Supporting information

Antioxidant self-healing hydrogel for 3D cell culture

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Experimental Section

1. Materials

All chemicals, reagents, and solvents were purchased from commercial sources and used without further purification. 2-(Acetoacetoxy) ethyl methacrylate (AEMA, Aladdin, 95%), 4-formylphenylboronic acid (FPBA, Heowns, 98%), thiourea (Aladdin, 98%), urea (Aladdin, 98%), magnesium chloride ($MgCl_2$, 99%, J&K chemical), ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl, 99%, J&K chemical), acetic acid (AcOH, Aladdin, 98%), 4-dimethylaminopyridine (DMAP, 98%, J&K chemical), 2-hydroxyethyl methacrylate (97%, J&K chemical), 4-carboxyphenylboronic acid (Aladdin, 98%), 2,2'-azobis(2,4-dimethyl)valeronitrile (ABVN, 98%, Energy), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$, 97%, J&K chemical), Roswell Park Memorial Institute-1640

(RPMI-1640) culture medium (Corning-Cellgro), phosphate buffered saline (PBS, pH~7.2-7.4, 0.01 M, Solarbio), fetal bovine serum (FBS, Gibco), penicillin-streptomycin solution (Gibco), trypsin-EDTA (Gibco, 0.25%), fluorescein diacetate (FDA, Sigma), propidium iodide (PI, 94%, Sigma) were used as purchased. 2,2'-Azobis(2-methylpropionitrile) (AIBN, J&K, 99.9%) was recrystallized from acetone twice prior to use.

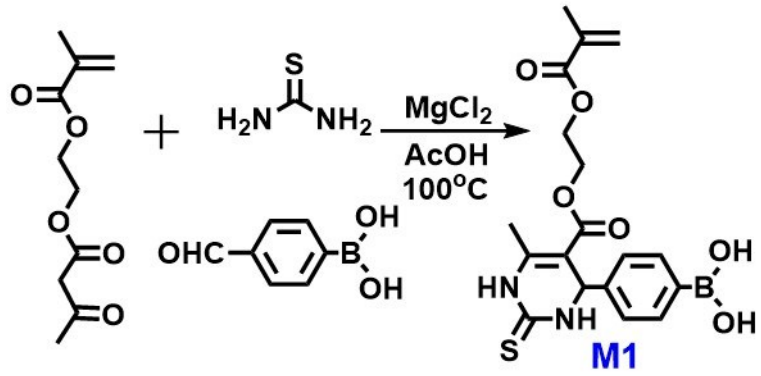
2. Instrumental Analysis

Gel permeation chromatography (GPC) analyses of polymers were performed using N,N-dimethyl formamide (DMF) containing 50 mM LiBr as an eluent. The GPC system was a Shimadzu LC-20AD pump system consisting of an auto injector, a MZ-Gel SDplus 10.0 μm guard column (50 × 8.0 mm, 10^2 Å) followed by a MZ-Gel SDplus 5.0 μm bead-size column (50 – 10^6 Å, linear), a Shimadzu RID-20A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 500 to 10^6 g mol^{-1}. ^{1}H NMR and ^{13}C NMR spectra were obtained using a JEOL JNM-ECA400 (400M Hz) spectrometer for all samples. The ESI-MS data were collected using a Micro TOF-QII Bruker. The FT-IR spectra were made in a transmission mode on a Perkin-Elmer Spectrum 100 spectrometer (Waltham, MA, USA). Rheology analyses were performed on a TA-AR G2 rheometer with parallel plate geometry (20 mm in diameter) at 25℃. SEM photos were taken from HITACHI SU-8010. The absorbance of $ABTS^{+\bullet}$ (734 nm) was collected using a Thermo Scientific™ Multiskan™ FC Microplate reader. CCK-8 analysis was tested by VICTOR™ X3 PerkinElmer 2030 Multilabel Plate Reader. Confocal microscopy

images are performed on a Zeiss710-3channel confocal microscope.

3. Methods

3.1. Synthesis of M1



In a 250 mL round-bottom flask, AEMA (32.1 g, 150 mmol), 4-formylphenylboronic acid (22.5 g, 150 mmol), thiourea (17.1 g, 225 mmol) and $MgCl_2$ (2.85 g, 30 mmol) were mixed in 75 mL of acetic acid and kept in a 100°C oil bath for 4 h. The mixture was precipitated in water for 3 times, then precipitated in diethyl ether for 3 times, and dried under vacuum to obtain the pure M1 (54.6 g, yield ~90%) for further polymerization.

^{1}H NMR (400 MHz, DMSO-d6) δ 10.39 (s, 1H), 9.68 (s, 1H), 8.01 (s, 2H), 7.70 (d, J = 8.0 Hz, 2H), 7.15 (d, J = 8.0 Hz, 2H), 5.94 (s, 1H), 5.75 – 5.56 (m, 1H), 5.15 (d, J = 3.6 Hz, 1H), 4.26 – 4.20 (m, 4H), 2.28 (s, 3H), 1.82 (s, 3H).

^{13}C NMR (101 MHz, DMSO-D6) δ 174.80, 166.83, 165.44, 146.28, 143.89, 136.06, 129.07, 128.21, 126.79, 126.62, 100.80, 62.98, 62.09, 54.43, 18.45, 17.75.

FT-IR cm^{-1}: 3182, 1720, 1701, 1572, 1413, 1355, 1177, 1134, 1096, 772, 653.

ESI-MS (m/z): [M+H^+] 405.1118 (theoretical value: 405.1115).

M2 was prepared by using urea instead of thiourea through the same procedure.

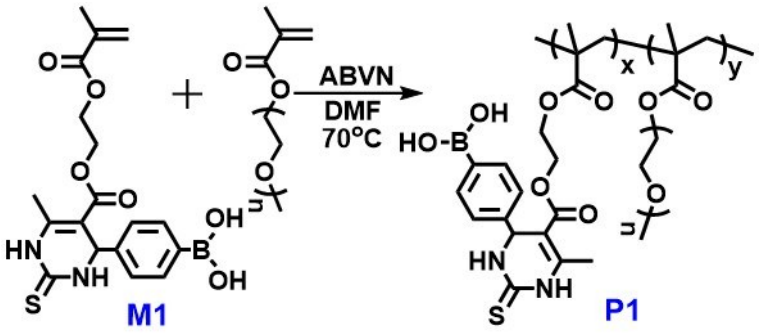
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.25 (d, J = 1.6 Hz, 1H), 7.98 (s, 2H), 7.75 (dd, J = 3.2, 2.1 Hz, 1H), 7.69 (d, J = 8.1Hz, 2H), 7.17 (d, J = 8.1 Hz, 2H), 5.95 (m, 1H), 5.65 (m, 1H), 5.12 (d, J = 3.3 Hz, 1H), 4.22 (dd, J = 8.2, 4.4 Hz, 4H), 2.24 (s, 2H), 1.83 (s, 3H).

^{13}C NMR (101 MHz, DMSO-D_6) δ 166.31, 165.09, 152.12, 149.09, 146.39, 135.53, 134.22, 126.02, 125.12, 98.79, 62.51, 61.21, 53.83, 17.91, 17.84.

FT-IR cm^{-1}: 3256, 3382, 1683, 1632, 1448, 1374, 1343, 1219, 1090, 758, 659.

ESI-MS (m/z): [M+H^{+}] 389.1421 (theoretical value: 389.1426).

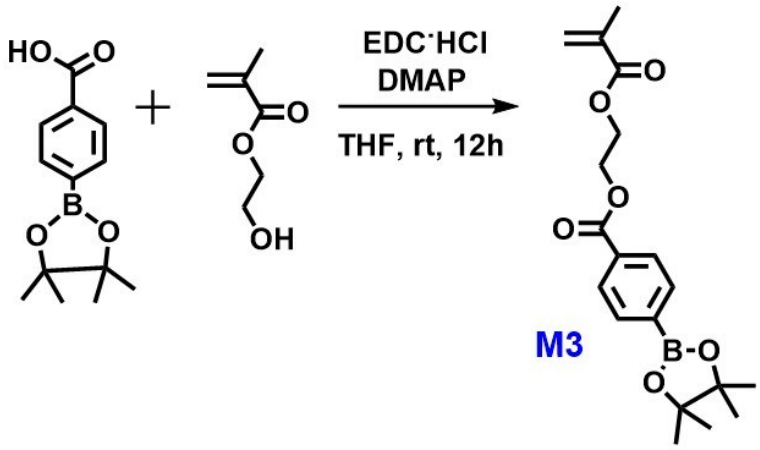
3.2. Preparation of P1



In a 250 mL round-bottom flask, M1 (20.2 g, 50 mmol), PEGMA (47.5 g, 50 mmol) and ABVN (0.25 g, 1mmol) were mixed in 50 mL DMF. The flask was sealed with a rubber septum and purged by nitrogen flow for 30 min, and kept in a 70°C oil bath for 12 h, sample was taken for the ^{1}H NMR and the conversion of polymerization was calculated as ~95%. The mixture was precipitated in diethyl ether for 3 times, and dried under vacuum to obtain the pure polymer (63.4 g, yield ~94%).

P2 was similarly prepared using M2.

3.3. Synthesis of a PBA-ester Monomer (M3)



In a 50 mL round-bottom flask, 2-hydroxyethyl methacrylate (1.3 g, 10 mmol), 4-carboxyphenylboronic acid pinacol ester (2.48 g, 10 mmol), EDC·HCl (3.8 g, 20 mmol) and DMAP (0.05 g, 0.4 mmol) were mixed in 40 mL of THF and kept at 25°C for 12 h. Then, the mixture was added H_2O (30 mL), and extracted with ethyl acetate (50 mL) for three times. The organic layer was separated and dried by $MgSO_4$. After removing the solvent by vacuum, the product was purified by a flash chromatography (ethyl acetate/petroleum ether: 1/4) as a pale yellow solid (3.0 g, 83%).

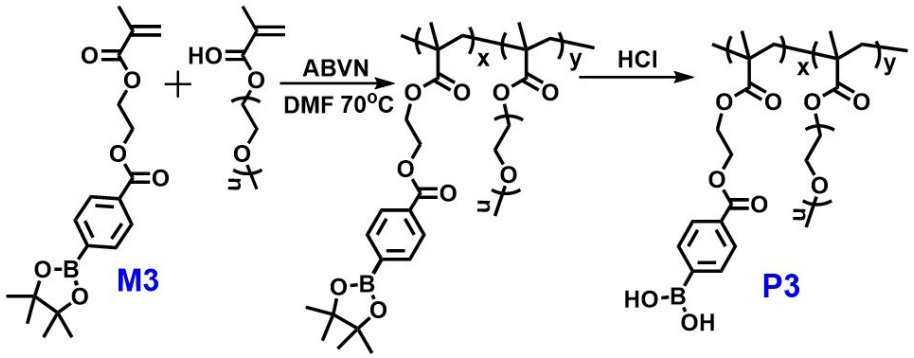
^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (d, J = 8.2 Hz, 2H), 7.81 (d, J = 8.2 Hz, 2H), 6.07 – 5.97 (t, J = 1.6 Hz, 1H), 5.68 (t, J = 1.6 Hz, 1H), 4.62 – 4.50 (m, 2H), 4.49 – 4.41 (m, 2H), 1.85 (s, 3H), 1.31 (s, 12H).

^{13}C NMR (101 MHz, DMSO-D_6) δ 166.39, 165.48, 135.61, 134.64, 131.79, 128.43, 126.11, 84.12, 62.78, 62.32, 24.66, 17.92.

FT-IR cm^{-1}: 2987, 1719, 1399, 1354, 1318, 1274, 1173, 1110, 852, 810, 709, 643.

ESI-MS (m/z): [M+H$^+$] 361.1721 (theoretical value: 361.1717).

3.4. Preparation of the P3



In a 25 mL Schleck tube, monomer M3 (1.8 g, 5 mmol), PEGMA (4.75 g, 5 mmol) and ABVN (0.025 g, 1mmol) were mixed in 5 mL DMF. The tube was sealed with a rubber septum and purged by nitrogen flow for 30 min, and kept in a 70°C oil bath for 12 h, sample was taken for the ^{1}H NMR and the conversion of polymerization was calculated as 98%. The mixture was precipitated in diethyl ether for 3 times, and dried under vacuum to obtain a P3-precursor copolymer (5.7 g, yield ~87%). Then, the P3-precursor polymer (3.0 g) was dissolved in 2M HCl (20 mL). The mixture was kept at 25°C for 2h, then dialyzed against H_2O and MeOH (MWCO: 3500) to get P3 quantitatively.

3.5. Antioxidative test of P1, P2 and P3

$ABTS^{+\bullet}$ solution was prepared by mixing aqueous solutions of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 3.84 mg/mL, 10 mL) and potassium persulfate (0.66 mg/mL, 10 mL), and kept overnight.

$ABTS^{+\bullet}$ solution was diluted by PBS solution to the absorbance of ~0.70, then added into a 96-well plate (~100 μL) followed by adding a P1 PBS solution (20 μL, 8 wt%). The absorbance was measured by a microplate reader at 734 nm. P2 and P3 were tested similarly. PBS solution was used as a blank solution. The results were presented as mean ± standard deviation (SD), n = 3.

3.6. Hydrogel preparation

The solution of PVA in PBS (7 wt%, pH ~ 7.4) was prepared by dissolving PVA powders (2.8 g) in PBS solution (37.2 g, pH ~ 7.4). A series of P1 solutions in PBS solution (6 wt%, 7 wt%, and 8 wt%) were prepared by dissolving P1 (0.18 g, 0.21 g, 0.24 g) in PBS solution (2.82 g, 2.79 g, and 2.76 g, pH ~ 7.4) respectively. As a typical example, Gel-6/7 was prepared by mixing equal volume solutions of P1 (6 wt%) and PVA (7 wt%), and a hydrogel was quickly formed in ~ 100 s. Gel-7/7 and Gel-8/7 were prepared similarly.

Other hydrogels (prepared by P2, P3) were prepared similarly.

3.7. Rheology analysis

Typically, a piece of hydrogel (~ 0.6 mL, ~ 2.0 cm diameters) was put on the measuring plate of a rheometer. The storage modulus (G') and loss modulus (G") of the hydrogel were recorded under frequency sweep step (strain = 1%).

All hydrogels were tested through the same process.

3.8. Self-healing test of the hydrogel

Typically, Gel-8/7 was prepared in a syringe, then injected into a glass bottle. Photos were taken to monitor the morphology changes of Gel-8/7 after injection.

Rheology analyses were further carried out to test the shear-thinning property of the hydrogels. Typically, Gel8/7 was prepared as described. The moduli of Gel8/7 under different strains were tested (frequency = 1 Hz). Then, alternative changed strains (1%, 400%) under the same frequency (1.0 Hz) were applied to Gel8/7. G' and G" values were recorded. The shear-thinning properties of Gel-8/8 and Gel-8/10 were

tested similarly.

3.9. Antioxidant ability of the P1-PVA hydrogels

A PBS solution of PVA (10 wt%, 400 μL, PH ~ 7.4) was added an $ABTS^{+\bullet}$ solution (absorbance ~3, 172 μL) to prepared PVA-$ABTS^{+\bullet}$ solution (7 wt%, absorbance ~3, pH ~ 7.4). Then P1 solution in PBS (572 μL, 8 wt%, pH~7.4) was added to get a P1-PVA-$ABTS^{+\bullet}$ hydrogel. Photos was taken at different time to monitor the colour change of $ABTS^{+\bullet}$ in hydrogel.

The absorbance of the P1-PVA- $ABTS^{+\bullet}$ hydrogel was measured in a microplate reader (734 nm). The results were presented as mean ± standard deviation (SD), n = 3.

P2-PVA hydrogels were prepared and tested similarly.

3.10. Cell culture

L929 cells were cultured in RPMI1640 medium (supplemented with 10% FBS, 1% penicillin and streptomycin) and incubated at 37°C, 5% CO_2. The medium was changed every two days to maintain the exponential growth of the cells. The cells were harvested with 0.025 (w/v)% trypsin and 0.01% EDTA, centrifuged and resuspended in the RPMI-1640 medium.

3.11. Cytotoxicity evaluation

The cytotoxicity to L929 cells was evaluated by a cell count kit-8 (CCK-8) assay.

L929 cells were seeded in 96-well microplates at a density of 1×10^5 cells/mL in 100 μL of respective well. After cell attachment for 24 h, the cells were incubated in 100 μL medium containing 1, 2, 4, 8, 16 mg/mL of P1 for 24 h. Then P1 was removed

and cells were washed with PBS for three times. Then the cells were incubated in 100 μL of culture medium containing 10% CCK-8 solution at 37℃ for 2 h. Plates were then analysed with a microplate reader (VICTOR^{TM} X3 PerkinElmer 2030 Multilabel Plate Reader). Absorbance was measured at 450 nm. The values were proportional to the number of live cells. The results were presented as mean ± standard deviation (SD), n = 5.

P2, P3 and PVA were also assayed using the same method.

3.12. 3D cell culture

L929 cells (1×10^6 cells/mL) were resuspended in a culture medium containing P1 (8 wt%). The L929/P1 medium (500 μL) was pipetted into a petri-dish, then PVA in medium (7 wt%, 500 μL) was pipetted into the same dish. The dish was gently shaken to form Gel8/7-L929 evenly. The gels were incubated at 37°C, 5% CO_2, and imaged either after 24 h or 48 h. To observe the survival of the cells inside, PBS-FDA-PI solution (FDA: 5 μg/mL; PI: 5 μg/mL, 1 mL) was added to the surface of Gel8/7-L929. After stored in the dark for 10 minutes, the cells were monitor by a confocal microscopy. The live and dead cells were observed through 450-490 nm and 515-560 nm band-pass excitation filters, respectively. The z-stacks were taken through the depth of the gels to detect the distribution of cells throughout. Viability is reflected by the percentage of FDA stained cells to total cells.

Gel8/7-L929 prepared as above was put in a syringe. Then, this cell-containing hydrogel was injected into a petri-dish through a 21G needle. The injected Gel8/7-L929 was incubated and monitored using the same method.

Supporting data

Table S1. Polymer parameters.

Name	M_n (GPC)[a]	PDI[a]
P1	126700	4.24
P2	72600	2.67
P3	55900	2.61

[a] Measured by gel permeation chromatography (GPC) using N, N-dimethylformamide (DMF) as eluent (1 mL/min).

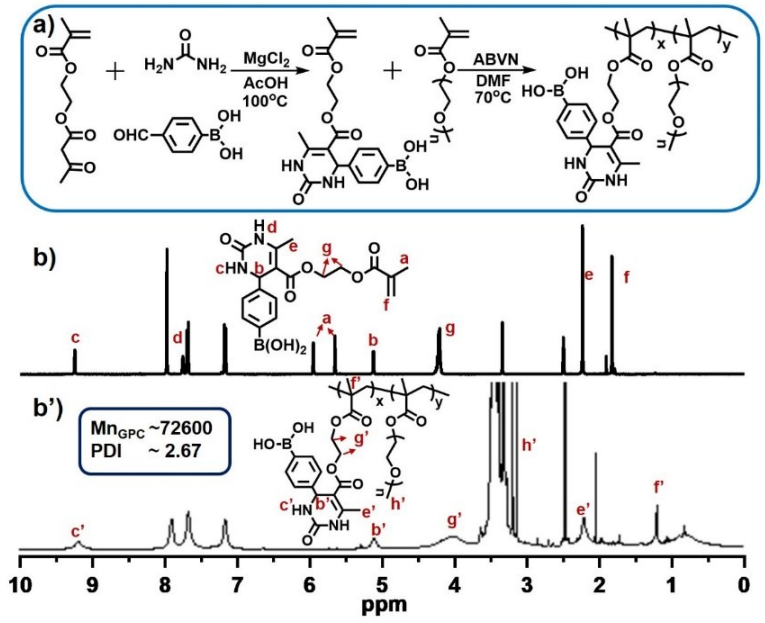


Figure S1. a) Preparation of M2 and P2.; b, c) ^{1}H NMR spectra (DMSO-d_6, 400 MHz) of M2 and P2 (b: M2; c: P2).

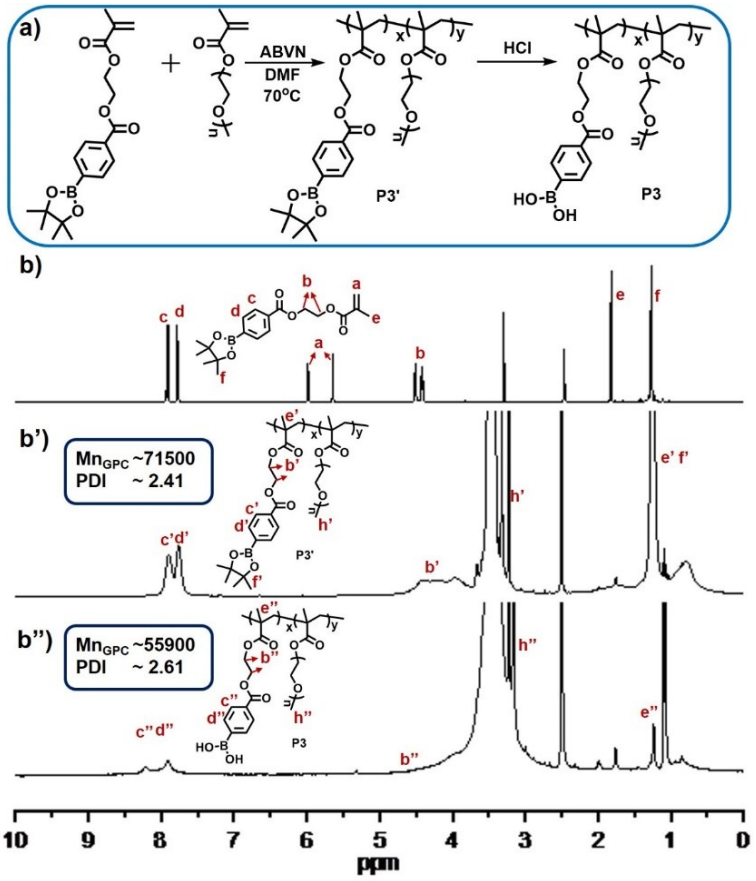


Figure S2. a) Preparation of M3 and P3; b, b', b'') ^{1}H NMR spectra (DMSO-d_6, 400 MHz) of M3, P3-precursor and P3 (b: M3; b': P3-precursor; b": P3).

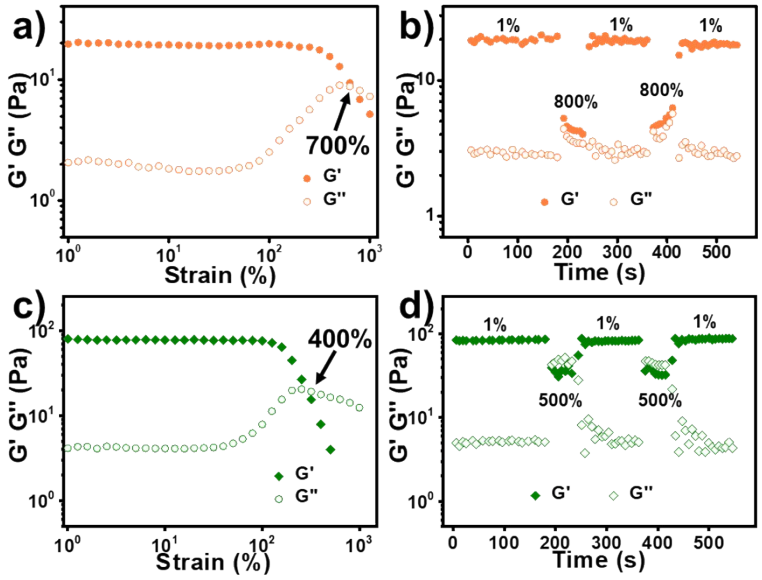


Figure S3. a) The storage modulus G' and loss modulus G'' of Gel-8/6 vs. different strains; b) G' and G'' of Gel-8/6 at alternating stains (1% and 800%); c) The storage modulus G' and loss modulus G'' of Gel-8/7 vs. different strains; d) G' and G'' of Gel-8/7 at alternating stains (1% and 500%).

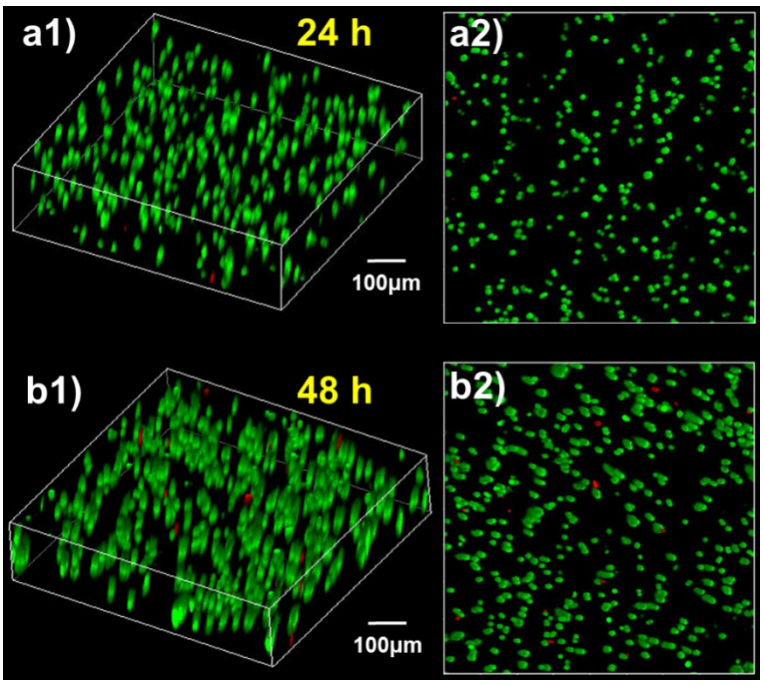


Figure S4. a, b) Fluorescein diacetate/propidium iodide (FDA/PI) double staining of cells in Gel-8/7 after injection (a: 24 h, b: 48 h); a2, b2: cell observations from the top of Gel-8/7.