Supporting information

Construction of spheroid array culture system on suspended permeable hydrogel membrane scaffold for improving expression of liver-specific function of HepG2 cells

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1. Materials

Hydroxypropyl cellulose (HPC, M.W. 100,000, #19188-4, Sigma-Aldrich Co.) was used as a water-soluble hydroxyl-rich polymer composing hydrogel membrane. 1,3,4,6-tetrakis (methoxymethyl) glycoluril (TMMGU, #T2058, Tokyo Chemical Industry Co., Ltd.) was used as an acid-catalyzed cross-linker. Poly (acrylic acid) (PAAc, M.W. 250,000, #169-18591, FUJIFILM Wako Pure Chemical Corp.) was used as a polymer composing sacrificial layer. Poly [(methyl methacrylate)-co-(7-(4-trifluoromethyl) coumarin) methacrylamide] (PCMM, #566241, Sigma-Aldrich Co.) was used as a polymer providing HPC hydrogel with local cell adhesiveness. Collagen (Cellmatrix Type I-C, Nitta Gelatin Inc.) was used to coat on the membrane and improve HepG2 cells adhesiveness. Polystyrene petri dish with 35 mm diameter (#3000-035, AGC TECHNO GLASS Co., Ltd.) was used as a basal substrate. HepG2 cells were provided by the RIKEN Bioresource center (Tsukuba, Ibaraki, Japan). D-PBS (-) (#045-29795, FUJIFILM Wako Pure Chemical Corp.) was used to wash cells and cross-linked polymer layer.

2. Apparatus

A spin-coater (ASS-301, Able Co., Ltd) was used for coating polymer solution. Pan type granulator (PZ-01R, AS ONE) was used for removing PAAc from the peripheral area of the dish. Irradiation unit (irradiation intensity: 280 mW, SOLIS-365C, Thorlabs, High-Power LED for Microscopy) was used for irradiating coated PCMM layer with UV light. Bright field images were taken with a cooled CCD camera system (VB-7000, Keyence CO.) installed in an inverted research microscope (IX70, Olympus Co.) through a 4X objective lens (Plan Apo 4X, Olympus Co.). 3-dimensional and fluorescent observation was carried out by using a confocal laser scanning microscope (LSM700, Carl Zeiss).

3. Cell incubation

HepG2 cells were maintained and repeatedly subcultured in Dulbecco's Modified Eagle Medium with high glucose (D-MEM, #045-30285, FUJIFILM Wako Pure Chemical Corp.) containing 10% fetal bovine serum (FBS) (#SH30396.03, Hyclone), penicillin-streptomycin (#168-23191, FUJIFILM Wako Pure Chemical Corp.), and MEM non-essential amino acids solution (#139-15651, FUJIFILM Wako Pure Chemical Corp.) at 37°C in the incubator (humidified 95% air, 5% CO_2 atmosphere).

4. Synthesis of fHPC

HPC and Fluorescein-4-isothiocyanate (FITC, #345-03663, Dojindo) were dissolved in tetrahydrofuran (THF). fHPC was synthesized by heating to dry the THF solution containing 0.98 wt% HPC and 0.086wt% FITC at 110˚C for 30 minutes. fHPC was dissolved in methanol and the solution was used to prepare pre-gel solution for **Fig. 3 (b)**.

5. Examination of HepG2 cells adhesiveness on the cross-linked PCMM

A 120 μL of 2,2,3,3-tetrafluoro-1-propanol (TFP) solution containing 3.0 wt% HPC, 0.15 wt% TMMGU and 0.015 wt% H_2SO_4 was spin-coated on the surface of PS petri dish at 2,000 rpm. The layer was baked at 85℃ for 1 h, 2 h, or 4 h. A 40 μL of trifluoroethanol (TFE) solution containing 1.0 wt% PCMM was over-coated on these cross-linked HPC layers at 1,000 rpm. The layer was irradiated with UV light in a polka-dot pattern for 60 s through the photomask. After flushing with ethanol/water mixture, cross-linked PCMM was obtained. The layers were soaked in 0.03 mg/mL collagen solution (pH:3) for 30 min. After flushing with D-PBS(-) and replacing for the D-MEM, 3.5 × 10^5 cells of HepG2 cells were seeded and incubated. The relationship between immobilization of cross-linked PCMM and HepG2 cells adhesiveness on the cross-linked HPC is shown in **Fig. S1**.

6. Evaluation of protein adsorption on the cross-linked HPC based on Bradford assay

A 100 μL of TFP solution containing 3.0 wt% HPC, 0.30 wt% TMMGU and 0.015 wt% H_2SO_4 was spin-coated on the surface of the PS dish at 2,000 rpm. The coated layer was baked at 85℃ for 2 h. A 30 μL of TFE solution containing 1.0 wt% PCMM was over-coated on these cross-linked HPC layers at 1,000 rpm. Some of these layers were irradiated with UV light in the whole area, and the others were not (denoted as "Light+" or "Light-", respectively). They were soaked in D-MEM containing 10% FBS (denoted as "FBS+") or D-PBS (denoted as "FBS-") for a day in the incubator. After flushing with D-PBS (-), they were soaked in the solution containing Coomassie Brilliant Blue G-250 (CBB) for 5 min, and flushed with water. Then, the absorbance measurement of the cross-linked HPC was carried out for each sample. By subtracting base line, we estimated the absorbance at 595 nm (A_{595}), which was attributed to the CBB binding to protein. The results of A_{595} of each sample are shown in **Fig. S2**.

7. Preparation of thin cross-linked HPC layer for constructing suspended spheroid array culture corresponding to Fig. 6(a) 1 and 3

A 50 μL of TFP solution containing 1.0 wt% HPC, 0.050 wt% TMMGU and 0.0050 wt% H_2SO_4 was spin-coated on the surface of PS petri dish at 1,500 rpm. The coated layer was baked at 85℃ for 1 h. After flushing with water, a thin cross-linked HPC layer was obtained. Spheroid and monolayer cultures on the suspended hydrogel membrane were constructed above this thin layer using the same method described in the main article.

8. Evaluation of respiratory metabolism

We measured glucose and lactate concentration in the culture medium collected at 15th day with Glucose Assay Kit-WST (#G264, Dojindo) and Lactate Assay Kit-WST (#L256, Dojindo) following each manufacturer's protocol. Glucose consumption was calculated by subtracting the measured glucose from the total amount of glucose of the D-MEM. Each result of glucose

consumption and lactate production of the cells is shown in **Fig. S5(a)** and **(b)**

9. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using RNeasy Plus Mini Kit (#74134, QIAGEN) following the manufacturer's protocol. Total RNA was reverse-transcribed using QuantiTect Rev. Transcription Kit (#205311, QIAGEN). qRT-PCR was conducted with QuantiFast SYBR Green PCR Kit (#204054, QIAGEN) by using thermal cycler Dice Real Time System TP8000 (Takara Bio). The PCR conditions were as follows: Denaturation at 95˚C for 5 min, amplification and quantification at 95˚C for 10 s and 60˚C for 30 s for 40 cycles. The threshold cycle was measured, and all gene expressions were normalized with the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following PCR primers produced by QIAGEN were used for detection of each gene expression level: CYP1A2(Hs_CYP1A2_1_SG QuantiTect Primer Assay, #249900, QIAGEN, NM_000761), CYP3A4 (Hs_CYP3A4_1_SG QuantiTect Primer Assay, #249900, QIAGEN, NM_001202855, NM_017460), and GAPDH (Hs_GAPDH _vb.1_SG QuantiTect Primer Assay, #249900, QIAGEN, NM_002046, NM_001289746, NM_001289745). The value of mRNA expression level for Immobilized-monolayer culture (**Fig. 6(a)** 4) was arbitrarily set to 1. All other expression values are fold expressions to relative this.

10. Statistical analysis

Data were expressed as mean ± standard deviation. Statistical comparison was performed using Student's *t*-test. Differences of $p < 0.05$ were considered to be statistically significant. All error bars were presented as standard deviation. An *f*-test was used to investigate whether variance of samples is equal or unequal.

11. Supporting movie

Movement of the patterned cell culture constructed on the flexible hydrogel scaffold suspended in the culture medium upon the shaking in planar direction can be seen in the movie "**Movie S1(A. Tsuyukubo et al., Biomaterials Science)"**.

12. Detailed condition

Detailed conditions (concentration of solutes, amount of coated solution and rotation speed for spin-coating etc.) corresponding to each suspended or immobilized membrane are summarized in **Table S1**.

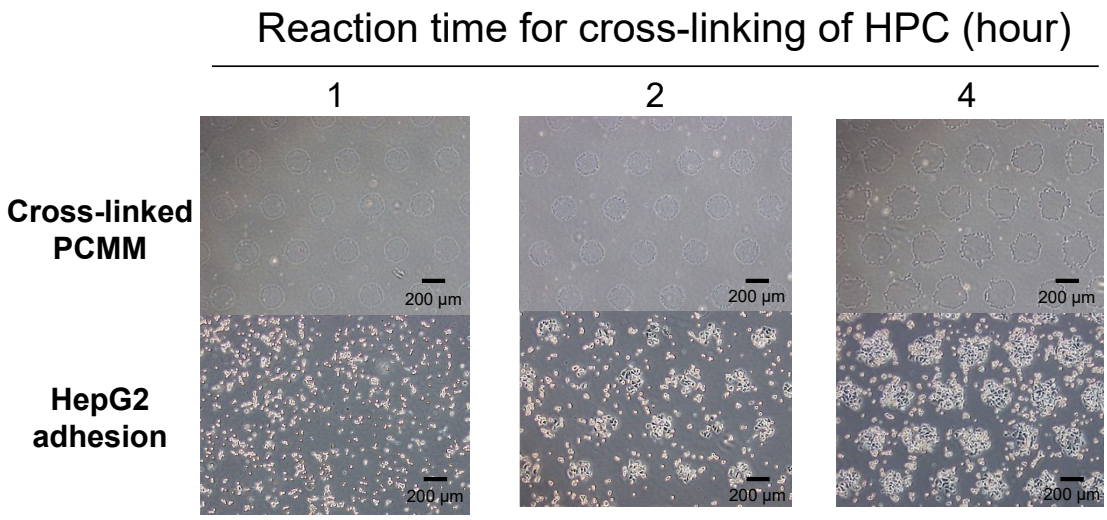

Fig. S1 Immobilization of PCMM and HepG2 cells adhesiveness on cross-linked HPC corresponding to cross-linking density.

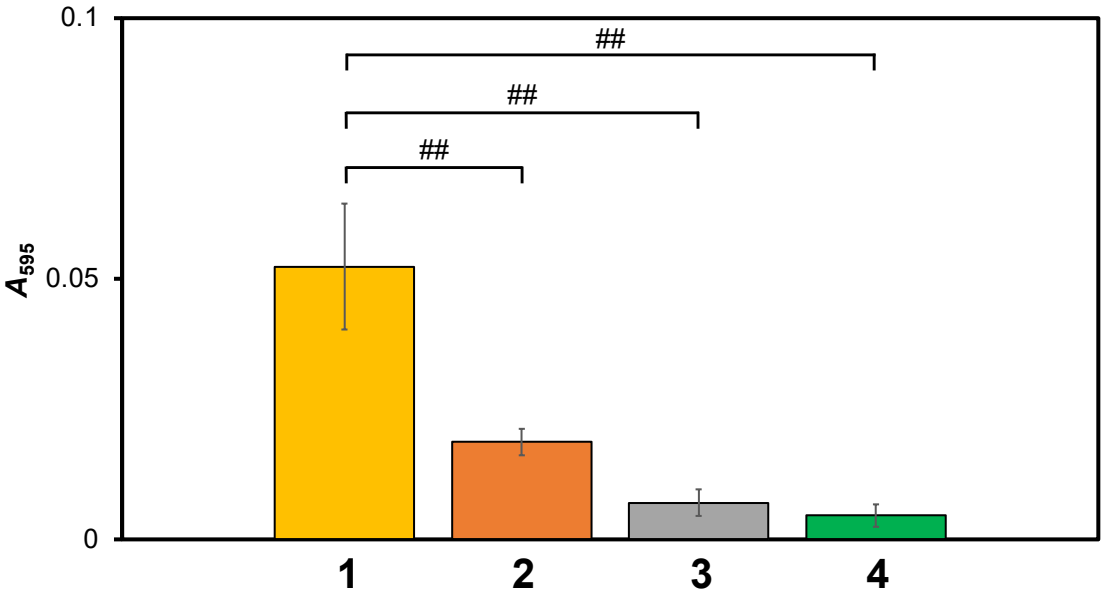

Fig. S2 A_{595} of CBB treated cross-linked HPC in the conditions with or without FBS and PCMM cross-linking. (1: Light+/FBS+, 2: Light+/FBS-, 3: Light-/FBS+, 4: Light-/FBS-, n = 9, ##$p < 0.0001$)

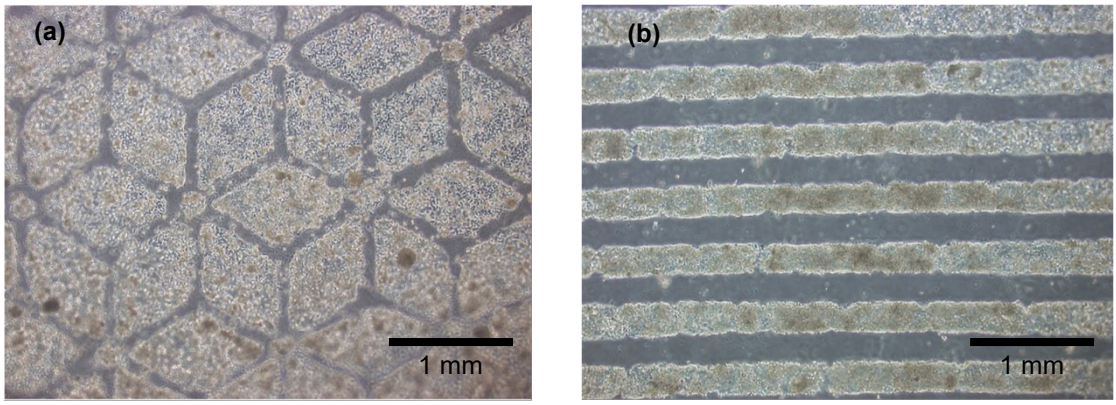

Fig. S3 Several cell patterning. (a) leaves of hemp, (b) strings.

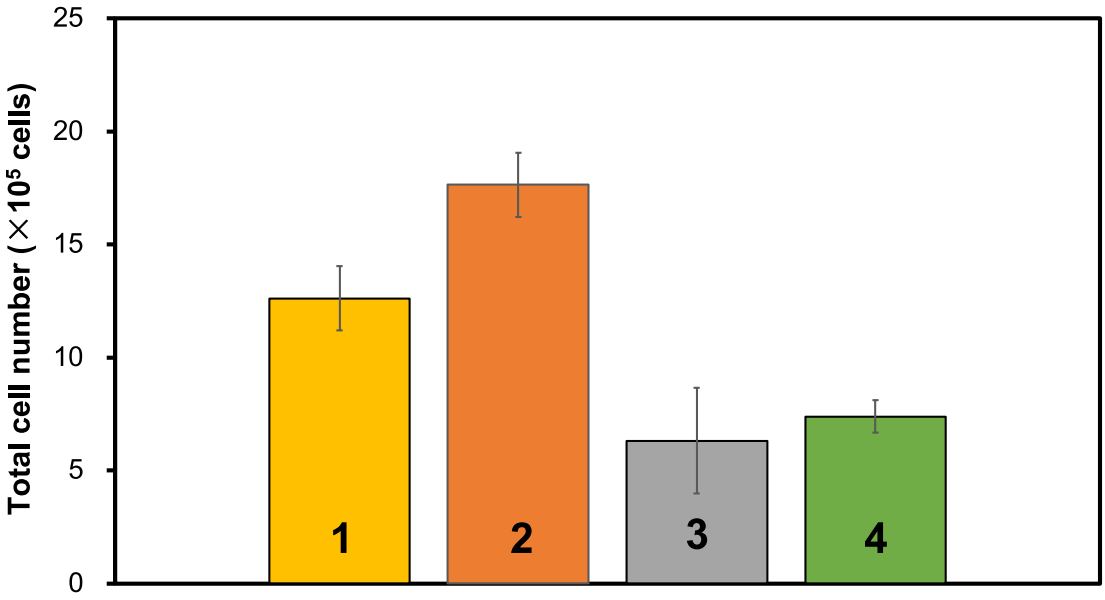

Fig. S4 Total cell number at 15th day. (1: Suspended-spheroid culture, 2: Immobilized-spheroid culture, 3: Suspended-monolayer culture, 4: Immobilized-monolayer culture, n = 4)

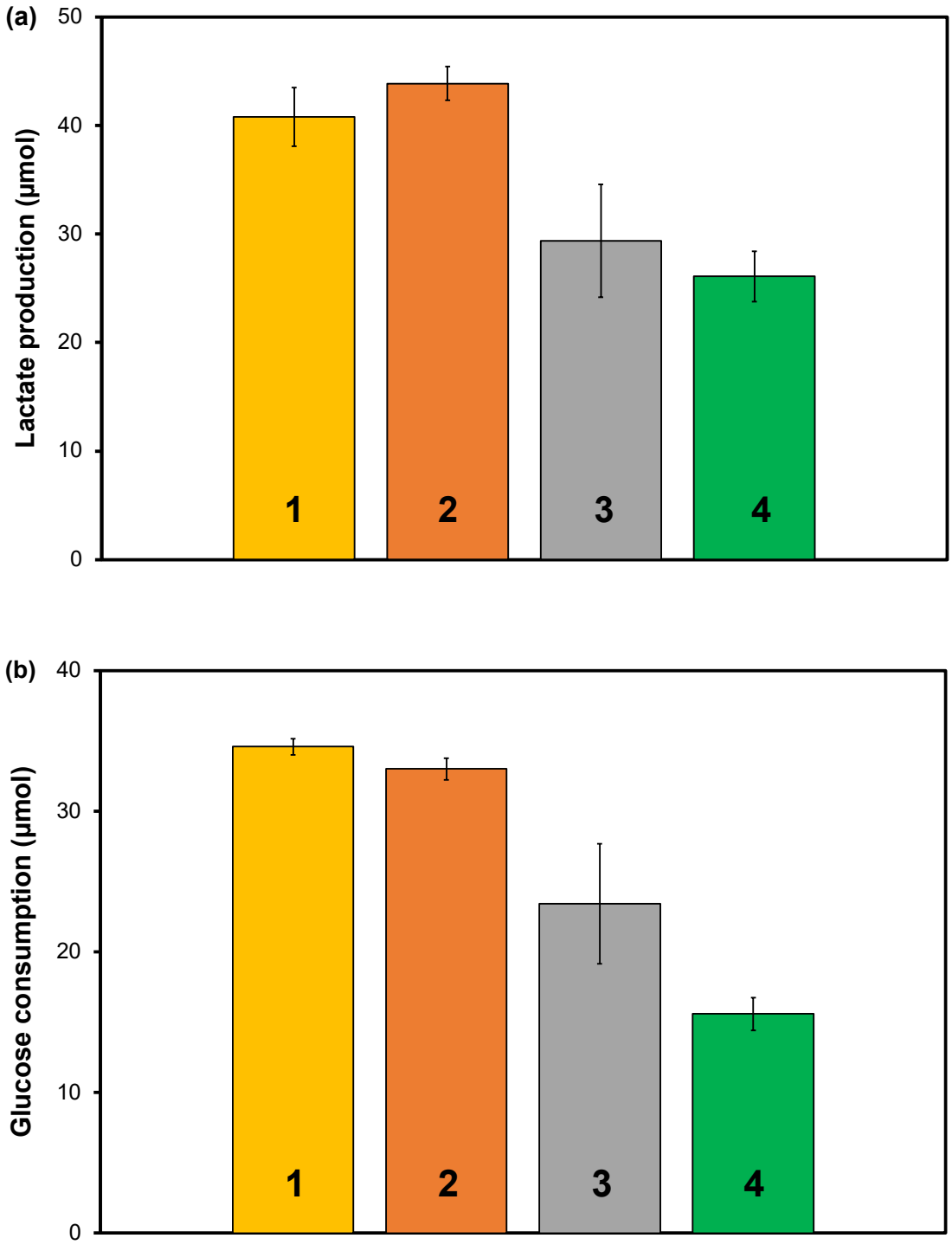

Fig. S5 (a) Lactate production and (b) glucose consumption of the cultured cells. (1: Suspended-spheroid culture, 2: Immobilized-spheroid culture, 3: Suspended-monolayer culture, 4: Immobilized-monolayer culture, n = 4)

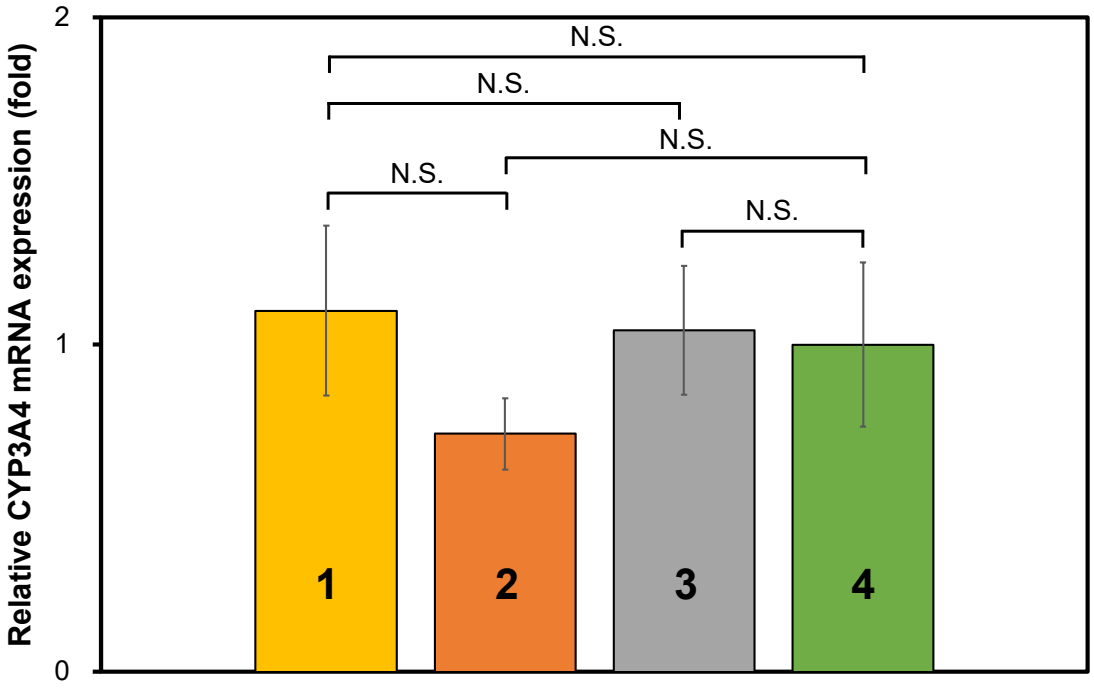

Fig. S6 Relative mRNA expression of CYP3A4, a liver-specific drug-metabolizing enzyme. (1: Suspended-spheroid culture, 2: Immobilized-spheroid culture, 3: Suspended-monolayer culture, 4: Immobilized-monolayer culture, n = 4, N.S. no significant)

Table S1 Detailed conditions for preparation of suspended or immobilized hydrogel membrane and cross-linking of PCMM

	PAAc layer			Cross-linked HPC layer							Cross-linked PCMM layer			
	PAAc concentration (wt%)	Amount of solution (μL)	Rotation speed (rpm)	Concentration of solutes (wt%)				Amount of solution (μL)	Rotation speed (rpm)	Baking time (min)	PCMM concentration (wt%)	Amount of solution (μL)	Rotation speed (rpm)	Irradiation time (s)
				HPC	fHPC	TMMGU	H_2SO_4							
1*	2.0	150	600	3.0		0.31	0.015	100	2,000	60				
2*	1.0	1.5		3.0	0.017	0.15	0.015	100	2,000	120				
3*	3.9	60	2,000	2.9		0.14	0.014	170	2,000	30	0.96	40	2,000	15
4*	3.9	60	2,000	2.9		0.15	0.015	170	2,000	20	0.96	40	2,000	15
5*	2.0	150	600	3.0		0.15	0.015	100	2,000	30	1.0	20	1,000	120
6*				3.0		0.15	0.015	100	2,000	30	1.0	20	1,000	30
7*	4.1	60	2,000	3.0		0.063	0.015	150	2,000	30	0.96	40	2,000	15

*1. Suspended hydrogel membrane corresponding to **Fig. 3(a)**.
*2. Small suspended hydrogel membrane with a few mm diameter in the culture medium (D-MEM) corresponding to **Fig. 3(b)**. PAAc solution was coated by not spin-coating, but casting.
*3. Suspended spheroid array culture system corresponding to **Fig. 5(a)**.
*4. Suspended spheroid array culture system corresponding to **Fig. 5(b)**.
*5. Suspended culture system corresponding to **Fig. 6 (a) 1(Suspended-spheroid) and 3(Suspended-monolayer)**.
*6. Immobilized culture system corresponding to **Fig. 6 (a) 2(Immobilized-spheroid) and 4(Immobilized-spheroid)**.
*7. Suspended culture system corresponding to **Fig. S3(a), (b) and Movie S1**.