Electronic Supplementary Information

Surface patterned hydrogel Film as flexible scaffold for 2D and 3D cell co-culture

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1. Experimental Section

Materials

Glycol chitosan (GC), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and acridine orange (AO) were obtained from Sigma-Aldrich Trading Company. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was bought from Beyotime (Shanghai, China). Cyclic (Arg-Gly-Asp-D-Phe-Lys) (RGD) was bought from China Peptides Co., Ltd. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Other compounds were obtained from J&K Chemical Ltd. (Beijing, China). Dual benzaldehyde capped poly(ethylene oxide) (OHC-PEO-CHO, MW = 2k Da) was synthesized according to literature.[1]

Synthesis of magnetic silica rods[2]

1) Synthesis of sulfonated polydivinylbenzene (PVDB) nanotubes. Polydivinylbenzene (PVDB) nanotubes were synthesized by according to the procedure reported in our previous report.[3] At room temperature e.g. 25 °C, a given amount (for example 150 mg) of BFEE (boron trifluoride diethyl etherate complex $BF_3{\cdot}O(Et)_2$) was immediately added into 150 ml of cyclohexane containing 4 wt% of monomer DVB under stirring to initiate the cationic polymerization. In order to monitor growth of the nanotubes at different stage, 10 g of ethanol was added to terminate the polymerization. The samples were filtered and washed with ethanol to remove residual initiator and monomer. After residual ethanol was evaporated, the nanotube powder was obtained. Then, 1 g of the PVDB powder was immersed in concentrated sulfuric acid (30 g) under stirring at 40 °C for desired time, e.g. 12 h. After the products were added slowly to a mixture of ice and water. After filtration, sulfonated PVDB nanotubes were obtained.

2) Synthesis of ferric oxide-hydroxides (β-FeOOH) composite PVDB nanotubes. The sulfonated PVDB in form of powder (0.1 g) was dispersed in 100 mL of water under ultrasonication at 25 °C for 15 min, followed by the addition of 0.6 g of $FeCl_3$. After stirring for another 10 min, 0.4 g of urea was added within a period of 10 min. The mixture was then heated at 85 °C for 6 h. The dispersion was filtered and the precipitate was washed with ethanol and water for several times. β-FeOOH composite tubes were then gained after lyophilization.

3) Synthesis of β-FeOOH@SiO_2 nanotubes. 0.1 g of β-FeOOH composite nanotubes, 81 mL of ethanol, 13.5 mL of water and 1.5 mL of ammonia were mixed by ultrasonication at 25 °C for 15 min. To this 100 μL of tetraethoxysilane (TEOS) was added, and the mixture was stirred at room temperature for 6 h. The products were harvested by filtration and were washed with ethanol and water.

4) Synthesis of Fe_2O_3@SiO_2 magnetic rods. The β-FeOOH@SiO_2 composite nanotubes were calcinated at 500 °C under nitrogen atmosphere for 3 h, and then calcinated at 500 °C under air atmosphere. Reddish brown product was obtained, in which the PDVB component was removed and the β-FeOOH was transformed to Fe_2O_3 particles. In addition, the resultant morphology of the residue magnetic silica materials changed from tubular to rod-shaped architecture due to the loss of the ductile polymer template. The average length and diameter of the rods are 1.5 μm and 150 nm respectively (SEM).

5) Synthesis of amine-modified magnetic silica rods. 20 mg of magnetic silica rods were dispersed in 10 mL of ethanol, followed by the addition of 20 μL of aminopropyltrimethoxysilane (APTMS). The dispersion was stirred for 12 h at 78 °C before the product was separated by magnet and washed with water for three times.

6) RGD functionalization of the magnetic silica rods. RGD (50 mg), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (125 mg) and N-hydroxysuccinimide (NHS) (150 mg) were dissolved in dimethyl sulphoxide (DMSO) and stirred for 1 h. Then the amine-modified magnetic silica -rods (20 mg) were added under stirring at 37 °C and stirred for 72 h. The functionalized nano-rods were collected via centrifugation (10000 rpm, 5 min) and washed with water for three times. The obtained solid was further freeze-dried for 24 h to remove residue water and stored at 4 °C for further use. The level of RGD grafting was checked using thermogravimetric analysis (TGA) which is 8 % to the total weight of the sample.

Cell culture

Mouse fibroblast cell L929 was a kind gift from School of Stomatology, Peking University and maintained in RPMI 1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine, antibiotics, penicillin and streptomycin at 37 °C, with 5% CO_2 in a 95% humidity atmosphere and subcultured every 24 h. Osteoblastic cell MC3T3-E1 was obtained from Cell Resource Center, IBMS, CAMS/PUMC. It was cultured at 37 °C, with 5% CO_2 in a 95% humidity atmosphere with the α-MEM medium (Cell Resource Center, IBMS, CAMS/PUMC) with 10% FBS. The subculture time is 72 h. The cells were cultured in 100 mm culture dish and harvested with 0.25% trypsin (Gibco, USA) before use.

Preparing dual cell loaded hydrogel film

The chemicals, i.e. GC, OHC-PEO-CHO, poly(acrylic acid) (PAA) and silica rods were sterilized under ultraviolet light for 1 h before the fabrication. GC was dispersed with MC3T3-E1 cells in culture medium and mixed by OHC-PEO-CHO medium solution, and the final concentration of GC and OHC-PEO-CHO were 1 % and 0.5 % (w/v) respectively. Typical the cell density is 2.5×10^5 cell/mL. The mixture of polymer solutions was stirred and poured into a mould to allow the gelation occurring within a few minutes. Silica rods

dispersion was prepared by dispersing in culture medium at desired concentration. 500 μL of the dispersion was dropped on the gel surface while magnetic field was applied to align the rods before the liquid phase was completely absorbed by the hydrogel substrate, which normally occurred within 4 h. In this procedure, 1 mL of PAA in culture media (100 μg/mL) was also added to the hydrogel surface for bridging the rods and the hydrogel via electrostatic interaction. The gelation and patterning process were all done under a condition of 37 °C, 5% CO_2 and a 95% humidity atmosphere. Following that, L929 cells, dispersed in culture medium, were pipetted onto the modified hydrogel surface at a typical level of 1×10^4 cell/cm^2. After 0.5 h, the culture dish was changed with fresh medium for incubation (37 °C, 5% CO_2 and 95% humidity atmosphere) and characterizations.

Characterizations

Transmission electron microscopy (TEM) was carried out on a JEM-1011 microscope at an operating voltage of 100 kV. The silica rods were dissolved in ethanol and ultrasounded for 5 min. Then the solution was dropped onto copper grids and then air dried. Scanning electron microscopy (SEM) images was taken on Hitachi S-4800 at 15 kV on freeze-dried composite hydrogel sample. TGA was taken by a PerkinElmer Pyris 1 instrument at a heating rate of 10 °C/min from room temperature (30 °C) to 700 °C in nitrogen. FTIR spectroscopy was performed using a Bruker Equinox 55 spectrometer with the sample pressed into KBr pellets. Optical micrograph images were performed using Olympus TH4-200 optical microscope. The mechanical property of the composite films was evaluated by compressive stress-strain measurements which were performed using INSTRON 3365 equipped with a 100 N load cell at a crosshead speed of 1 mm/min. Cylinder shaped hydrogel films with 10 mm in diameter and 8 mm in thickness were prepared in mould and used for the measurement.

AO/PI stain and CLSM observation

At desired incubation duration, the cell loaded hydrogel films were cut into pieces and stained by 1 mL 4',6-diamidino-2-phenylindole (DAPI, 5 μg/mL), acridine orange (AO, 2 μg/mL) and propidium iodide (PI, 3 μg/mL) for 20 min, followed by washing with PBS for three times. It was noted that the washing procedure caused very limited detachment of cells during the entire process. The samples were transferred to CLSM observation which was done using a FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan), with an excitation wavelength of 405 nm, 488 nm and 559 nm for DAPI, AO and PI, respectively. The emission of DAPI is 425-475 nm, while AO emits green colour, i.e. 500-580 nm, from living cells and PI emits red colour, i.e. 600-650 nm, from dead cells.[4]

Cytotoxicity

Cytotoxicity was accessed by CCK-8 assay. L929 cells were seeded in 96-well plates at a density of 1×10^4 cell/well. After 24 h incubation, the culture media was removed by silica rod dispersions at different concentration (50 - 1600 μg/mL), and the plates were incubated at 37 °C for 24 h. Then 100 μL of freshly prepared medium with 10% CCK-8

reagent solution was added to each well and incubated for 1 h before it was measured at the absorbance of 450 nm using a microplate reader (Thermo MULTISKAN MK3).

Proliferation measurement

Dual cell loaded hydrogel films were put in 24-well plates and incubated for predesigned time intervals. The initial cell density of MC3T3-E1 and L929 was 2.5×10^5 cell/mL and 1×10^4 cell/cm^2. Cell proliferation was measured by CCK-8 assay following the procedure given in the Technical Manual of the kit, which was similar to that described in the above section. For the reading, the optical density (OD) value for the number of seeded cells was set as 100%. The proliferation was then defined as the percentage of OD at the desired incubation time over that recorded from the amount of seeded cells. For each sample, the measurement was conducted in triplicates.

Handling property of the hydrogel film

After the hydrogel films were prepared, with L929 on the patterned surface, and incubated in culture medium in a 24-well plate for 4 h (37 °C, 5% CO_2 and 95% humidity atmosphere). The culture medium was then pipetted out of wells to let the hydrogel film to expose to air for up to 4 h. A control was also prepared on adherent cells seeded on the well-plate directly. The plates were put on a clean bench, protecting from light, at room temperature (25 °C) and 30% of humidity. The viability was then accessed by CCK-8 assay and compared with that treated by normal procedure.

References

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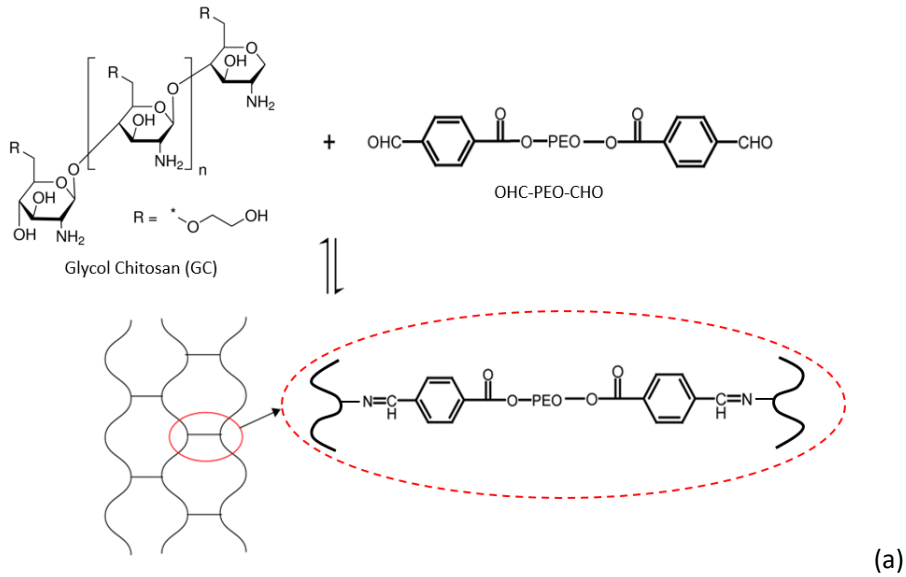
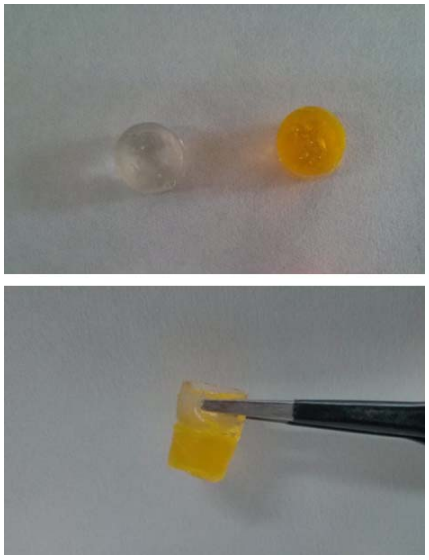
2. Y. Chen, Z. Liu, X. Qu, F. Liang, Z. Yang, Chem. Asian J. 2016, DOI:10.1002/asia.201600435.
3. W. Ni, F. Liang, J. Liu, X. Qu, Z. Yang, Chem. Commun., 2011, 47, 4727.
4. T. Kanzawa, Y. Kondo, H. Ito, S. Kondo, I. Germano, Cancer Res., 2003, 63, 2103.

2. Supplementary scheme and figures

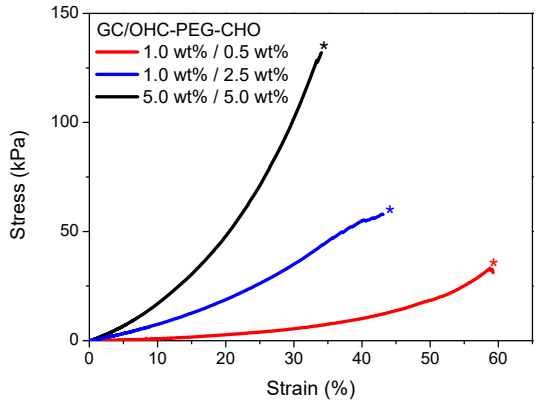


(a)

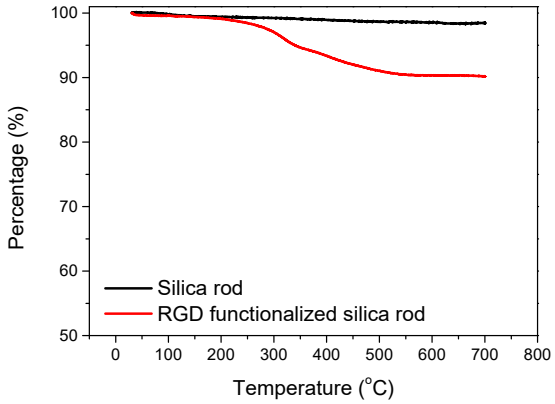


(b)

Scheme S1. (a) Mechanism of in situ gelation of GC and OHC-PEO-CHO. (b) A demonstration of the self-healing behavior of the GC/OHC-PEO-CHO hydrogel.



(a)



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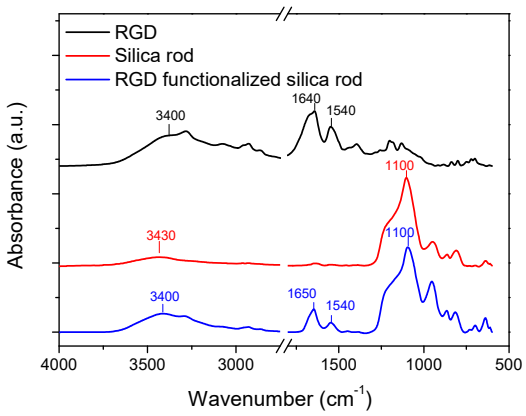

(c)

Figure S1. (a) Compressive stress-stain curves of GC/OHC-PEO-CHO hydrogels with different chemical composition. * Failure point. (b) Thermogravimetric analysis and (c) FTIR spectra of the RGD functionalized silica rods.

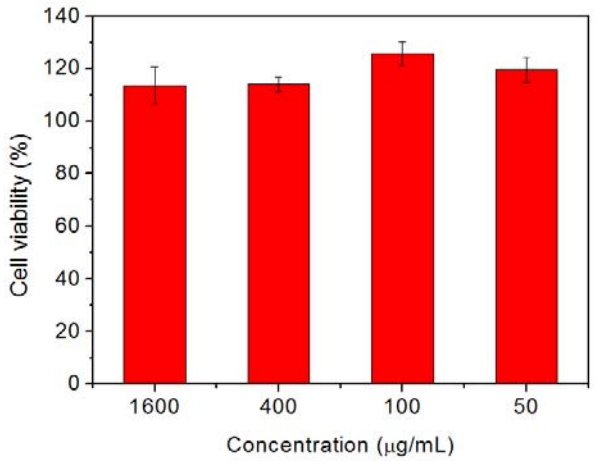

Figure S2. Cytotoxicity of the silica rods measured on L929 cell line for a 24 h incubation.

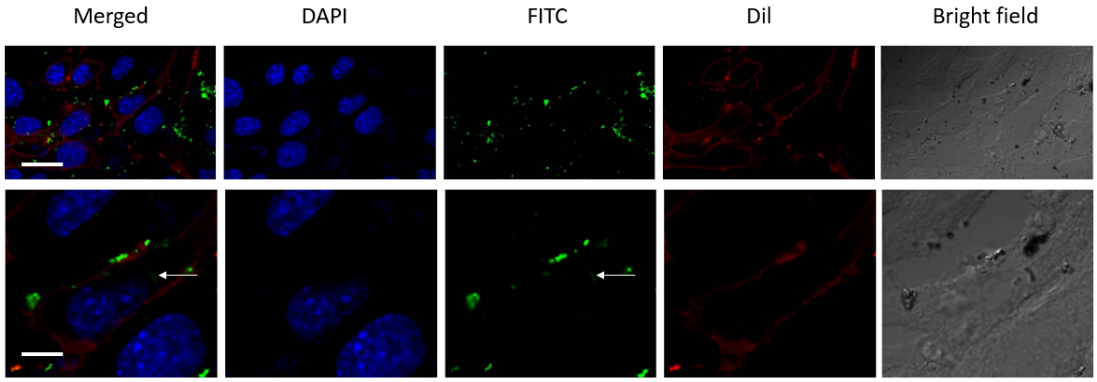

Figure S3. Distribution of the silica rods after incubation with L929 cells. The cells were seeded in microscope slides in a culture dish and cultured in 1640 medium. After 24 h, the media was removed by a dispersion of FITC labeled rods at a concentration of 100 μg/mL. The cells were incubated for another 24 h. Subsequently, the cells were washed by PBS for three times and fixed by 4% formaldehyde for 20 min. Then, Dil solution (2 μg/mL) was used to stain the membrane of the cells for 20 min following the stain of nuclear using DAPI (5 μg/mL). After a further wash with PBS for three times, images were then taken using a confocal laser scanning microscopy (Olympus, Japan). The arrows indicate the FITC fluorescence in the cytoplasm of cell. Scale bar: 20 μm (top row), 10 μm (bottom row). The magnitude is the same for each row.

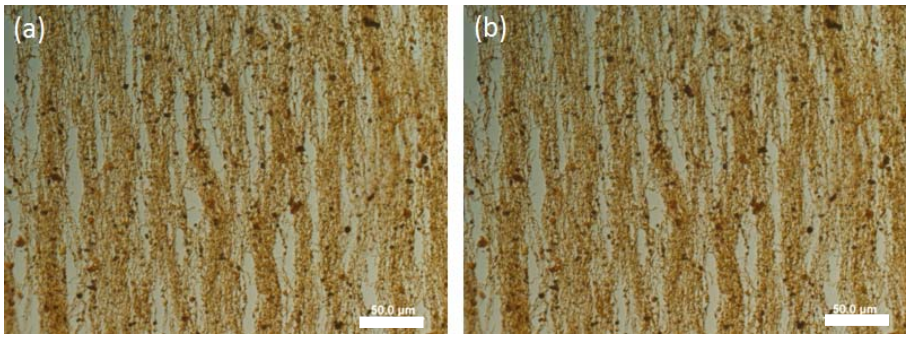

Figure S4. Firmness of the adhered silica rods on the GC/OHC-PEO-CHO hydrogel surface monitored by optical microscope before (a) and after (b) the surface was washed by PBS for three times. Scale bar: 50 μm.

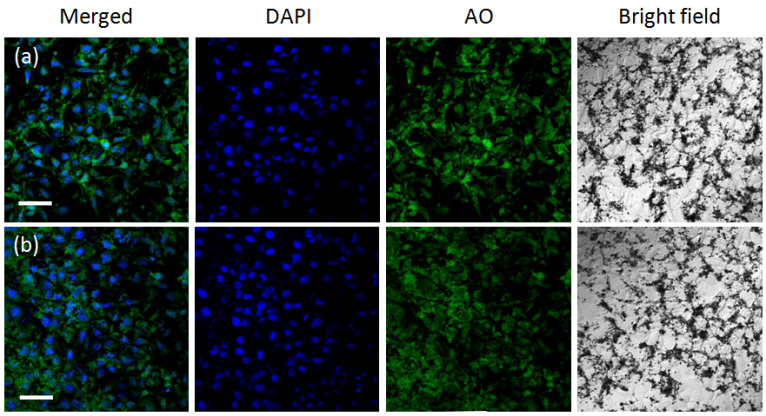

Figure S5. CLSM of L929 cells proliferated on the composite hydrogel with randomly oriented patterned surface for 4 (a) and 7 days (b). The PI channel showed no fluorescence in cells under the experimental condition. The bright field images were taken after the focus was set on the rod layer between the L929 cells and hydrogel surface to visualize the status of the silica rods. Scale bar: 50 μm. The magnitude is the same for each row.

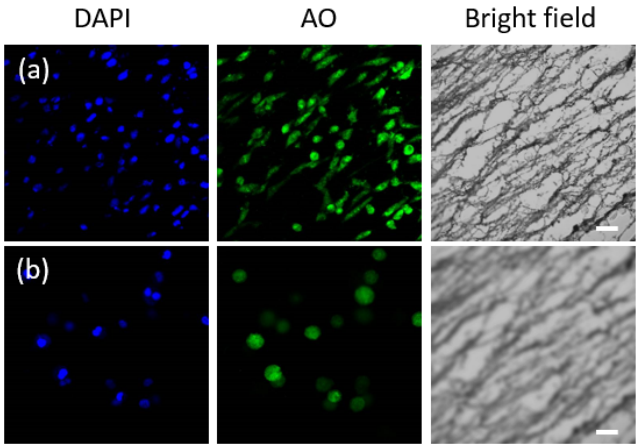

Figure S6. CLSM images of DAPI, AO channels and the bright field picture of Figure 3a and b, observed from the cell loaded composite hydrogel film after incubated for 4 h. (a) L929 on the patterned surface. (b) MC3T3-E1 in the hydrogel matrix. The PI channel showed no fluorescence in cells under the experimental condition. Scale bar 50 μm. The magnitude is the same for all images.

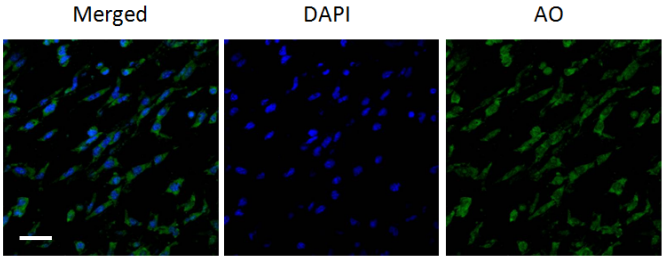

Figure S7. CLSM images of the L929 cells on surface patterned hydrogel film after being exposed to air for 4 h. Scale bar: 50 μm. The magnitude is the same for all the images.