Precise Construction of Cell-instructive 3D Microenvironments by Photopatterning a Biodegradable Hydrogel

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1. Materials and Characterizations

1.1. Materials

All chemical reagents were purchased from commercial available sources such as Sigma and Aladdin. Deionized water was used to prepare all aqueous systems. Unless specifically mentioned, the buffer used in the experiments is 0.01 M phosphate buffer saline (PBS, pH = 7.2). Matrix metalloproteinase substrate peptide Ac-GCRDGPQGIWGQDRCG-NH₂, cyclo(Arg-Gly-Asp-d-Phe-Lys) (c(RGDfK)), cyclo(Arg-Gly-Asp-d-Phe-Cys) (c(RGDfC)) were purchased from Apeptide Co., Ltd., Shanghai, China. Dulbecco’s modified eagle’s medium (DMEM, Gibco), Live/Dead imaging kit (Invitrogen), Alexa Fluor 488-phalloidin, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific. NB-1, bovium serum albumin (BSA), fetal bovine serum (FBS) and BOC-Lys are commercial available and purchased from Sigma.

1.2. Characterizations

Proton spectra (¹H NMR) were recorded on a Bruker Avance (600 MHz) spectrometer. Chemical shifts were reported in parts per million (ppm) downfield from the Me₄Si resonance which was used as the internal standard when recording ¹H NMR spectra. Absorption spectra were recorded on a Shimadzu UV-2550 UV-Vis spectrometer. Attenuated total reflectance FTIR (ATR-FTIR) spectra were recorded on a NICOLET 5700
FT-IR spectrometer. Mechanical measurements were recorded on a HAAKE MARS III rheometer. The confocal fluorescence images were performed on a confocal laser scanning microscope (CLSM, Leica-TCS-SP8) with 5×, 10× and 40× objective lens.

2. Synthesis and Characterization of HA-NB-VS

2.1. Synthesis of HA-NB-VS

![Reaction Scheme]

**Figure S1.** The synthetic procedure for the HA derivatives, including HA-NB, HA-NB-SH, and HA-NB-VS. The reaction conditions: i) 1, Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM), DMSO/MES buffer (10.0 mM, pH = 5.5), and RT for 12 h; ii) 2, DMTMM, MES buffer (pH = 5.5), and RT for 12 h, then Tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) and RT for 3h; iii) divinyl sulfone, triethanolamine buffer (TEOA, 300 mM, pH = 8.0) and RT for 10 h.

2.2. Characterization of HA-NB
Figure S2. $^1$H NMR spectrum (600 MHz, D$_2$O) of HA-NB.

The substitution degree was calculated by the NMR integral analysis using the following formula $Y\% = \frac{I_{[a]}}{(I_{[c]} ÷ 3)} × 100\%$.

2.3. Characterization of HA-NB-VS

Figure S3. $^1$H NMR spectrum (600 MHz, D$_2$O) of HA-NB-VS.

The substitution degree was calculated by the NMR integral analysis using the following formula $Y\% = \frac{I_{[c]}}{(I_{[f]} ÷ 3)} × 100\%$.

3. In situ formation of HA-MMP hydrogel via thiol-Michael reaction
Figure S4. a-b) *In situ* rheological analysis for gel formation and the effects of hydrogel formulation on final moduli. For data in b), four sets of independent samples were monitored.

4. NB photolysis and its ability to conjugate protein in hydrogel

4.1. The stability of imine-bond in NB-3 generated from PAAL

Figure S5. a) The evolution of HPLC spectra of NB-3 kept in acetonitrile-water solution (4/1, v/v) over time. b) The calculated normalized remaining ratio of NB-3 from a).

4.2. Photolysis of HA-NB and HA-MMP hydrogel
Figure S6. UV-vis spectra evolution for the photolysis of HA-NB (2 mg mL⁻¹) in PBS a) and HA-MMP hydrogel film b) upon 365 nm LED light irradiation (10 mW cm⁻²).

4.3. FTIR-ATR spectra for protein photoimmobilization in hydrogel

Figure S7. The ATR-FTIR spectra for photolysis analysis and BSA immobilization in HA-PEG. Up images are the hydrogels of HA-PEG, HA-PEG+hν and HA-PEG +BSA-Rho+hν (BSA-Rho was used for better visual).

“HA-PEG+hν” means the xerogel from the irradiated HA-PEG hydrogel, the disappeared peak at 1320 cm⁻¹ and 1520 cm⁻¹ (-NO₂ stretching vibration) confirmed the photolysis of the NB group.

“HA-PEG+BSA +hν” means the xerogel from the irradiated HA-PEG hydrogel with BSA-Rho premix, the increased peak at 1644 cm⁻¹ (amide stretching vibration of BSA) confirmed the BSA immobilization in hydrogel.

5. Photopatterning of protein and cells

5.1. 3D photopatterning of BSA-Rho

BSA-Rho and BSA-FITC were used as protein models in the assay and prepared as reported.¹
Figure S8. BSA-Rho 3D patterning in HA-MMP hydrogels by photomasks after 3 min irradiation (10 mW cm$^{-2}$): a) stripes with 200 µm width and spacing and b) frames with 100 µm extra and 50 µm intra side-length. Images represent 100 µm depth at z-projection, scale bars are 200 µm. The protein concentration was fixed at 10 µM.

5.2. Sequential photopatterning of BSA-FITC and BSA-Rho in HA-MMP hydrogel

First, HA-MMP hydrogels were soaked in BSA-Rho solution (10 µM) and patterned by photomask with strip lines (365 nm LED light source, 10 mW cm$^{-2}$ for 3 mins). After washing in PBS overnight, the hydrogel was resoaked in BSA-FITC solution (10 µM) and followed by photopatterning with the mask in perpendicular way and washing process. The patterned hydrogel was imaged by using A1R Nikon confocal microscope from two channels, rhodamine channel $\lambda_{ex} = 561$ nm (red) and FITC channel $\lambda_{ex} = 488$ nm (green).
**Figure S9.** 3D and 2D confocal images for sequential protein patterns of BSA-Rho and BSA-FITC in HA-MMP hydrogel after soaking in PBS for a) 1 day and b) 7 days.

**5.3. Cell adhesion on hydrogel mediated by peptide or protein patterning**
Figure S10. Confocal micrographs (Calcein channel, $\lambda_{ex}=488$ nm) and quantifications of HDF cells numbers on patterned HA-MMP hydrogels. a) photopatterning of c(RGDfK) at different concentrations, b) photopatterning of BSA and BSA-c(RGDfC). c) Data representation of the adhered cell densities in a) and b). “+hv” means cells in the irradiated areas, “-hv” means cells in the nonirradiated areas. A total of $5 \times 10^4$ cells were seeded on the hydrogels and co-cultured for 12 h. *** denotes p < 0.001.

5.4. Synthesis and determination of the substituted degree of BSA-c(RGDfC)

The synthesis procedure of BSA-c(RGDfC) was performed as previously described.2 Briefly, 50 mg BSA (0.75 μmol) was dissolved in 4 mL PBS (100 mM, pH = 7.2) and then activated by dropping sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, 5.0 mg in 1 mL warm PBS). After stirring for 1 h at room temperature, the solution was desalted with a Sephadex G-50 column for 1
h and incubated with cyclo(Arg-Gly-Asp-d-Phe-Cys) (c(RGDfC), 1.0 mg mL\(^{-1}\)) overnight at room temperature. Then, the mixture was transferred to a dialysis bag (MWCO 3500, Spectrum®) and dialyzed against deionized water at 4 °C for 3 d. The newly purified protein solution was freeze-dried and stored at −20 °C prior to use. MALDI-TOF and SDS-PAGE analyses were conducted to characterize the modified protein BSA-c(RGDfC), and the substitution degree of c(RGDfC) was determined by MALDI-TOF.

**Figure S11.** The synthetic procedure for BSA-c(RGDfC). Structure of BSA from PDB 4F5S.

From above procedure, the number of substituents (n) can be determined using following equation:

\[
Mw_{BSA-c(RGDfC)} = Mw_{BSA} + 797.32 \times n
\]

Based on the molecular weight from the mass spectra, n can be determined as 5.65, then the substitution degree can be determined as 9.4% (relative to 60 amine groups in a BSA molecule).

5.5. The effects of the photoirradiation of hydrogel on cell adhesion
Figure S12. Confocal images of cells adhesion on photoirradiated HA-MMP hydrogels. A total of $5 \times 10^4$ cells were seeded on the hydrogels and photoirradiated for 3 min (365 nm LED, 10 mW cm$^{-2}$) and co-cultured for 12 h.

6. AND logic gate for 3D cell culture

6.1 Light cytotoxicity for the AND logic gate manipulation

![Graph showing cell viability over time](image)

Figure S13. The cytotoxicity of irradiation by 365 nm LED light.

6.2 The longitudinal depth of photoimmobilization protein in hydrogel.

Given that light attenuation would lead to protein longitudinal gradient in hydrogel, BSA-Rho was used as the model to explore the depth of photopatterning. The irradiation condition was same as above (365 nm LED light source, 10 mW cm$^{-2}$ for 3 mins) by using a square-shaped photomask.
Figure S14. a) A representative image of the BSA-Rho 3D photopatterning constructed from a series of XY cross-sectional micrographs over a 0.85 mm depth. b) The longitudinal fluorescence intensity profile along the central axis of the square.

6.3 AND logic gate for cell viability and spreading

Figure S15. Confocal images of cells in the user-programmed hydrogels at day 1, 4, 7 and 12. (0, 0) means BSA+HA-PEG hydrogel, (1, 0) means BSA-c(RGDfC)+HA-
PEG hydrogel, (0, 1) means BSA+HA-MMP hydrogel, and (1, 1) means BSA-c(RGDFC)+HA-MMP hydrogel.

6.4 AND logic gate for 3D cell migration

Figure S16. The microscopic images for HDF cells migration in hydrogels after 12 d culture: a) BSA+HA-PEG hydrogel, b) BSA-c(RGDFC)+HA-PEG hydrogel, and c) BSA+HA-MMP hydrogel.

7. Photo-modulated 3D cell culture and migration in HA-MMP hydrogels

Figure S17. Photo-modulated 3D HDF cell culture and migration in BSA-c(RGDFC)+HA-MMP hydrogels. a) Z-stacked confocal images (200 μm depth) of encapsulated cells stained by a live/dead assay after 12 d of culture. b) Evaluation of cell viability in photopatterned hydrogels. ***denotes p < 0.001.
Figure S18. Time dependent analysis of a) microscopic images and b) cell migration distance for HDF cells in patterned BSA-c(RGDfC)+HA-MMP hydrogels.

Figure S19. The bright field image for cell migration in BSA patterned hydrogels after 12 d of culture.

8. References
