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Electronic Supplementary Information

Decorating Protein Hydrogels Reversibly Enables Dynamic Presentation and Release of Functional Protein Ligands on Protein Hydrogels

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Table S1. Protein sequence

GRG_5RG_4R-IG_N MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDA TKTFTVTERS GGRPSDSYGAPGGGNRS $(MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDA$ $TKTFTVTERS)_5$ GGRPSDSYGAPGGGNRS $(MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDA$ $TKTFTVTERS)_4$ GGRPSDSYGAPGGGNRS LIEVEKPLYGVEVFVGETAHFEIELSEPDVHGQFKLKGQPLAASPDCEIIEDG KKHILILHNCQLGMTGEVSFQAANTKSAANLKVKELRS MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVGCGLG
G_CI-ECFP[-$(FnIII)_2$] CGDGEWTYDDATKTFTVTERS LIEVEKPLYGVEVFVGETAHFEIELSEPDVHGQFKLKGQPLAASPDCEIIEDG KKHILILHNCQLGMTGEVSFQAANTKSAANLKVKELRS MVSKGEELFTGVVPILVELDGDVNGHRFSVSGEGEGDATYGKLTLKFICTT GKLPVPWPTLVTTLTWGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFF KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNAISDNVY ITADKQKNGIKAHFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST QSALSKDPNEKRDHMVLLEFVTAAGITRS [(RLDAPSQIEVKDVTVTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTE DENQYSIGNLKPDTEYEVSLISRRGD MSSNPAKETFTTRS$)_2$]

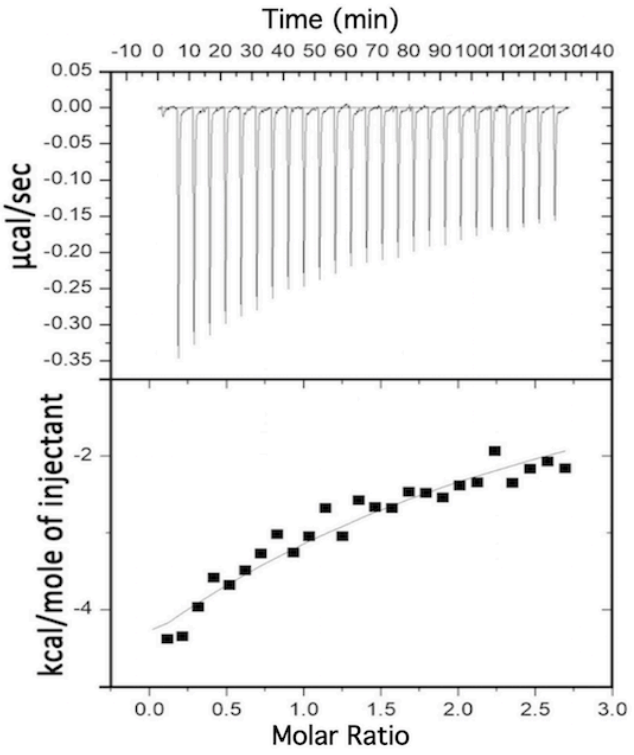

Figure S1. ITC plot of the reconstitution of G_C-I27 and I27-G_N fragments. Titration of 280 µM G_C-I27 solution into 25 µM I27-G_N solution was carried out in sodium phosphate buffer (pH 7.4) at 10 ℃. Fitting of the binding curve revealed that the binding ratio of I27-G_N and G_C-I27 is close to the complex stoichiometry of 1:1 with Kd of 4.1×10^{-5} M.

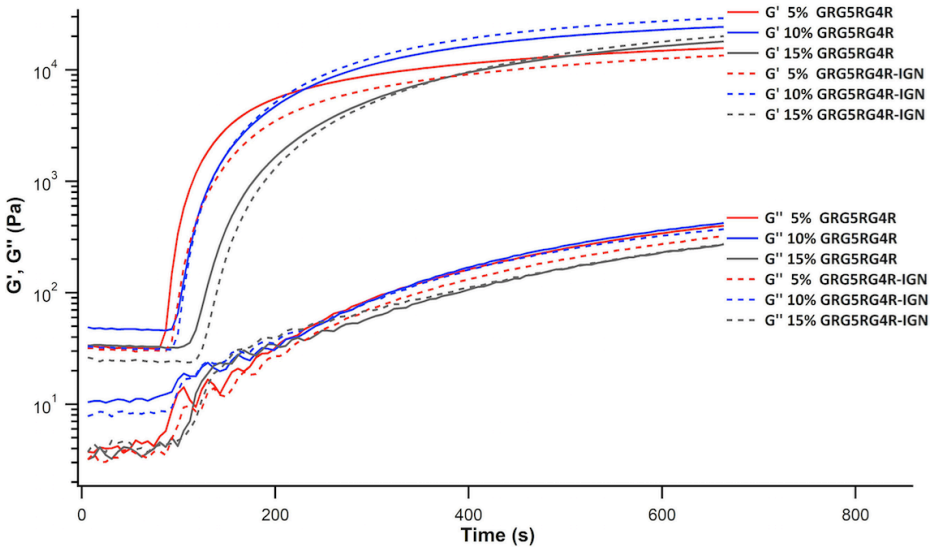

Figure S2. Viscoelastic properties of GRG_5RG_4R and GRG_5RG_4R-IG_N hydrogels. A) Time evolution of the storage and loss moduli of GRG_5RG_4R and GRG_5RG_4R-IG_N hydrogels during photochemical crosslinking.

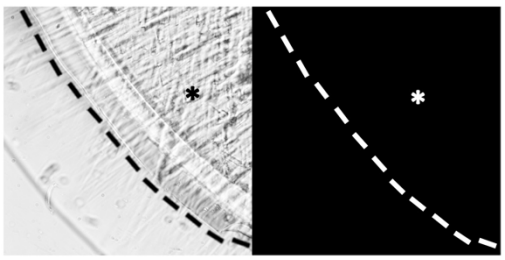

Figure S3. Photographs of GRG_5RG_4R-based hydrogels after incubating in G_CI-EGFP (left: bright field image; right: fluorescence image). * is a reference point on the hydrogel, and dotted lines indicate the edge of the hydrogel.

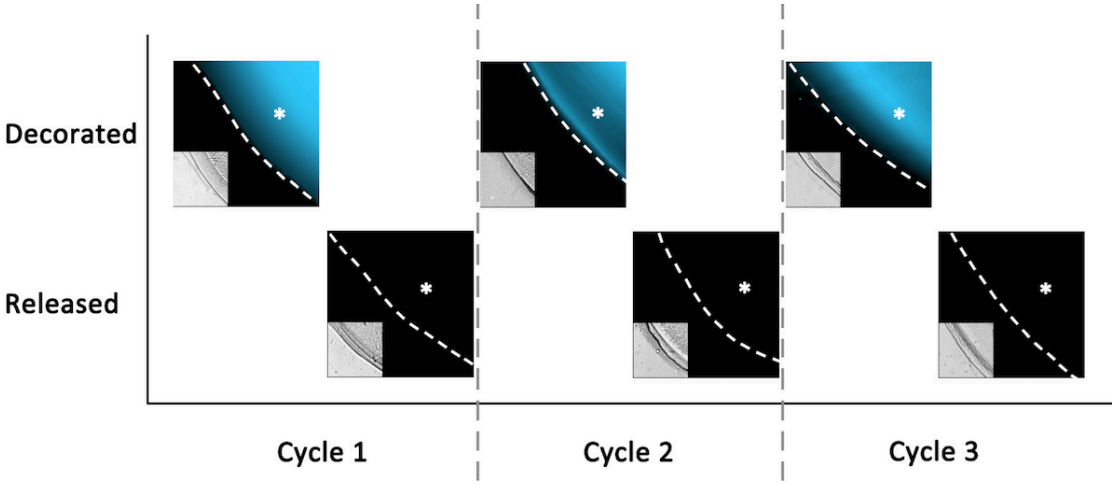

Figure S4. Functional protein ligands can be reversibly and repeatedly conjugated to and released from GRG_5RG_4R-IG_N based protein hydrogels via temperature and redox potential-controlled PFR. Fluorescence images are shown. The insets are the bright field images of the same hydrogels (after decoration with ECFP and release of ECFP, respectively). Dotted lines indicate the edge of the hydrogels, and * are reference points on the hydrogels. The release ratio of the ECFP from the hydrogel for the three cycles is 89%, 82% and 85%, respectively.

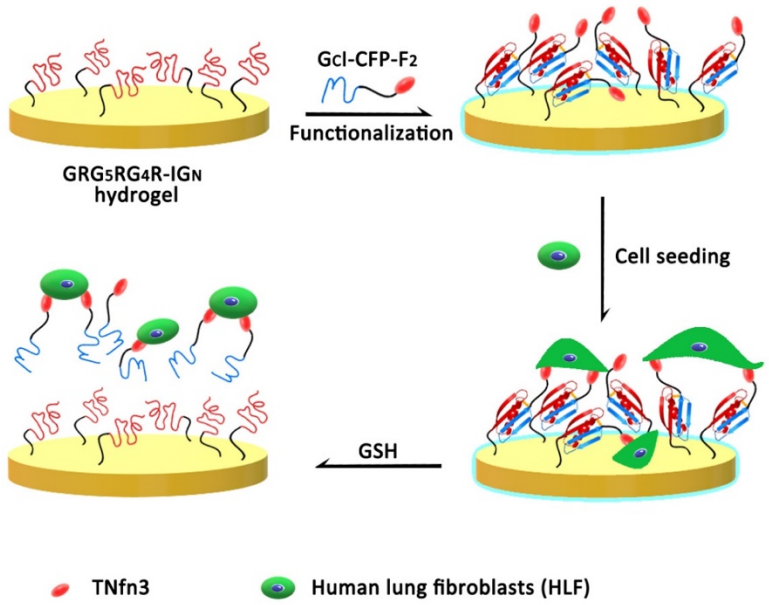

Figure S5. Schematic design of cell culture and release matrix by presenting ligand on and releasing from hydrogels via PFR in a well-controlled fashion.

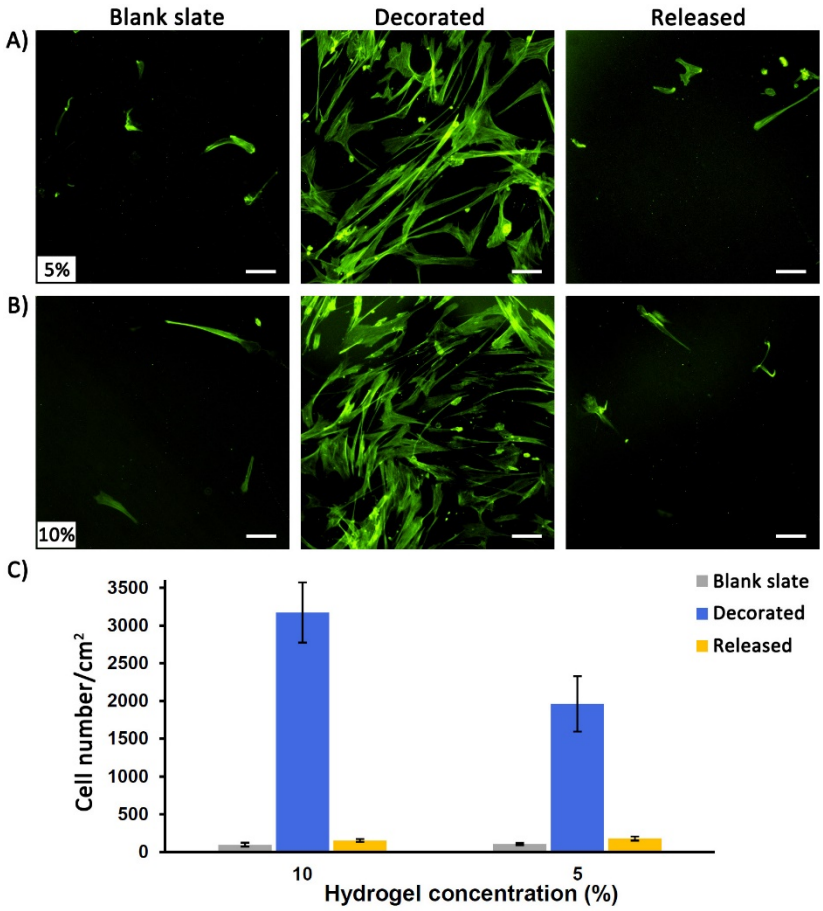

Figure S6. Culture and release of HLFs on GRG_5RG_4R-IG_N based hydrogels: A-B) Fluorescence images of HLFs on 5% (A) and 10% (B) protein hydrogels. It is evident that HLFs can adhere well on protein hydrogels after decoration of TNfn3 ligands via PFR, and HLFs can be released from hydrogel surface after TNfn3 ligands are released from hydrogels. C) Cell number of HLFs on protein hydrogels. The cell numbers were quantified from full-field fluorescence microscopy images. The scale bars represented 100 μm.

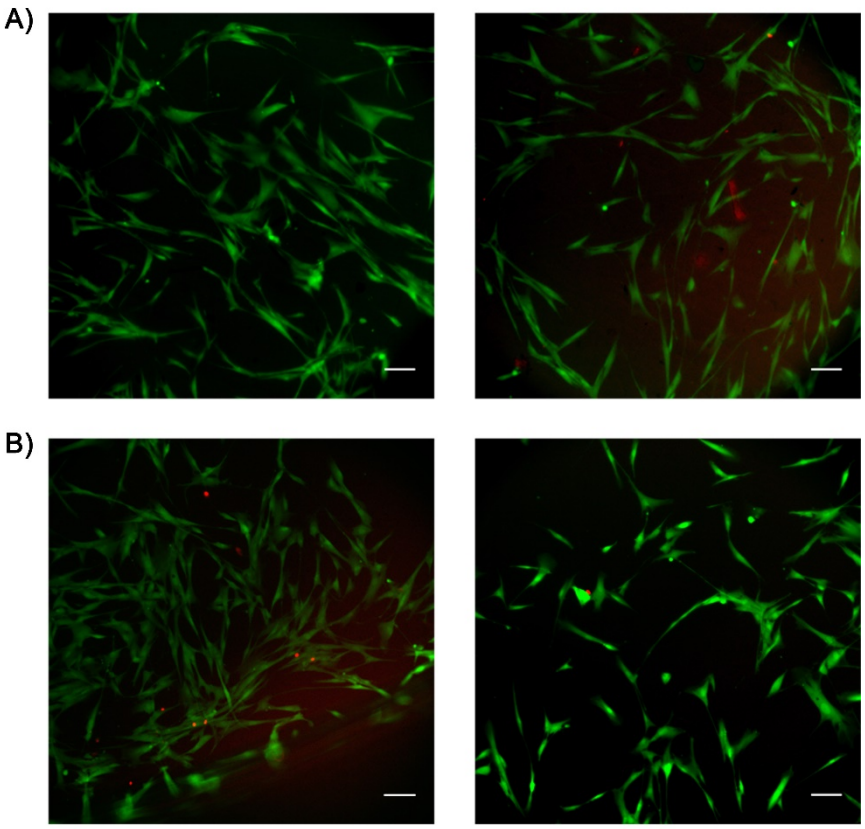

Figure S7. Live&Dead assay revealed that the cells cultured on (A) and released from (B) the GRG_5RG_4R-IG_N based hydrogels. Live cells are in green and dead cells are in red. The scale bar is 100 μm. The cell viability is 96% for cells cultures on the hydrogel, and 93% for cells released from the hydrogel.

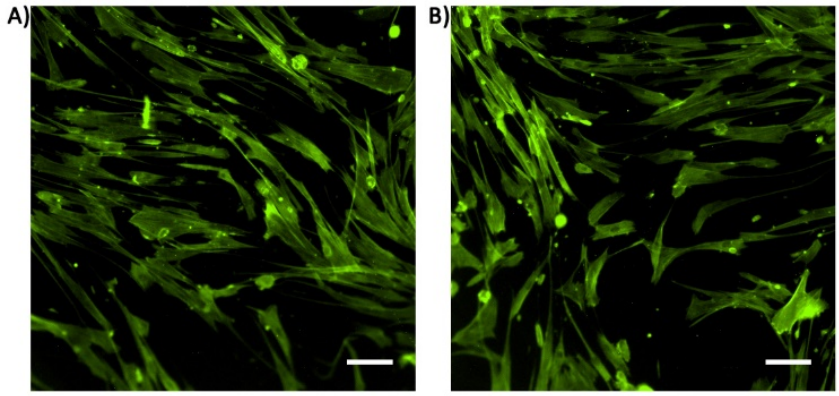

Figure S8. HLF density on 7% FRF_4RF_4R hydrogels does not change in response to redox potential. Fluoresce microscopy imaging of HLFs on FRF_4RF_4R hydrogels: A) HLFs were cultured in standard DMEM and B) in DMEM containing 20 mM GSH. Scale bars represented 100 μm.

Materials and methods

Protein engineering

The genes encoding GRG_5RG_4R, I27-G_N, G_C-I27, ECFP and $(FnIII)_2$ were constructed in expression vector pQE80L containing a 5' BamHI, 3' BglII and KpnI restriction site as we described previously.[1] Digesting I27-G_N with restriction endonuclease BamHI and KpnI resulted in I27-G_N insert with sticky ends, then ligated it into digested pQE80L-GRG_5RG_4R vector with BglII and KpnI to form pQE80L-GRG_5RG_4R-I27-G_N. In a similar way, we also obtained pQE80L-G_C-I27-ECFP and pQE80L-G_C-I27-ECFP-$(FnIII)_2$ plasmid.

Protein overexpression was carried out in *Escherichia coli* strain DH5α. The overnight culture was inoculated into 2.5% Luria-Bertani broth (LB) liquid medium with 100 ug/mL Amp, and incubated until OD600 is about 0.6-0.8 at 37 °C. Then, 1 mM isopropyl-1-β-D-thiogalactoside was added to the culture to induce the protein overexpression. Protein overexpression continued for about 4 hours. The cells were harvested by centrifugation at 4000rpm, 4 °C for 10 min and frozen at -80 °C. Lysed cells with 100 mg/mL lysozyme for 30 minutes and removed precipitated section by centrifugation at 12000 rpm for 60 minutes. Proteins were purified from the supernatant using Co^{2+} affinity chromatography. Excessive salts in the eluted proteins samples were removed by dialysis against water for 24 hours and the dialyzed protein samples were then lyophilized. Amino acid sequences of all the constructs used in this study are detailed in Table S1.

Isothermal titration calorimetry (ITC) experiments

Calorimetric experiments were carried out on an VP-ITC micro-calorimeter (MicroCal). Prior to the titration, the calorimeter was calibrated by known heat pulse as recommended by the manufacture. Titrations of 280 μM G_C-I27 to 25 μM I27-G_N were performed in 20 mM sodium phosphate buffer containing 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), pH 7.4. The reference cell was filled with MilliQ water, the sample cell was filled with 1.43 mL buffer or I27-G_N solution and syringe contained 290 μL G_C-I27 solution. All samples were degassed for 20 minutes before use. Titrations were performed as follows: a very preliminary injection of 6 μL was followed by 25 injections of 10 μL ligands. The delay time between the injections was 5 min. The resulting mixture kept stirring at 300 rpm during titration. Experimental data were plotted as the amount of heat evolved per second following each injection of G_C-I27 into the I27-G_N solution along with time and blank titration of G_C-I27 into buffer were performed to correct for heat generated by dilution and mixing.

Hydrogel preparation and decoration with protein ligands

Gelation of the polyprotein is based on the well-established photochemical crosslinking strategy.[2, 3] Lyophilized proteins GRG_5RG_4R, GRG_5RG_4R-IG_N and FRF_4RF_4R were dissolved in PBS (pH 7.4) and mixed with ammonium persulfate (APS, 1M) and $[Ru(II)(bpy)_3]^{2+}$ (20 mM) at final concentrations of 50 mM and 260 μM respectively. The protein solution was pipetted into a mold then illuminated by a 200W

fiber optic white light source at a height of 10 cm for 10 min. The hydrogel was gently taken off from the mold and soaked in excessive G_C-I27-ECFP or G_C-I27-ECFP-$(FnIII)_2$ solution including 20 mM GSH then treated with air oxidation overnight at 4 °C to facilitate ligand decoration and disulfide bonds formation in reconstituted GL5CC. Afterwards, the hydrogel was rinsed thoroughly with PBS for several times in order to remove remaining crosslinking reagents and unconjugated protein ligands.

For cell culture experiments on protein hydrogels, all procedures were operated under sterile conditions. To prepare the hydrogel for cell culture, the protein solution with appropriate amount of APS and $[Ru(II)(bpy)_3]^{2+}$ was sandwiched between a clean hydrophobic surface and a glass coverslip for photochemical crosslinking. The resultant hydrogel is ~0.8 mm thick and ~0.5 cm^2 in surface area.

Rheology experiments

Viscoelastic properties of the GRG_5RG_4R and GRG_5RG_4R-IGN hydrogels were determined by measuring viscoelastic moduli (storage modulus G′ and loss modulus G″) using a stress-controlled rheometer (TA Instruments) equipped with an 8 mm flat plate and a fiber optical illumination system.[4] Measurements were carried out at room temperature and the light was turned on at 60 s. At each time, 20 μL sample was loaded and a solvent trap was applied around samples to prevent dry drying artifacts.

Cell culture

Human lung fibroblasts (HLF) were first cultured in T75 tissue culture flasks with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin/Streptomycin mix at 37 °C. 9.3×10^4 cells were seeded on the top of hydrogels in triplicate in each tissue culture dish and cultivated in regular medium at 37 °C for 24 hours to undergo cell adhesion onto control and pre-functionalized hydrogels. For cell release, pre-functionalized hydrogels continued to be cultured for another 24 hours by replacing the medium with fresh medium containing 20 mM GSH.

Cell staining and imaging

Samples were first washed twice with pre-warmed sterilized PBS (pH 7.4, 37 °C) to remove non-adherent cells and fixed with a 4% paraformaldehyde solution in PBS for 15 mins at room temperature. Fixed cells were permeabilized by exposure to 0.1% Triton X 100 in PBS for 5 mins. Then cells were stained with Alexa Fluor 488 phalloidin for 20 minutes. After staining, samples were washed with PBS to remove excess staining dye. Images were taken at ×10 magnifications using an inverted fluorescence microscope (Nikon, Eclipse Ti) equipped with an EM-CCD camera (Andor).

Cell viability experiments (Live & Dead assay)

A LIVE/DEAD Viability Kit for Mammalian Cells (Thermofisher Scientific) was used to assess the viability of cells on hydrogels. LIVE/DEAD Assay staining solution was prepared with 2 μM Calcein AM and 4 μM ethidium homodimer-1 in sterile PBS.

Culturing medium was removed from the cells. Hydrogel samples were then incubated in the staining solution for 30 mins at room temperature. After staining, hydrogels were soak-washed with PBS for 3 times.

For the cell viability of released HLF from hydrogels, cells released from the hydrogel surface were collected from the culturing medium by centrifugation. After removing the supernatant, released cells were resuspended in normal DMEM medium and transferred to a 24-well plate. After overnight culturing, LIVE/DEAD staining and imaging were carried out.

Images were taken at ×10 magnifications using an Olympus Fluorescence Microscope. Live cells were captured with GFP filter and colored green in images (Ex/Em: 494/517 nm), dead cells were imaged with RFP and colored red (Ex/Em: 528/617 nm).

Reference

1. S. Lv, D. M. Dudek, Y. Cao, M. M. Balamurali, J. Gosline and H. Li, *Nature*, 2010, **465**, 69.
2. C. M. Elvin, A. G. Carr, M. G. Huson, J. M. Maxwell, R. D. Pearson, T. Vuocolo, N. E. Liyou, D. C. C. Wong, D. J. Merritt and N. E. Dixon, *Nature*, 2005, **437**, 999.
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4. X. Gao, S. Lyu and H. Li, *Biomacromolecules*, 2017, **18**, 3726.