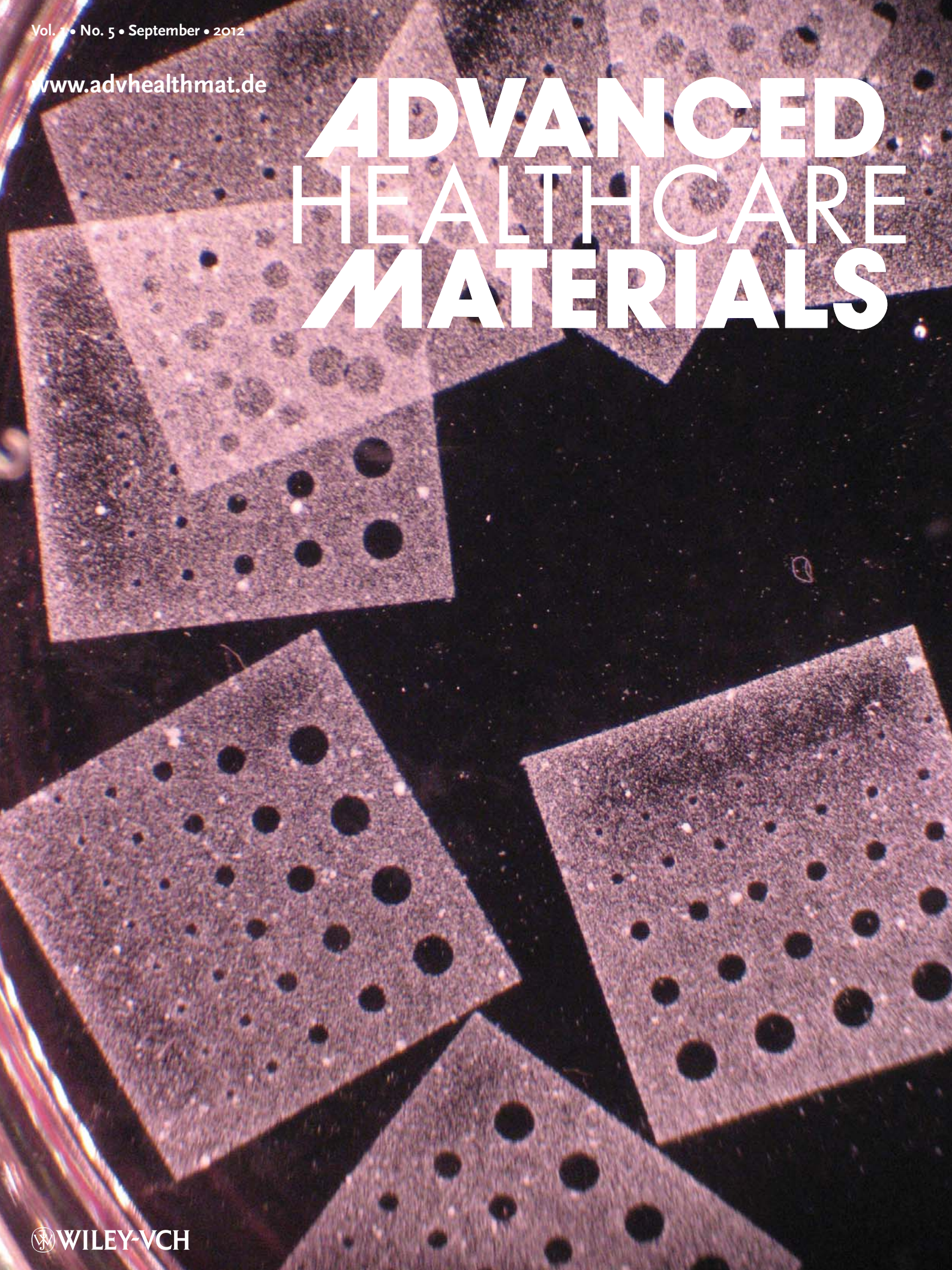

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Cellular Hydrogel Biopaper for Patterned 3D Cell Culture and Modular Tissue Reconstruction

*Wonhye Lee, Chae Yun Bae, Seyong Kwon, Jaejung Son, Jinho Kim, Yong Jeong, Seung-Schik Yoo, and Je-Kyun Park**

In vitro biofabrication of tissue composites is promising for the reduction or elimination of animal experiments in basic biology[1] and pharmacological assays,[2] also enabling partial or total replacement of defective tissues.[3] To generate realistic tissues, the cellular scale environment must precisely duplicate in vivo conditions, including cell-to-cell and cell-to-extracellular matrix (ECM) interactions[4] and spatiotemporal control of physicochemical stimuli.[5,6] Recently, a new approach based on modular tissue engineering has emerged for the reconstruction of tissues, thus paving the way to solving the challenges of poor cell penetration or uneven cell seeding into scaffolds and to engineering complex tissues with multiple cell types and unique ECM compositions.[7] The basic forms for modular tissue engineering typically are cellular "microunit," "microfiber," and "sheet" modules for reconstruction of realistic three-dimensional (3D) tissue with precise control of the cellular scale environment. The cellular modules can be categorized as "cell-only modules," "cell-attached solid modules," or "cell-containing hydrogel modules."

Until now, the "cell-only modules" used to build cellular structures have taken the form of cell spheroids,[8] cell microfibers,[9] and cell sheets.[10] To form "cell-attached solid modules," cells have been attached to solid microbeads,[11] solid threads,[12] or solid sheets,[13] and assembled to build cell-attached solid scaffolds. One of the major disadvantages of the "cell-only" or "cell-attached solid" modules is that they have limited capability to duplicate the in vivo environment. Tight cell-to-cell junctions[10] or the solidity of the used materials[13] can prevent the crucial formation of the ECM environment or cell-to-cell interactions. In the case of "cell-containing hydrogel modules," cell-hydrogel microunits[14,15] and cell-hydrogel microfibers[6] have been fabricated and assembled to reconstruct tissues. Hydrogels are promising biomaterials for their intrinsic diffusion permeability to nutrients, metabolites, and oxygen, and for their tailorability to resemble native ECM;[7,16] in addition, they are more feasible than other modular 3D cell culture approaches for in vivo–like tissue reconstruction. However, existing "cell-containing hydrogel modules" of microunits or microfibers require specialized engineering tools and techniques for fabrication and assembly.[6,14,17] In this Communication, we present the development of a new microarchitectured, freestanding, cell-containing hydrogel biopaper based on simple lamination techniques. To the best of our knowledge, fabrication and assembly of cellular hydrogel modules in the form of "freestanding biopaper" has not been previously reported. The "microunit" or "fiber" modules are formed into "sheet" modules midway through the assembly;[9,18] characterization of the biopaper is indispensable for tissue reconstruction based on these modules. In this study, we also demonstrate artificial 3D hepatic tissue reconstruction by fabricating and assembling hepatic hydrogel sheet modules based on the newly developed cellular hydrogel biopaper.

In consideration of both limitation of diffusion into tissues and mechanical stability as a building module, the cellular hydrogel biopaper (10 mm × 10 mm) was designed to have a thickness of 50–200 μm. To achieve these dimensions, we selected alginate hydrogel as the starting material for several reasons,[5] including easy and rapid processability with calcium ions for cross-linking, biocompatibility for high cell viability, mechanical stability during the replication of microarchitectures, and high diffusive permeability to metabolites and nutrients. To fabricate the hydrogel biopaper as freestanding modules, we used a facile fabrication method based on sol–gel transitional hydrogels as previously reported.[19] As shown in **Figure 1**a, the fabrication method was based on a) the use of a hydrophilic substrate to form a thin layer of hydrogel precursor without cumbersome sandwich or injection micromolding[5,20] techniques and b) the direct delivery of nebulized gelling agent on top of the hydrogel precursor for cross-linking without morphological distortions.[21] We evaluated the mechanical stability of the fabricated biopaper (with 0.5%–2% alginate hydrogels, cross-linked with nebulized 100 mM calcium chloride, containing 10^5–10^7cells mL^{-1}) during the freestanding culturing (Figure 1) and handling processes of release (Supporting Information, Video 1), harvesting, transfer and assembly (Supporting Information, Figure S1); the biopaper was mechanically flexible and stable throughout all the experimental procedures.

To create an in vivo–like 3D environment requires not only that the hydrogel microarchitectures mimic the ECM, but also

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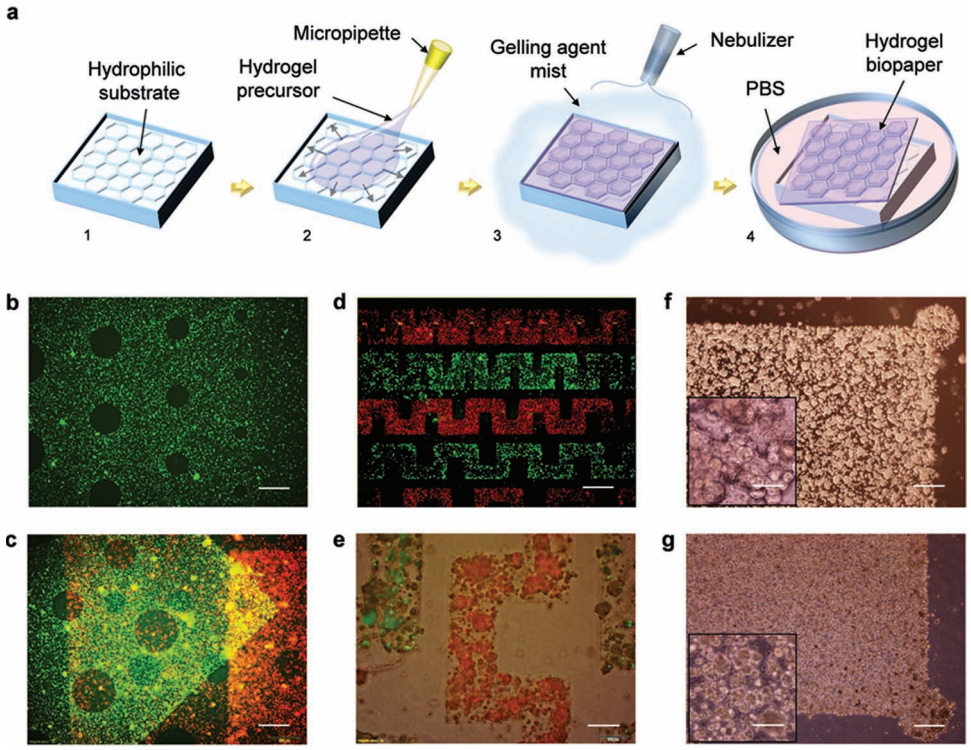

Figure 1. Fabrication schematics of the microarchitectured freestanding hydrogel biopaper and demonstration of cell patterning with long-term culturing capabilities in the biopaper. a) Fabrication schematics of the hydrogel biopaper. Hydrophilic substrates are prepared with desired microstructures (1), and the hydrogel precursor is dispensed by micropipette and coated as a thin film over the substrates (2). The thin film of hydrogel precursor is cross-linked with a fine mist of a gelling agent to prevent morphological distortions of the gel (3). Sheets of cross-linked hydrogel biopaper are submerged in PBS and released as freestanding structures from the substrates (4). b,c) Spontaneous cell patterning via a micropillar array on the substrate. Fluorescent images of viable HepG2 cells in the hydrogel biopaper with microhole perforations, immediately after the fabrication on the substrate (b) and at day 7 of freestanding culturing (c). d,e) Capillary force–based patterning of multiple types of cells via filling of open-faced microfluidic channels on the substrate. A fluorescent image of viable HepG2 cells patterned in microcomb-like architectures of the hydrogel biopaper, immediately after the fabrication on the hydrophilic substrate (d), and a phase contrast image of the biopaper at day 7 of freestanding culturing, overlaid with fluorescent images of viable cells (e). f,g) Phase contrast images of HepG2 cell morphologies with an initial cell seeding density of ~5×10^6 cells mL^{-1}, at day 5 (f) of culturing in the freestanding cellular hydrogel biopaper of calcium alginate (inset: magnified view) and at day 11 (g) of culturing in the freestanding cellular hydrogel biopaper of alginate/gelatin blend (inset: magnified view). Scale bars: 500 μm (b–d,f,g), 200 μm (e), and 100 μm (insets of (f) and (g)).

"on-demand" cell positioning for organized cellular structures. Through the fabrication of a cellular hydrogel biopaper on a substrate with an array of pillars, human hepatocellular carcinoma (HepG2) cells and hydrogels were spontaneously patterned with microhole perforations (Figure 1b). The microhole-perforated biopaper could be cultured freestanding, and the cells aggregated randomly in the biopaper without morphological distortion of the microholes during 7 days of culturing (Figure 1c). As shown in Figure 1d, HepG2 cells (in alginate precursor) stained with a red or green fluorescence indicator (CellTracker; Molecular Probes) were alternately filled into open-faced parallel channels on a hydrophilic substrate and subsequently cross-linked. The multiple microcomb-like cellular hydrogel microfibers were embedded in the hydrogel biopaper and cultured as a single freestanding module. This result indicated that multiple types of cells could be patterned in the hydrogel biopaper. As shown in Figure 1e, the HepG2 cells in the biopaper proliferated and aggregated, while the initial microcomb-like cellular structures were still observable at day 7 of freestanding culturing. This feature may be applicable to maintain initial cell patterning during long-term culturing without the use of biofouling materials. As a 3D cell culture module, the cell (HepG2) viability in the biopaper was 95.6 ± 1.0% (mean ± S.D., $n = 23$) at day 1 of culturing, as assayed using calcein-AM and ethidium homodimer-1. As shown in Figure 1f, the HepG2 biopaper with cell concentration of 5×10^6 cells mL^{-1} exhibited cell proliferation and aggregation for 5 days without morphological distortion or disruption of the biopaper. The aggregated HepG2 cells, however, escaped from the biopaper through freestanding culturing beyond 8 days (data not shown). To mitigate cell escape, we added gelatin (a derivative of collagen, a thermosensitive hydrogel) to the hydrogel biopaper, with the rationale that gelatin is a cell adhesive ECM material. In particular, at a 1:1 mixture of 1% alginate and 5% gelatin, we were able to reduce the incidence of cells escaping from the biopaper sufficiently to enable long-term freestanding culturing beyond 10 days (Figure 1g). However, the speed of the cell proliferation and aggregation (Figure 1g, inset) was slower than that observed for the biopaper without gelatin. Further study is required to elucidate the full effects of the gelatin additive.

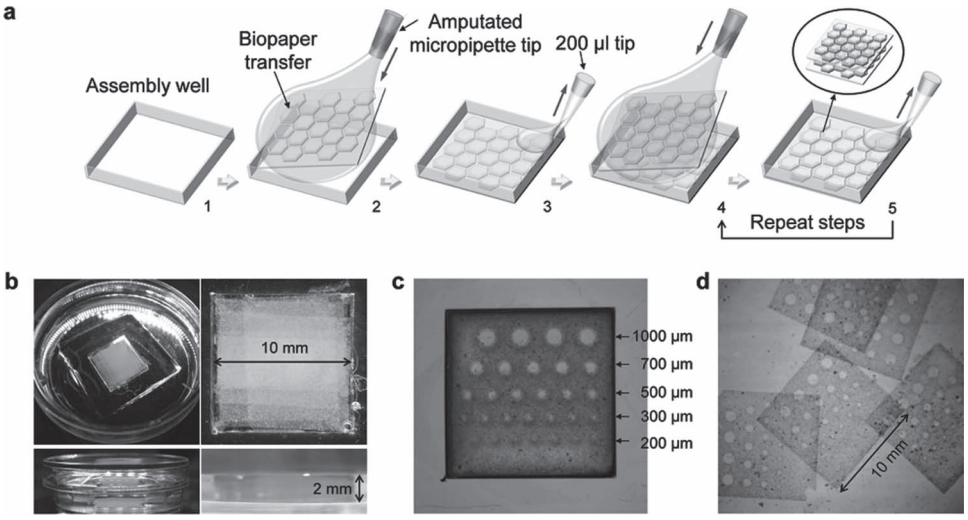

Figure 2. Schematics and demonstrations of the freestanding cellular hydrogel biopaper assembly. a) Schematics of the biopaper stacking assembly procedure. Into an assembly well (1), a cellular hydrogel is transferred with PBS (2) and the surrounding PBS is thoroughly aspirated using micropipettes (3) to place the biopaper at the bottom of the well. The next sheet of biopaper is transferred (4) and stacked on the previous sheet by means of PBS aspiration (5). By repeating steps (4) and (5), multiple sheets of biopaper are stacked. b) Top and side views of the multilayered cellular hydrogel biopaper of HepG2 cells obtained by stacking in a square assembly well measuring 10 mm (width) × 10 mm (length) × 2 mm (depth). The right-hand panels in the figure show magnified views of the pictures in the left-hand panels. c,d) Stereomicroscopic images of stacked and de-stacked microhole-perforated cellular hydrogel biopaper of HepG2 cells. Five pieces of the microhole-perforated biopaper measuring 10 mm (width) × 10 mm (length) were stacked with guided alignment in the size-fitting square assembly well. The microholes with diameters of 200, 300, 500, 700, and 1000 µm were observable (c). The stacked cellular hydrogel biopaper was de-stacked without structural destruction (d).

The ability to generate multilayered cellular hydrogel composites is critical to the engineering of spatially complex biological tissues. Thus, development of reliable handling and assembly techniques, including biopaper harvesting, transfer, and stacking, are necessary for modular tissue reconstruction (**Figure 2**a). The biopaper was mechanically stable in a liquid, but difficult to manipulate with tweezers. Therefore, an amputated micropipette tip was used to harvest the biopaper. The biopaper was harvested in a randomly folded configuration with the surrounding medium, and it spontaneously unfolded when transferred out of the tip. During these harvesting and transfer processes, the embedded cells were viable, as shown in Figures 1c and 1e. As the surrounding medium was aspirated, the transferred sheets of biopaper sank to the bottom of the container and stacked. These harvesting, transfer, and stacking steps were repeated to laminate multiple layers of biopaper (Figure 2b). More than 10 layers of biopaper could be stacked using the proposed techniques. Additionally, biopaper containing patterned cellular structures could be transferred to a nonplanar substrate as a vehicle for transfer of the patterned mature cell structures onto arbitrary substrates with on-demand timing. For alignment of multiple layers of biopaper, a size-fitting assembly well was utilized. Through layer-by-layer stacking of biopaper into the well, each sheet of biopaper (10 mm × 10 mm) was aligned using the size-fitting well of 10 mm (width) × 10 mm (length) × 2 mm (depth) as a guide. Alignment of multiple layers of biopaper was examined through stacking of microhole-perforated layers of hydrogel biopaper of HepG2 cells. Figure 2c shows a top view of a stack of five layers of biopaper with observable 200 µm microholes, demonstrating that the multiple layers of biopaper could be assembled with an alignment resolution of 200–300 µm. The stacked biopaper layers could be delaminated with gentle pipetting, thereby guaranteeing intact biopaper during the lamination and delamination processes (Figure 2d); this feature may be useful for downstream studies.[13]

We further examined the biopaper's applicability to hepatic tissue reconstruction. First, hepatic hydrogel sheet modules containing HepG2 cells were fabricated either with hexagonal microarchitectures (Hexa biopaper; **Figure 3**a) or without any microarchitecture (Flat biopaper). As biofunctional assays, albumin secretion (as a surrogate marker of hepatic tissue functions) and cell proliferation assays were conducted on Hexa and Flat biopaper, using conventional culturing (2D culturing) as a control. One-way analysis of variance (ANOVA) with Tukey's test was used for statistical analysis. As shown in Figure 3b, Flat biopaper showed significantly increased cumulative albumin secretion during the initial 2 days of culturing compared to 2D culturing ($p = 0.0067$, $n = 3$). Moreover, during days 2–4 of culturing, both Hexa and Flat biopaper showed significantly increased cumulative albumin secretion compared to 2D culturing ($p = 0.0050$, 0.0110, $n = 3$, respectively), and that of Hexa biopaper was even higher than that of Flat biopaper. Further study is required to elucidate the full effects of the increase of the albumin secretion in the hepatic biopaper, but the albumin assay results indicated that the hepatic hydrogel sheet modules could be employed as a functional culturing system for hepatic cells to obtain increased expression levels of drug-metabolizing enzymes. In the proliferation assay (Figure 3c), as previously reported,[22] a 2D culture showed significantly higher proliferation than 3D cultures of Flat and Hexa biopaper at days 2–5 of culturing ($p = 0.04423$, 0.0019, 0.0024, 0.0024,

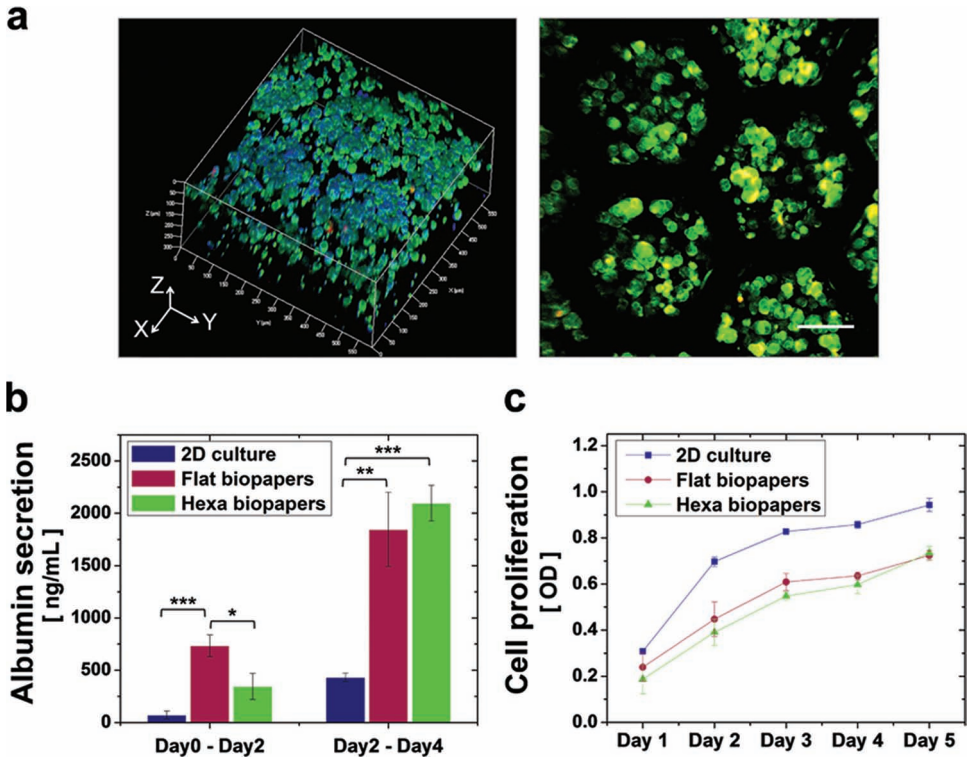

Figure 3. Biofunctional assays of the hepatic hydrogel sheet modules for hepatic tissue reconstruction. a) *Z*-Stack imaging inspection of the hepatic hydrogel sheet module with hexagonal microarchitectures at day 1 of cell culturing: 3D rendering (left), and an image slice at a focal point (right). Viable cells were stained with green fluorescence indicator (calcein-AM), and dead cells were stained with red (ethidium homodimer-1). Scale bars and the lengths of the *X*-, *Y*-, and *Z*-axes are 100 µm. b,c) Results of albumin secretion assay (b) and cell proliferation assay (c) with mean ± standard error for the three groups of hepatic hydrogel sheet modules with hexagonal microarchitectures (Hexa biopaper), the hepatic hydrogel sheet modules without any microarchitecture (Flat biopaper), and conventional culturing of HepG2 cells (2D culturing) as a control. Albumin secretion from the hepatic hydrogel sheet modules (Hexa biopaper and Flat biopaper) increased significantly compared to that from the conventional cultures. HepG2 cell proliferation of the 2D culture was higher than for 3D cultures of the hepatic hydrogel sheet modules. The statistical analysis was conducted with ANOVA followed by Tukey's test, $*p < 0.1$, $**p < 0.05$, $***p < 0.01$, $n = 3$.

p = 0.0188, 0.0005, 0.0011, 0.0032, n = 3, daily, respectively), and no significant differences were observed between Flat and Hexa biopaper.

In this Communication, we have presented novel, freestanding cellular hydrogel biopaper for patterning suspended 3D cell cultures or complex tissue reconstruction. The biopaper features both the desired hydrogel microarchitectures and the organized cellular arrangements that can duplicate the native cellular environment. The cell-containing biopaper was mechanically stable without morphological distortion or disruption during freestanding cell culturing, and long-term freestanding culturing was enabled upon addition of the cell adhesive gelatin to the biopaper. We also developed new and facile handling and stacking techniques for the biopaper, and we utilized the techniques to construct stratified hepatic tissue modules by laminating the fabricated hepatic hydrogel biopaper, which had increased liver function. To the best of our knowledge, this is the first study that creates cellular hydrogel modules in the form of "freestanding biopaper" for 3D cell culturing and tissue reconstruction. As a result of its simplicity and versatility in fabrication, culturing, handling, and assembly, the microarchitectured freestanding cellular hydrogel biopaper could provide unprecedented tools to study cell–ECM interactions, structure–function relationships, tissue morphogenesis, and modular tissue reconstructions.

Experimental Section

Materials: A chemically cross-linkable hydrogel of 0.5% or 1% (w/v) sodium alginate precursor (from brown algae, Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS; Gibco/Life Technologies, Carlsbad, CA) and a thermosensitive hydrogel of 5% (w/v) gelatin (porcine skin Type A, Sigma-Aldrich) in PBS were used. As a cross-linking agent for sodium alginate, 100 mM calcium chloride ($CaCl_2$; Sigma-Aldrich) in distilled water was used. All solutions, including alginate hydrogel precursor, gelatin solution, and $CaCl_2$ reagent, were pre-filtered through a 0.22 µm pore–sized bacterial filter (Millipore, Billerica, MA) and sterilized overnight by germicidal ultraviolet (UV) irradiation inside a clean bench. The sterilized hydrogel precursor of alginate and the alginate/gelatin blend were stored in a laboratory refrigerator (4 °C) and warmed in a water bath (37 °C) before the hydrogel architectures were fabricated. For cellular studies, human cell lines of HepG2 cells were used (see Supporting Information).

Freestanding Culturing of Cellular Hydrogel Biopaper: Fabricated cellular hydrogel biopaper was exfoliated as freestanding units, harvested by pipetting with surrounding medium (or PBS) using an amputated 1 mL micropipette tip (Supporting Information, Figure S1), and transferred to the target locations. Multiple cellular hydrogel biopaper could be

gathered and cultured in a Petri dish, or individual pieces of hydrogel biopaper could be separately cultured using 6- or 12-well plates. The cellular hydrogel biopaper of HepG2 cells was cultured with the same medium as used for the conventional cultures in a 5% CO_2-humidified incubator at 37 °C. The culture medium was exchanged every day (or every other day) with careful aspiration of the used medium to avoid removal of the freestanding cellular hydrogel biopaper. The exchange period of culture medium was made adjustable by changing the number of pieces of cellular hydrogel biopaper in each tissue culture dish. The cellular hydrogel biopaper could be used with on-demand timing during the freestanding culturing.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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[1] D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin, D. E. Ingber, *Science* **2010**, *328*, 1662.
[2] L. G. Griffith, M. A. Swartz, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 211.
[3] R. Langer, J. P. Vacanti, *Science* **1993**, *260*, 920.
[4] a) D. R. Albrecht, G. H. Underhill, T. B. Wassermann, R. L. Sah, S. N. Bhatia, *Nat. Methods* **2006**, *3*, 369; b) E. Cukierman, R. Pankov, D. R. Stevens, K. M. Yamada, *Science* **2001**, *294*, 1708; c) C. M. Nelson, M. M. VanDuijn, J. L. Inman, D. A. Fletcher, M. J. Bissell, *Science* **2006**, *314*, 298.
[5] N. W. Choi, M. Cabodi, B. Held, J. P. Gleghorn, L. J. Bonassar, A. D. Stroock, *Nat. Mater.* **2007**, *6*, 908.
[6] E. Kang, G. S. Jeong, Y. Y. Choi, K. H. Lee, A. Khademhosseini, S.-H. Lee, *Nat. Mater.* **2011**, *10*, 877.
[7] H. Geckil, F. Xu, X. Zhang, S. Moon, U. Demirci, *Nanomedicine* **2010**, *5*, 469.
[8] a) D. M. Dean, A. P. Napolitano, J. Youssef, J. R. Morgan, *FASEB J.* **2007**, *21*, 4005; b) G. R. Souza, J. R. Molina, R. M. Raphael, M. G. Ozawa, D. J. Stark, C. S. Levin, L. F. Bronk, J. S. Ananta, J. Mandelin, M.-M. Georgescu, J. A. Bankson, J. G. Gelovani, T. C. Killian, W. Arap, R. Pasqualini, *Nat. Nanotechol.* **2010**, *5*, 291.
[9] H. Onoe, R. Gojo, Y. Tsuda, D. Kiriya, M. Kato-Negishi, S. Takeuchi, in *Proc. of the 14th Int. Conf. on Miniaturized Systems for Chemistry and Life Sciences*, (Eds: S. Verpoorte, H. Andersson-Svahn, J. Emnéus, N. Pamme), The Chemical and Biological Microsystems Society, San Diego, CA **2010**.
[10] K. Ohashi, T. Yokoyama, M. Yamato, H. Kuge, H. Kanehiro, M. Tsutsumi, T. Amanuma, H. Iwata, J. Yang, T. Okano, Y. Nakajima, *Nat. Med.* **2007**, *13*, 880.
[11] S. Pautot, C. Wyart, E. Y. Isacoff, *Nat. Methods* **2008**, *5*, 735.
[12] K. H. Lee, S. J. Shin, C.-B. Kim, J. K. Kim, Y. W. Cho, B. G. Chung, S.-H. Lee, *Lab Chip* **2010**, *10*, 1328.
[13] a) B. J. Papenburg, J. Liu, G. A. Higuera, A. M. C. Barradas, J. de Boer, C. A. van Blitterswijk, M. Wessling, D. Stamatialis, *Biomaterials* **2009**, *30*, 6228; b) R. Derda, A. Laromaine, A. Mammoto, S. K. Y. Tang, T. Mammoto, D. E. Ingber, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18457.
[14] Y. Du, E. Lo, S. Ali, A. Khademhosseini, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 9522.
[15] a) J. G. Fernandez, A. Khademhosseini, *Adv. Mater.* **2010**, *22*, 2538; b) Y. T. Matsunaga, Y. Morimoto, S. Takeuchi, *Adv. Mater.* **2011**, *23*, H90.
[16] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337.
[17] S. E. Chung, W. Park, S. Shin, S. A. Lee, S. Kwon, *Nat. Mater.* **2008**, *7*, 581.
[18] F. Yanagawa, H. Kaji, Y.-H. Jang, H. Bae, D. Yanan, J. Fukuda, H. Qi, A. Khademhosseini, *J. Biomed. Mater. Res. A* **2011**, *97A*, 93.
[19] W. Lee, J. Son, S.-S. Yoo, J.-K. Park, *Biomacromolecules* **2011**, *12*, 14.
[20] B. M. Gillette, J. A. Jensen, B. Tang, G. J. Yang, A. Bazargan-Lari, M. Zhong, S. K. Sia, *Nat. Mater.* **2008**, *7*, 636.
[21] W. Lee, J. C. Debasitis, V. K. Lee, J.-H. Lee, K. Fischer, K. Edminster, J.-K. Park, S.-S. Yoo, *Biomaterials* **2009**, *30*, 1587.
[22] S.-F. Lan, B. Safiejko-Mroczka, B. Starly, *Toxicol. In Vitro* **2010**, *24*, 1314.

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Cellular Hydrogel Biopaper for Patterned 3D Cell Culture and Modular Tissue Reconstruction

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Cellular Hydrogel Biopaper for Patterned Three-Dimensional Cell Culture and Modular Tissue Reconstruction

*Wonhye Lee, Chae Yun Bae, Seyong Kwon, Jaejung Son, Jinho Kim, Yong Jeong, Seung-Schik Yoo, and Je-Kyun Park**

Mammalian Cell Culture: A human hepatocellular carcinoma cell line, HepG2, was purchased from the American Type Culture Collection (ATCC, Manassas, VA) as a frozen vial. HepG2 was cultured with Dulbecco's modified Eagle's media (DMEM; Gibco/Life Technologies) with high glucose, l-glutamine, and sodium pyruvate, and was supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The cell culture was maintained in a 5% CO_2-humidified incubator at 37°C, and the culture media was exchanged every other day with a gentle rinse of phosphate-buffered saline (PBS; Gibco/Life Technologies, Carlsbad, CA). Cultured confluent HepG2 cells were split in a ratio of 1:4 at day 5 of culture.

Preparation of Cell Suspensions in Hydrogel Precursor: To prepare the cell suspension in the hydrogel precursor of alginate or alginate/gelatin blend, 80% confluent HepG2 cells on tissue culture dishes were treated with 0.25% trypsin–EDTA for 3 min, harvested, and centrifuged at 1000 rpm for 3 min. After supernatant removal, the remaining cell pellet of HepG2 cells was resuspended in the hydrogel precursor (prewarmed in a 37°C water bath) with gentle pipetting up and down. The hydrogel cell suspensions were prepared in concentrations of ~5×10^6 cells mL^{-1}. The prepared hydrogel cell suspensions were kept in a 5% CO_2-humidified incubator at 37°C. Immediately before the biopaper fabrication, the stored hydrogel cell suspensions were gently resuspended by pipetting up and down to

maintain a uniform cell concentration among multiple cellular hydrogel biopapers during the fabrication procedures.

Preparation of Hydrophilic Substrates: A hydrophilic surface of poly(dimethylsiloxane) (PDMS) was utilized as a substrate for the preparation of a thin film of hydrogel precursor. Rectangular PDMS slabs (top surface area of 10 mm × 10 mm) with desired microarchitectures were prepared by conventional PDMS replica molding techniques[1] and placed open-faced in a Petri dish (8–20 slabs in each dish). The PDMS slabs were reversibly attached to the dish and used as a package for the subsequent experiments. The PDMS substrates were sterilized with 70% ethyl alcohol, rinsed in distilled water, and then coated with 6% (w/v) Pluronic F-127 (a gift from BASF, Ludwigshafen, Germany) solution in PBS for 1 h (or overnight) on a laboratory rocker. The binding of Pluronic F-127 to the PDMS surface reduces protein absorption and cell adhesion. The PDMS substrates coated with Pluronic F-127 were gently rinsed with distilled water, dried, and then stored in a clean bench. Used hydrophilic substrates could be reclaimed for use by briefly washing with tap water followed by recoating with Pluronic F-127; substrates treated in this manner could be reused more than 10 times over 6 months.

Fabrication of Freestanding Cellular Hydrogel Biopapers: To fabricate cellular hydrogel biopapers that were thinner than the diffusion depth limitation (~200 μm) in biological tissues, a controlled volume (10–20 μL) of cell hydrogel suspension was evenly spread and coated as a thin film over each hydrophilic substrate (10 mm × 10 mm). To construct the hydrophilic substrates, the PDMS slabs were rendered hydrophilic immediately before coating with the hydrogel precursor *via* treatment with oxygen plasma for 30–90 s (using a plasma cleaner set at medium power; Harrick Scientific, Ithaca, NY). Next, a mist of gelling agent was directly delivered *via* a nebulizer with ultrasonic transducer (MH-300A; M-Tech, Seoul, Korea) to coat the top surface of coated hydrogel precursor film, thereby cross-linking the hydrogel as a

thin biopaper without morphological distortions at both the macro- and microscale.[2] The quantity of the delivered gelling agent was controlled by varying the nebulizing time from 10 s to 5 min with changes in the experimental conditions such as concentration of the cross-linking agent, the nebulizing performance of the nebulizer used, or both. This nebulization technique eliminates the unavoidable use of a semipermeable physical barrier to prevent the morphological distortion of hydrogel microarchitectures[3] commonly obtained using conventional methods. After the hydrogel cross-linking, the cellular hydrogel biopapers were exfoliated from the hydrophilic PDMS substrates as freestanding units. For the exfoliation, PBS or culture medium was added to submerge the substrates with overlaid biopapers, and then gentle pipetting up and down around the overlaid biopapers induced exfoliation (Supporting Video 1).

Cell Patterning in Hydrogel Biopapers: Midway through the fabrication of cellular hydrogel biopapers, cell-patterning techniques using obstacles, channels, or microwell arrays on hydrophilic PDMS substrates were introduced to demonstrate the fabrication versatility of the freestanding cellular hydrogel biopapers. For cell patterning using obstacles, PDMS substrates were prepared with obstacles (height of ~300 μm) such as those provided by a pillar array. Through the fabrication of the thin (~200 μm) cellular hydrogel biopaper on the PDMS substrates, cells and gels were spontaneously patterned *via* the obstacles (Figure 1b). For cell patterning using channels (Figure 1d), open-faced microfluidic channels on hydrophilic PDMS substrates were filled with a hydrogel cell suspension. When the hydrogel cell suspension (loaded in a 20-μL micropipette tip) made contact at one end of an open-faced microfluidic channel, the hydrophilic channel was spontaneously filled with the hydrogel cell suspension *via* capillary force. The hydrogel cell suspension in the channels was then cross-linked by the presence of a mist of cross-linking agent. To combine the patterned cellular architectures in separate multiple channels in a single hydrogel biopaper, a thin layer of

hydrogel precursor was coated over the PDMS substrates containing the cellular hydrogel pattern, which was subsequently cross-linked and released as a freestanding hydrogel biopaper that maintained the cellular architectures. For cell patterning using a microwell array (Figure 3a), a hydrogel cell suspension was coated on the PDMS substrates with a microwell array, and the coated hydrogel cell suspension was gently wiped three to five times before hydrogel cross-linking using a cell scraper. The wiping step localized cells into microwells on the substrate, which consequently generated a cellular microarray in the hydrogel biopapers after cross-linking.

Fluorescent Staining for Cell Viability and Long-Term Cell Tracking: A cell viability assay was performed using a commercially available mammalian cell viability and cytotoxicity assay kit (Molecular Probes, Carlsbad, CA). The samples were incubated for 40 min in a solution of calcein-AM (5 μL) and ethidium homodimer-1 (20 μL) in PBS (10 mL). For long-term observation of the viable cells, commercially available probes (green and red fluorescent staining) for long-term tracing of living cells (CellTracker; Molecular Probes) were used. The samples were incubated for 30 min at 37°C in a solution of CellTracker (5 μM) in PBS. Cellular fluorescence was observed using inverted epifluorescence microscopes (IX51; Olympus, Tokyo, Japan and Axiovert 25; Carl Zeiss, Oberkochen, Germany) and a multi-photon laser scanning microscope (LSM 510 NLO; Carl Zeiss).

Albumin Secretion Measurement via An Enzyme-Linked Immunosorbent Assay (ELISA): Albumin secretion was quantified from the used culture media for HepG2 cells in three different groups of conventional cultures (2D), cellular hydrogel biopapers of HepG2, without any microarchitectures (Flat biopapers), and with hexagonal microarchitectures (Hexa biopapers) at days 2 and 4 of culture. The cultures were conducted in 12-multiwell plates, and the hepatic hydrogel sheet modules were cultured as freestanding structures in culture media (2 mL). The culture media (200 μL) was sampled daily from each culture group (n = 3 for

each group) and stored in a –80ºC deep freezer. The stored media were assayed using a commercial albumin ELISA kit (Immundiagnostik AG, Bensheim, Germany). Albumin was quantified with goat antihuman albumin and horseradish peroxidase (HRP)-conjugated antibody. Microtiter plates were pre-coated with the goat antihuman albumin in the kit. The plates were washed three times with a washing buffer [PBS including 0.05% (v/v) Tween 20], and blocked by a blocking solution [containing tris(hydroxymethyl)aminomethane (Tris) (50 mM), NaCl (0.14 M), and 1% bovine serum albumin, pH 8] for 30 min at room temperature. After washing the plate three times with the washing buffer, the (diluted) samples of albumin standards (100 µL, as calibrators) and albumin controls (100 µL, concentration known sample) were added to the plates and incubated at room temperature for 1 h. The sampled culture media was diluted in a sample dilution buffer in a ratio of 1:50. After washing the plates five times with the washing buffer, HRP detection antibody (100 µL) was added to each plate and incubated at room temperature for 1 h. After washing, a 1:1 solution of tetramethylbenzidine and H_2O_2 was added and allowed to react for 5 min before a stop solution was added to each plate. All the blocking and incubation steps were conducted on a horizontal mixer. Color-changed solutions were read by a microtiter plate reader (SPECTRAmax 250; Molecular Devices, Sunnyvale, CA) at 450 nm and normalized with a reference wavelength of 690 nm. For the evaluation of the test results, a four-parameter algorithm was used to obtain calibration curve fitting and data analysis.

Cell Proliferation Assay Using Prestoblue Reagent: A HepG2 cell proliferation assay was conducted for three different groups of conventional cultures (2D culture), hepatic hydrogel sheet modules of HepG2, without any microarchitectures (Flat biopapers), and with hexagonal microarchitectures (Hexa biopapers) during a 5-day culture period. The cultures were conducted in 12-multiwell plates, and biopapers were cultured as freestanding structures in culture media (2 mL). At each culture day, commercially available resazurin-based cell

viability reagent (200 µL, PrestoBlue; Invitrogen, Bedford, MA) was added to each well of the culture groups (n = 3 for each group) and incubated for 6 h. After the incubation, color-changed culture media (200 µL) was sampled from the wells and dispensed into a 96-multiwell plate. The absorbances of the color-changed sampled media were measured by a microtiter plate reader at 570 nm and normalized with a reference wavelength of 600 nm. The normalized absorbance values (optical density, OD) demonstrate metabolic activity of cells, hence quantitatively measuring the cell viability and proliferation.

[1] G. M. Whitesides, E. Ostuni, S. Takayama, X. Jiang, D. E. Ingber, *Ann. Rev. Biomed. Eng.* **2001**, *3*, 335.

[2] W. Lee, J. Son, S.-S. Yoo, J.-K. Park, *Biomacromolecules* **2011**, *12*, 14.

[3] G. T. Franzesi, B. Ni, Y. Ling, A. Khademhosseini, *J. Am. Chem. Soc.* **2006**, *128*, 15064.

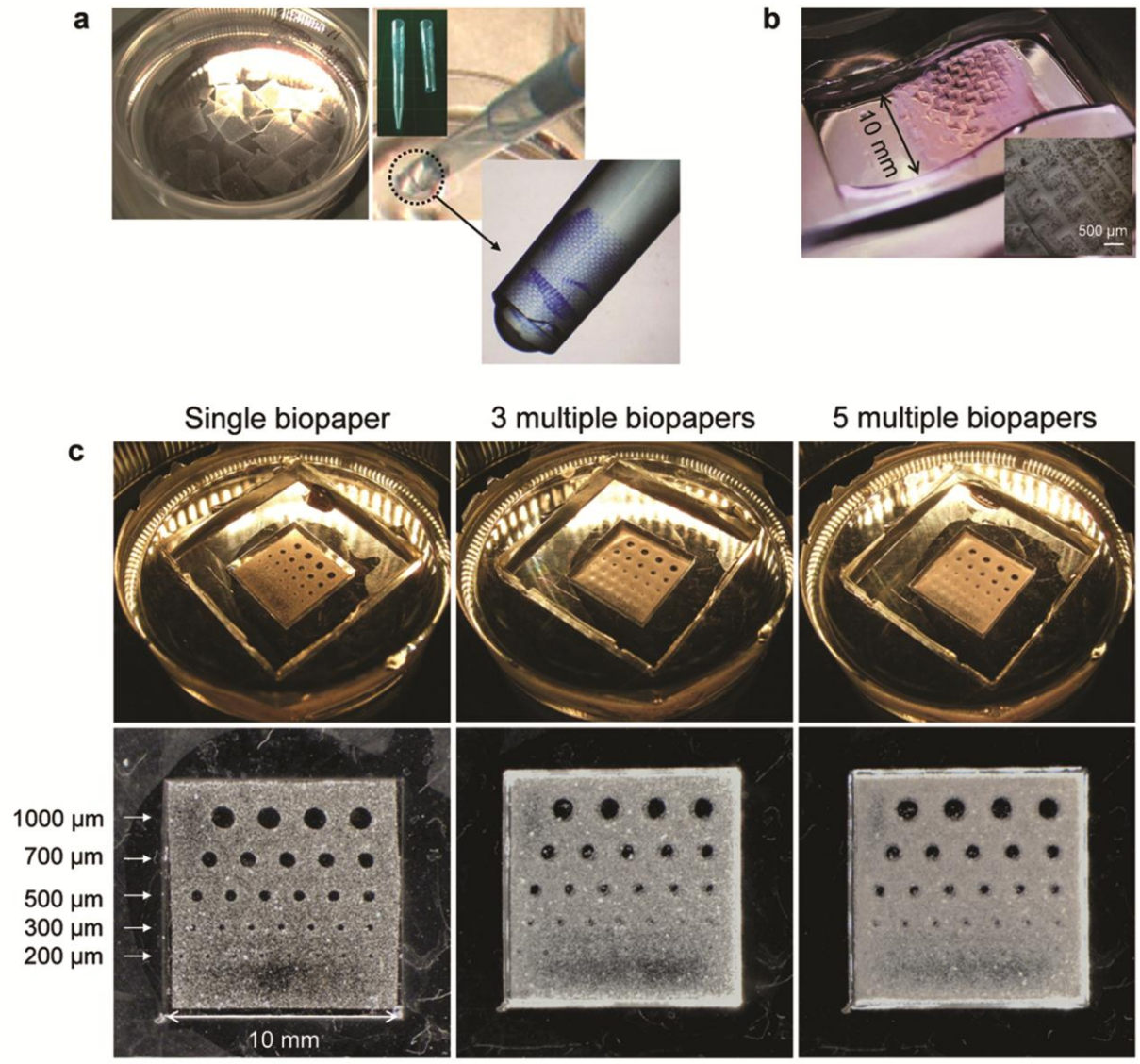

Figure 1. Handling, harvest, and transfer of the freestanding cellular hydrogel biopapers and alignment examination through layer-by-layer stacking of microhole-perforated cellular hydrogel biopapers of HepG2 cells using the proposed assembly method. **a**, Multiple freestanding cellular hydrogel biopapers of HepG2 cells cultured in a 60-mm culture dish (left), and the use of an amputated 1-mL micropipette tip to harvest and transfer the biopapers (center, inset: normal and amputated 1-mL micropipette tips). A harvested microarchitectured hydrogel biopaper as a randomly folded structure (right bottom). **b**, Transfer of the cellular hydrogel biopaper of HepG2 cells with microcomb-like architectures to a nonplanar substrate at day 12 of the freestanding culture (inset: a stereomicroscopic view of the transferred biopapers, scale bar: 500 µm). **c**, The microholes with diameters of 300, 500, 700, and 1000 µm were observable throughout stacking of the one-, three-, and five-multilayer biopapers. The lower images are stereomicroscopic dark-field top view of the stacked biopapers.

Supporting Video 1: The movie shows real-time exfoliation of the cellular hydrogel biopapers of HepG2 cells from the hydrophilic PDMS substrates. The substrates overlaid with the cross-linked cellular hydrogel biopapers were submerged in PBS. Gentle pipetting up-and-down of the liquid around the biopapers easily induced exfoliation of the biopapers.