Electronic Supplementary Information (ESI)
Experimental details for the communication

"Doubly Stimulus-Induced Stem Cell Aggregation During Differentiation on Biopolymer Hydrogel Substrate"

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Materials: HA sodium (MW ~1.6×10^6), chitosan (deacetylation degree: 88%, M_η: 4.2×10^5), adipic dihydrazide (ADH), 1-hydroxybenzotriazole hydrate (HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), tris (2-carboxyethyl) phosphine (TCEP), sodium periodate, *t*-butyl carbazate, trinitrobenzenesulfonic acid (TNBS), bovine serum albumin (BSA), coumermycin, novobiocin, trypsin, collagenase type II and fluorescein diacetate were purchased from Sigma. Maleimide PEG NHS Ester (Mal-PEG-NHS) (MW 3500) was purchased from JenKem Technology USA. Recombinant human BMP-2 (rhBMP-2) and the BMP-2 Quantikine ELISA Kit were obtained from R&D Systems (Minneapolis, MN, USA). All other reagents and solvents are of analytical grade and used as received.

Synthesis of GyrB-functionalized HA-NH_2 (GyrB-HA-NH_2): Aminated HA (HA-NH_2) was synthesized following previously described procedures [1]. Mal-PEG-NHS solution (5 mg/mL in DMSO) was added into a HA-NH_2 solution (5 mg/mL in nanopure H_2O) and incubated at room temperature for 6 h. Maleimide-functionalized Mal-HA-NH_2 was obtained as a white foam by freeze-drying after exhaustive dialysis against nanopure water (MWCO 12,000). The conversion of end groups as confirmed by ^{1}H NMR (300 MHz, Bruker Avance) was found to be at least 88.5%. The cysteine-tagged GyrB-Cys was prepared as described previously [2]. A 20-fold molar excess of TCEP was added to the GyrB-Cys eluate (100 mg/mL) and incubated at room temperature for 1 h followed by a buffer exchange to 50×10^{-3} M NaH_2PO_4, 150×10^{-3} M NaCl, 2×10^{-3} M EDTA pH 7.4 by ultrafiltration (MWCO 10,000) under nitrogen atmosphere leading to an 8000 dilution of TCEP and a final GyrB-Cys concentration of 150 mg/mL. Subsequently, Mal-HA-NH_2 was added at a ratio of 1.5 GyrB-Cys per Mal group and incubated for 6 h at 37 ^{o}C. Unbound GyrB-Cys was removed by dialysis for 3 days. The GyrB-HA-NH_2 was finally freeze dried at -50 ^{o}C and stored at 4 ^{o}C. The substitution degree of GyrB-HA-NH_2 (~36.1%) is indicated by the GyrB contentas determined by the Bradford method with BSA as standard.

Synthesis of GyrB-functionalized GC (GyrB-GC): GC was synthesized according to the reference [3-4]. 0.3 g Chitosan and 0.6 g lactose were dissolved in 20 mL of a 1:2 mixture of methanol and 3% (v/v) acetic acid (pH 4.5), into which 0.1 g sodium cyanoborohydride was added. The solution was stirred at room temperature for 24 h. The resulting product was exhaustively dialyzed against nanopure water and then freeze-dried. The GC was characterized by FTIR (Nicolet Avatar 360, USA). By

comparing with chitosan, the spectrum of GC shows a new absorption peak around 1555 cm^{-1} corresponding to the secondary amine group, which suggests the linkages between -CH_2- groups of lactose and -NH_2 of chitosan. The extent of derivatization of GC was determined by ^1H NMR spectroscopy (300 MHz, Bruker Avance). From the ratio between the area of the peak assigned to H1 of chitosan-NH_2 and to H1 of *N*-alkylated galactose-NH, about 58.3% of the available amino groups of chitosan have reacted with lactose.

Following the similar procedure to synthesize GyrB-HA-NH_2, GyrB-GC was prepared as a white foam. The substitution degree of GyrB-GC (~28.7%) is indicated by the GyrB content as determined by the Bradford method with BSA as standard.

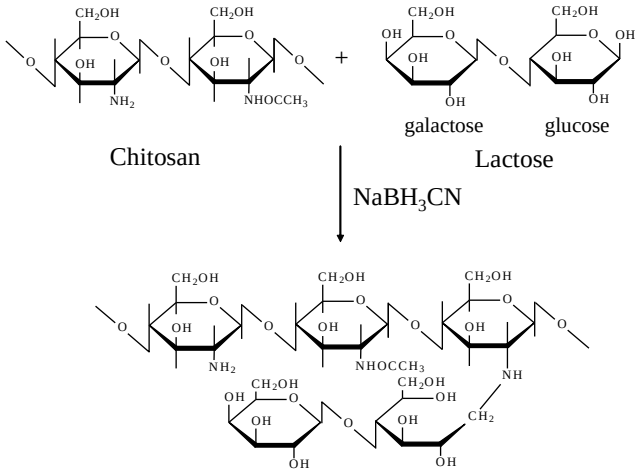

Fig. S1 synthesis route and molecular structure of GC [4].

Preparation of GyrB-functionalized Gel-Substrate: An aqueous solution of sodium periodate (0.5 M, 5mL) was added dropwise into HA sodium solution (10 mg/mL in nanopure H_2O), and the reaction was stirred for 2 h at room temperature in the dark. The dry aldehyde HA (HA-CHO) was obtained by freeze-drying after exhaustive dialysis against nanopure water. GyrB-HA-NH_2 and HA-CHO were dissolved in PBS separately at a concentration of 20 mg/mL. The GyrB-functionalized HA hydrogels were formed by mixing of GyrB-HA-NH_2/HA-CHO solutions at volume ratio of 2/1 at room temperature (Fig. S2). 600 μL of the mixtures was injected into a 24-well culture plate. The gelation time was recorded under sealed conditions at room temperature when the solution lost its fluidity. For preparation of GFs-loaded gel-substrate, BMP-2 was add into GyrB-HA-NH_2 solution, and mixed with HA-CHO, which resulted in formation of hydrogels. The final concentration of BMP-2 was fixed with 200 ng/mL in gels.

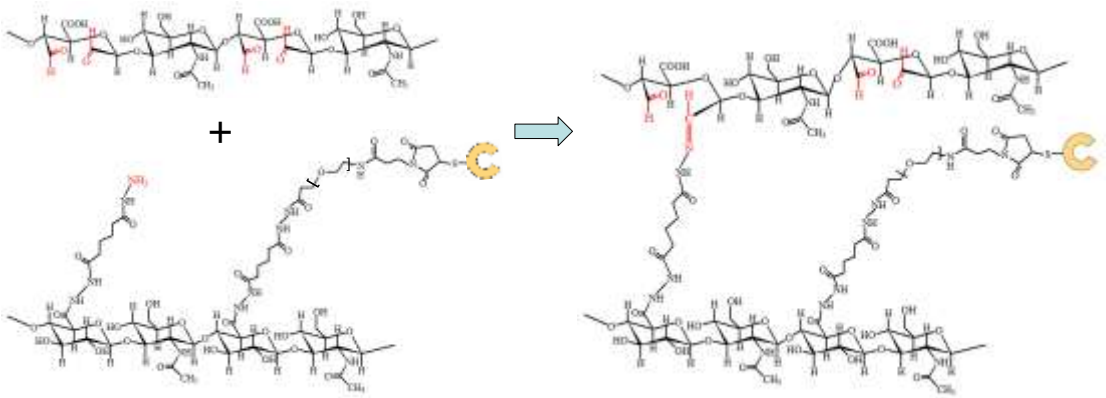

Fig. S2 Fabrication of HA gel-substrate via the Schiff-base reaction.

Fabrication of Biological Stimulus Gel-Substrate: For stimulus substrate formation, GyrB-GC was first dissolved in PBS at a concentration of 5 mg/mL. Subsequently, coumermycin (used as 50 mg/mL stock solution in DMSO) was mixed with GyrB-GC which was applied on at a molar ratio of GyrB:coumermycin = 2:1. The mixture was added onto the gel-substrate and incubated at 37 ^{o}C.

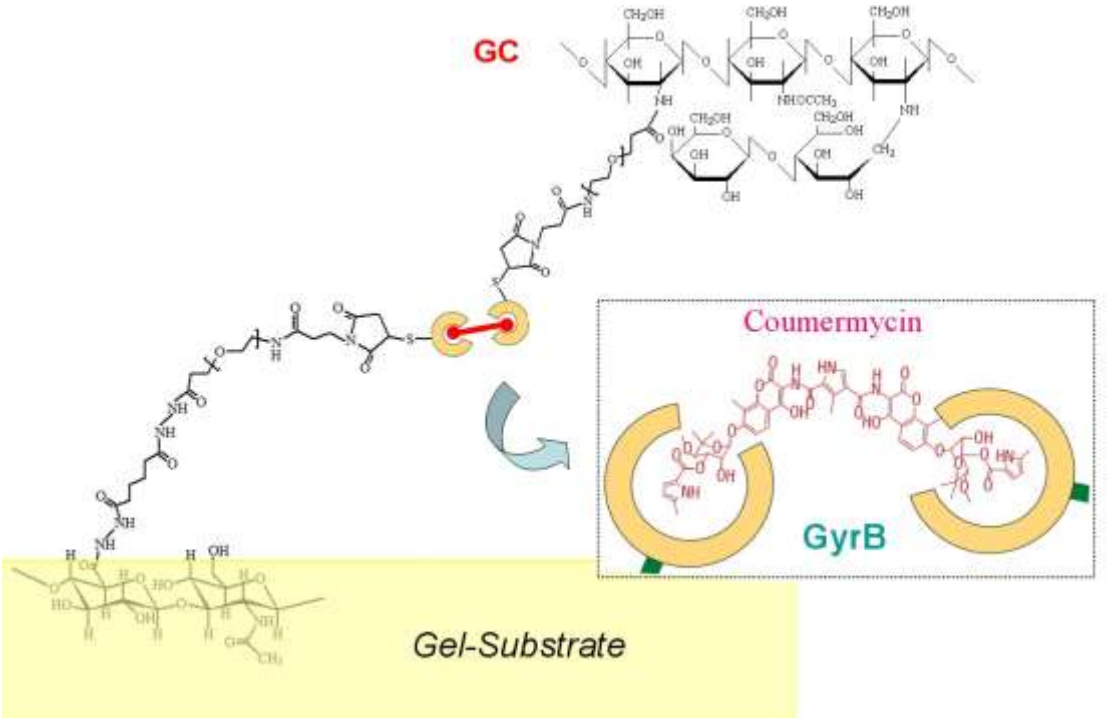

Fig. S3 Fabrication of biological stimulus-substrate.

Trigger-Inducible Stimulus Dissolution: For dissolution experiments, stimulus gel-substrates were incubated in PBS in the presence of the indicated novobiocin concentrations. The stimulus dissolution was monitored by the quantification of retained GyrB-GC in the buffer. GC amount was quantitatively detected by UV-vis spectroscopy after GyrB-GC was hydrolyzed in HCl solution [5].

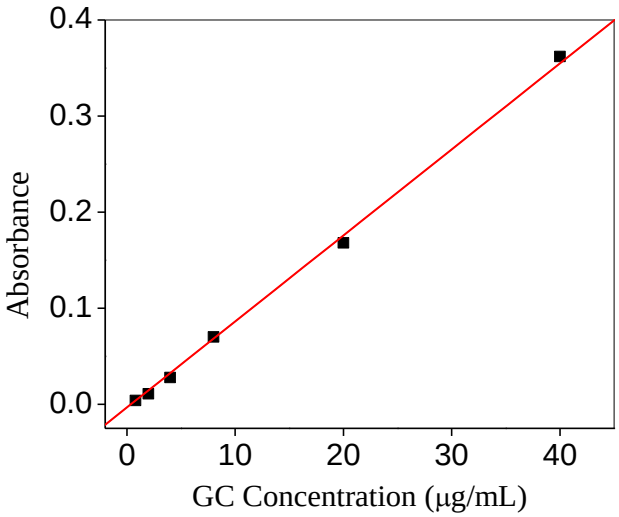

Fig. S4 The calibrated curve of GC concentration.

Compressive Modulus: Mixtures of solutions described above were injected into a 24-well culture plate to obtain columned hydrogels (16.5 mm diameter, 5.0 mm height). Compressive modulus of elasticity was measured in the elastic region of hydrogel using a dynamic mechanical analyzer (DMA-7, Perkin-Elmer) in unconfined compression at a constant stress rate of 40 mN/min up to 20% strain at room temperature. Values reported are an average n = 5, ± standard deviation.

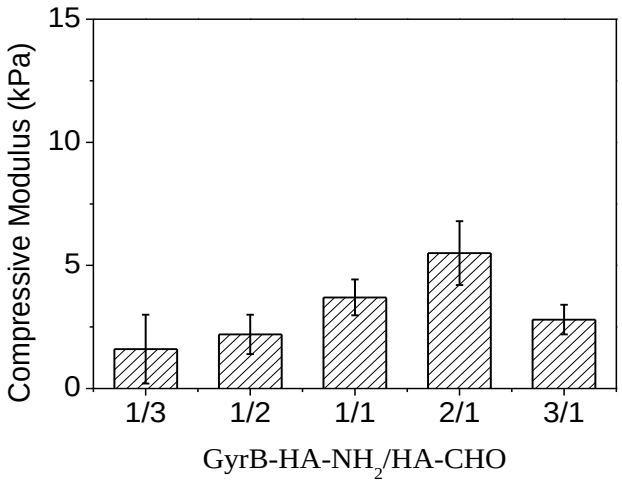

Fig. S5 Compressive modulus of gel-substrates at different ratios of HA-CHO.

The mechanical properties of gel-substrates were characterized after coupling and subsequent dissociation of GyrB-GC by a dynamic mechanical analysis method (Fig.S6). With the 2/1 volume ratio of GyrB-HA-NH_2/HA-CHO, the gel-substrate showed the best stability characterized by the highest value of 5.5 kPa for the compressive modulus. With the conjugation of BMP-2 and sequential coupling of GyrB-GC, the bio-functionalized substrates displayed similar compressive modulus and no significant difference was found ($p > 0.05$).

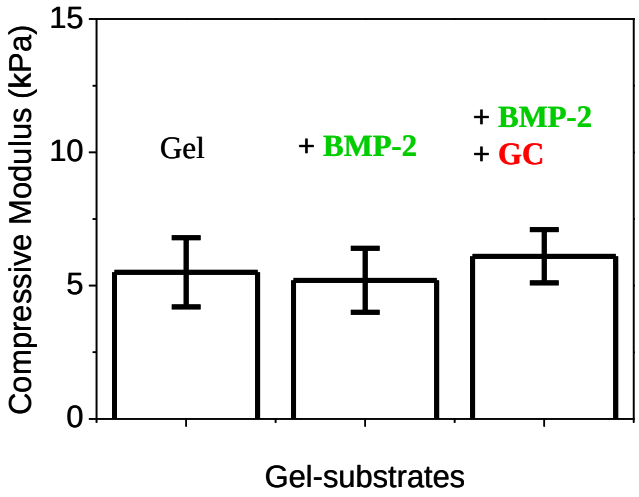

Fig. S6 Compressive modulus of gel-substrates at room temperature.

Substrate Morphologies: The surface morphologies of the gel-substrates were observed after critical point drying by atomic force microscopy (AFM) on a scanning probe microscope (SPA3800N, Japan) with a dynamic force mode.

Swelling Properties: The swelling property of the gel-substrates was determined in PBS at 37 ^{o}C. A known weight (W_0) of the gel-substrates was incubated in PBS for a predetermined time interval at 37 ^{o}C. The wet weight (W_t) of the gel-substrates was determined immediately after the surface-adsorbed water was removed by filter papers. The swelling ratio was defined as $(W_t-W_0)/W_0$.

BMP Release: In vitro BMP-2 release was performed according to the reported procedure [6]. 600 μL of gel-substrates was incubated in 3 mL of the binding buffer (0.1 vol.% BSA, 1 vol.% penicillin-streptomycin in PBS) at 37 ^{o}C. The supernatant was collected and the released amount of BMP-2 was measured using a BMP-2 Quantikine ELISA Kit.

Cell Culture: Human adipose-derived stem cells (ASCs) were isolated from human adipose tissue obtained from elective cosmetic surgery procedures [1,7]. The fat tissues were minced with scissors in the collagenase solution consisted of Hanks' balanced salt solution (3.0 mL/g of fat) (Sigma-Aldrich), bovine serum albumin (fatty acid free, pH 7.0, 3.5 g/100 mL Hanks') (Intergen Company, Purchase, NY) and 1% type II collagenase (3.0 mg/g of fat) (Worthington Biochemical Corporation, Lakewood, NJ). The centrifuge tubes were shaken at 100 rpm for 50 min at 37 °C. Following digestion, the content of each tube was filtered through double-layered sterile gauze. The filtrates were then centrifuged at 1000 rpm for 10 min at 37 °C, and a three layer suspension, consisting of a fatty layer on the top, a serum layer in the middle, and a cellular pellet at the bottom of each tube, was obtained. The fatty layer and most of the supernatant was aspirated off, leaving the pellet intact at the bottom. The pellet in each tube was then suspended in 10 mL of erythrocyte lysis buffer (pH =

7.4), vortexed, and centrifuged again at 1000 rpm for 10 min at 37 °C. The pellets were suspended in the plating medium consisted of DMEM/F12 with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 1% Fungizone (all products obtained from Gibco, Invitrogen Corporation, Carlsbad, CA). Adherent ASCs were expanded for a period of 5–8 days at 37 °C, and the medium was changed every other day until the cells achieved 80% confluence.

The gel-substrates were prepared with sterile PBS on the bottoms of 24-well tissue culture plates. The ASCs were seeded at a density of 8×10^4 cells/well and cultured statically in DMEM culture medium. The ASCs on the gel-substrates were observed under confocal laser scanning microscopy (CLSM, Bio-Rad Radiance 2100) after FDA staining. To assess the kinetics of cellular aggregation, the gel-substrates in 24-well culture plates were rinsed gently with PBS and treated with trypsinization and collagenase type II after a period of time. The detached cellular aggregates were then harvested and counted under a haemocytometer. The cell and aggregate viabilities were measured by the MTT assay.

References

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