Supporting Information

Directed Self-assembly of Polypeptide-engineered Physical Microgels for Building Porous Cell-laden Hydrogels

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Materials and Methods

1 Materials

Restriction endonuclease BamHI, NheI, SpeI, and T4 DNA ligase were obtained from New England Biolabs Inc. (Beijing, China). Tris(2-carboxyethyl)phosphine (TCEP), *β*–mercaptoethanol, isopropyl-*β*-D-thiogalactoside (IPTG), ampicillin, kanamycin, Ethidium homodimer-1 and Calcein AM were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Ni-NTA separation column was purchased from Qiagen China (Shanghai) Co., Ltd. Polyethylene glycol (PEG, molecular weight: 6 kDa) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Acryloyl chloride was obtained from Aladdin Inc. (Shanghai, China). Photoinitiator 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959) was a kind gift from Ciba Inc. (Tarrytown, NY). Tri-distilled water was used for all solutions.

2 Synthesis and purification of the coil-coiled polypeptides

PQE9P plasmid was a gift from Prof. David Tirrell at the California Institute of Technology, Pasadena, CA. The gene segment encoding polypeptide Pcys containing BamHI, NheI, and SpeI restriction sites was synthesized by the method of polymerase chain reaction (PCR) which used PQE9P plasmid as the template. The PQE9Pcys plasmid was constructed from Pcys and PQE9P plasmid through DNA recombinant manipulation. The Pcys segment and the PQE9P plasmid were digested by BamHI to yield cohesive ends. Digested Pcys segment and vector were ligated together with T4 DNA ligase to construct PQE9Pcys plasmid. The segment encoding RGD containing NheI and SpeI restriction sites was also acquired by PCR. Digested RGD segment with NheI and SpeI was inserted into the NheI restriction site of PQE9Pcys to construct PQE9RGDPcys plasmid. The sequence of PQE9Pcys and PQE9RGDPcys were verified at the DNA sequencing core facility of Sunny Institute at

Shanghai. PQE9RGDPcys plasmid was transformed into *E. coli* strain M15. Cultures of PQE9RGDPcys cells were grown at 37 ºC in 1 L of 2xYT media supplemented with 50 mg L^{-1} of ampicillin and 25 mg L^{-1} of kanamycin, respectively. The culture was induced with 1 mM IPTG when it reached an optical density (600 nm) of 0.7. Protein expression continued for 4 h. Cells were harvested by centrifugation (30 min, 6,000 g). The cell pellet was re-suspended in 8 M urea (pH = 8.0) and frozen at -80 ºC. The thawed lysate was centrifuged at 12,000 g for 30 min and the supernatant was collected for purification. A 6×Histidine tag encoded in pQE9 vector allows the polypeptide to be purified by affinity chromatography on a nickel nitrilotriacetic acid resin following the denaturing protocol provided by Qiagen. To prevent sulfhydryl oxidation and non-specific disulfide bond formation during purification of RGDPcys, 14 mM *β*–mercaptoethanol was added to the washing and elution buffers. The eluted fractions were dialyzed against sterile tri-distilled water for three days at room temperature, frozen, and lyophilized. The purified polypeptide was analyzed on a Bruker Reflex III reflectron MALDI-TOF mass spectrometer, RGDPcys (MS: 8483.5 Da, the theoretical calculation of molecular weight: 8487.2 Da).

3 Synthesis of PEGDA

Photosensitive polymers PEGDA were synthesized from linear PEG with an average molecular weight of 6 kDa. Briefly, a solution of PEG in dichloromethane was reacted under argon with acryloyl chloride and triethylamine at an acryloyl chloride: OH molar ratio of 4:1. The product was precipitated in ice-cold diethyl ether, dried under vacuum, and stored at -20 ºC under the protection of argon. The final yield of PEGDA was more than 85%. High degree of substitution (> 95%) was confirmed by ^{1}H NMR (Varian Unity spectrometer). ^{1}H NMR (300 MHz, $CDCl_3$, 25 ºC, TMS): δ = 6.2 (d, 2H), 6.0 (d, 2H), 5.7 (d, 2H), 4.1 (t, 4H), 3.4 ppm (m, 539H).

4 Preparation of RGDP-PEG-acrylate

The precursor (RGDP-PEG-acrylate) of the photo-cross-linked microgel was synthesized by conjugating the engineered polypeptide RGDPcys (having a C-terminal cysteine residue) to PEGDA through the Michael-type addition reaction. RGDPcys (230 mg, 27 μmol) was dissolved in 2.7 mL 8 M (pH = 8) urea buffer followed by the addition of 300 μL TCEP (150 mM). After incubation at room temperature for 1.5 h, 270 μmol PEGDA and 27 mL 8 M (pH = 8) urea were added. The pH of the mixture was adjusted to 8.0. The mixture was stirred at room temperature for 24 h under dark condition. SDS-PAGE was used to monitor the degree of reaction. The excess PEGDA was removed by Ni-NTA affinity column. The purified product RGDP-PEG-acrylate was dialyzed against sterile tri-distilled water for 3 days under dark condition, frozen, and lyophilized. The products were stored at -20 ºC under the protection of argon.

5 Cytotoxicity of RGD-PEG-acrylate hydrogel

RGD-PEG-acrylate solution (10% w/v) containing 0.2% photoinitiator Irgacure 2959 was pipette in 96-well plate and exposed to UV light (365 nm, 12.5 mW cm^{-2}) for 5 min to form hydrogels. NIH 3T3 fibroblasts were seeded on the hydrogel at 5000 cells per well and cultured at 37 ºC, 5% CO_2 in cell incubator for 48 h. Tests under all conditions were run in triplicate. Cells were stained with the calcein AM/ethidium homodimer for 20 min and examined on an inverted fluorescent microscope (Olympus IX71, Japan). Stained cells were trypsinized and counted using a hemocytometer.

6 Fabrication of microgel units

RGDP-PEG-acrylate solution (8%, w/v) containing 0.2% photoinititor Irgacure 2959 was prepared in phosphate-buffered saline (PBS) or Dulbecco's Modified Eagle Medium (DMEM).

Microgels having predefined morphological features were photolithographically fabricated. Photomasks with square, circle, and star (dimensions: 500 × 500 μm) patterns were designed using Auto CAD 2010 software and made in Kunshan Kaisheng Electronics Co., Ltd (Kunshan, China) at a resolution of 1200 dpi. The prepolymer solution was pipette into a space separated by two pieces of cover glass (thickness 200 μm), and another coverslip and a photomask were placed sequentially on the top of the pipette prepolymer solution. After exposure under UV light (365 nm, 12.5 mW cm^{-2}) for 30 s, the coverslip was removed slowly. Microgels were harvested by washing with PBS or DMEM. By using photomasks with different shape (circle, square, star) and dimension (500 × 500 μm^2), microgels with different shape and size (500 × 500 × 200 μm^3) were fabricated.

7 Assembly of microgels

To make sure microgels close enough to assemble into a construct by self-healing, the microgels (circle-shaped) were collected into the barrel of a 1 mL syringe (with the outlet sealed) and centrifuged at 225 × g for 10 min. The sample was incubated at 25 ºC for 10 min to allow further assembly.

Constructs consisted of distinct layers were prepared by sequential assembly of different microgels. Two hydrophobic dyes, the yellow dye 2-amino-4,6-bis-[(4-N,N-diphenylamino)styryl] pyrimidine and red dye 1,5-diphenylthiocarbazone, were encapsulated into two batches of microgels, respectively. These encapsulated hydrophobic dyes could not diffuse out from the microgels. The microgels stained with different dyes were assembled in a tube through layer-by-layer self-assembly.

8 Fabrication and assembly of cell-laden microgels

NIH 3T3 fibroblasts were used as a model in this study. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37 ºC.

To encapsulate cells within the microgels, the cells were trypsinized and re-suspended in RGDP-PEG-acrylate (8%, w/v) containing 1% photoinitiator I-2959 at a concentration of 1×10^6 cells per mL. Cell-laden microgels were fabricated and assembled as described in sections **6** and **7**. The viabilities of cells in the cell-laden microgels and the assembled constructs were examined by staining the samples with the live/dead assay reagents (Calcein AM and Ethidium homodimer-1, Invitrogen). The samples were examined using a 10 × objective on an Olympus FV1000 confocal microscopy for fluorescence imaging. XYZ scanning mode was adopted for 2-D and 3-D images of cells in the microgels.

9 Penetration rate of assembled constructs

To compare the penetration rate of assembled construct and nonporous hydrogels, construct assembled from circle-shaped RGDP-PEG-acrylate microgels (8% w/v, 40 μL), nonporous RGDP-PEG-acrylate hydrogel (8% w/v, 40 μL), and nonporous PEGDA hydrogel (8% w/v, 40 μL), were prepared in a PCR tube with a little hole in the bottom, respectively. Rhodamine 6G (70 μL) was added on the hydrogels. The tubes were maintained vertically throughout the whole experiment.

10 Perfusion culture of assembled cell-laden constructs

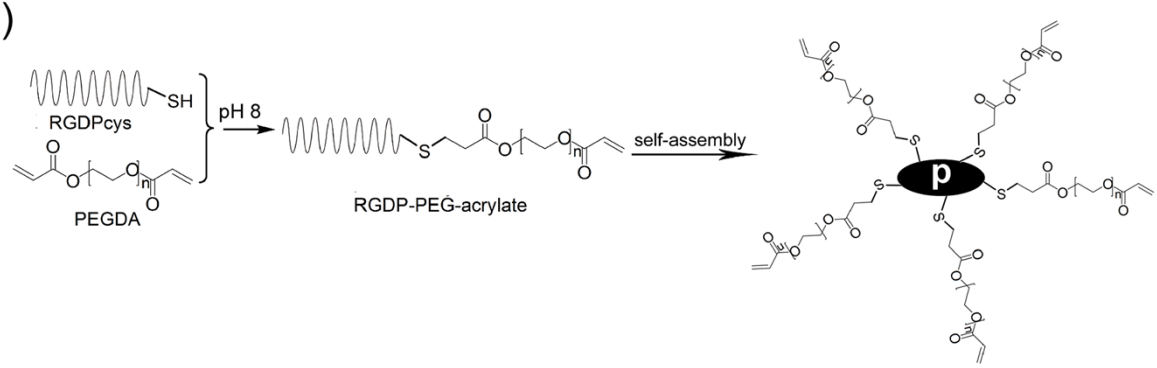
Perfusion culture was performed according to the literature method.[1] In brief, circle-shaped cell-laden microgels were fabricated in DMEM supplemented with penicillin-streptomycin (100 units mL^{-1}) and FBS (10%) and assembled into a construct in the barrel of a 1 mL syringe. The construct was incubated in a tissue culture incubator for 4 days through perfusion culture. Nonporous hydrogels (8% w/v, isopyknic RGDP-PEG-acrylate hydrogel) were statically cultured in a 5% CO_2 humidified incubator at 37 ºC for 4 days. The DMEM was refreshed every day. After 4 days of culturing, cell viabilities in both the perfused porous constructs and the statically cultured nonporous hydrogels were examined. Each sample was

cut into 3 mm thick slices, and each slice was stained with the live/dead labeling reagents (Calcein AM and Ethidium homodimer-1, Invitrogen). The samples were examined using a 10 × objective on the Olympus FV1000 confocal microscopy for fluorescence imaging. XYZ scanning mode was adopted for 3-D images.

References:

1 B. Liu, Y. Liu, A. K. Lewis and W. Shen, *Biomaterials,* 2010, **31**, 4918-4925.

(a)



(b)

RGDPcys: MRGS **[6H]**GSDASYAVTG**RGD**SPASSTS**[P]**TSMGGC

Abbreviations for peptide domains:

[6H]: HHHHHH

[P]: APQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMESDAS

a b c d e f g a b c d e f g a b c d e f g a b c d e f g a b c d e f g

37 40 44 47 51 54 58 61 65 68

Fig. S1 (a): Illustration of the formation of self-assembled multi-functional macromers. (b): Amino acid sequences of RGDPcys.

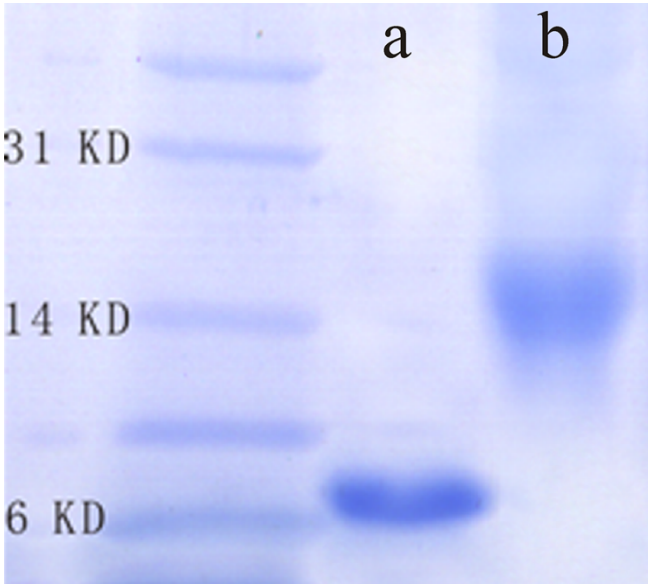


Fig. S2 SDS-PAGE of RGDPcys and RGDP-PEG-acrylate: a, RGDPcys; b, RGDP-PEG-acrylate. The molar ratio of RGDPcys and PEGDA is 1:10.

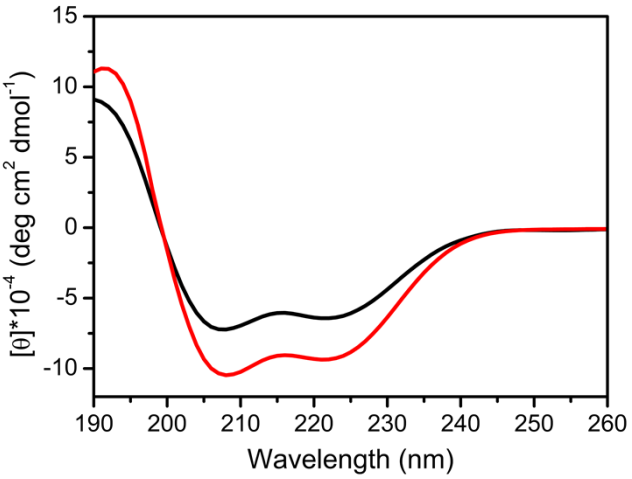


Fig. S3 Circular dichroism (CD) spectroscopy of RGDPcys and RGDP-PEG-acrylate was conducted on a Jasco J-810 CD spectrometer. Both samples were at a concentration of 5 μM in 10 mM sodium phosphate buffer, pH 7.4. The wavelength spectrum was measured over a range from 190 to 260 nm with a step size of 1 nm at room temperature (25 ºC). All scans were made in triplicate and averaged. The black and red lines represent RGDPcys and RGDP-PEG-acrylate, respectively.

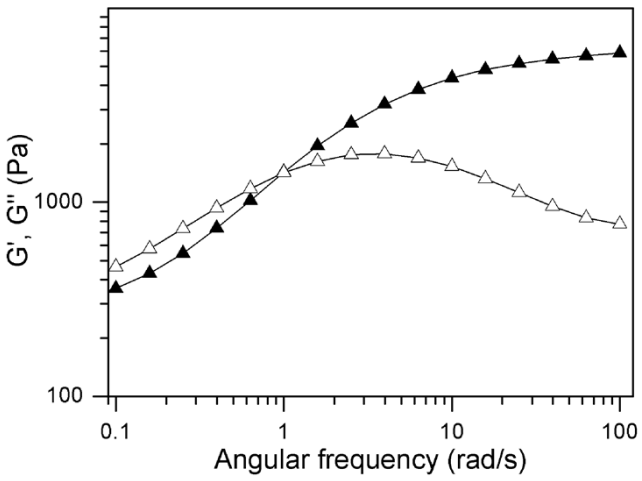


Fig. S4 Rheological oscillatory shear measurement of 8% w/v photo-cross-linked RGDP-PEG-acrylate hydrogel. Storage modulus G': filled symbols; loss modulus G'': open symbols. Measurement was performed at 1% strain, pH 7.4, and 37 °C.

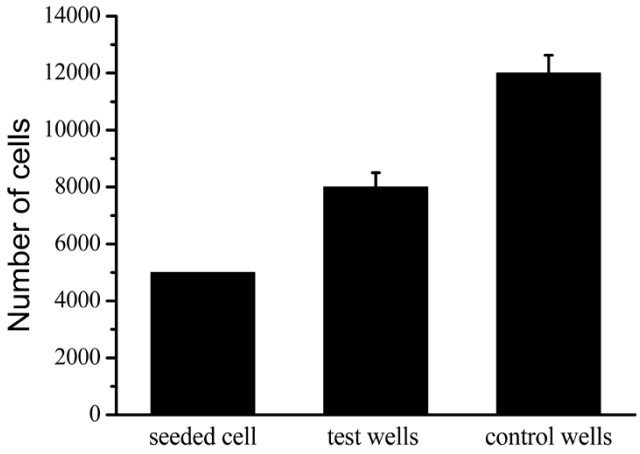


Fig. S5 Cytotoxicity of 8% w/v photo-cross-linked RGDP-PEG-acrylate hydrogel. NIH 3T3 fibroblasts were cultured on the surface of hydrogel for 48 h.

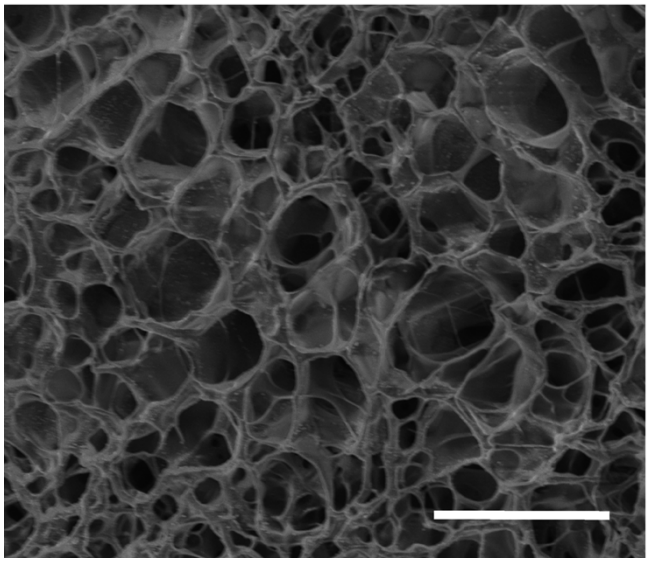


Fig. S6 SEM of 8% w/v photo-cross-linked RGDP-PEG-acrylate hydrogel. The scale bar is 40 μm.