Electronic Supplementary Information

Facile formation of microporous chitosan hydrogel based on self-crosslinking

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1. Materials and methods

1.1. Materials

Oligochitosan (MW = 5~10 kDa, Cat. No.: P312015) was purchased from Dibo Chemicals Co., Ltd. (Shanghai, China). N-(β-maleimidopropyloxy)succinimide ester (BMPS, Cat. No.: F01022) was ordered from Highfine Biotech (Suzhou, China). Fluorescein isothiocyanate (FTIC) (Cat. No.: 255493), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Cat. No.: 560965), β-glycerophosphate disodium salt tetrahydrate (Cat. No.: G0195) and dexamethasone 21-acetate (Cat. No.: 278250) were provided by J&K Scientific (Hong Kong, China). Phosphate buffered saline (PBS) powder (Cat. No.: P3813) and ethanol absolute (AR, Cat. No.: 20821.330) were obtained from Sigma-Aldrich and VWR Chemicals (Fontenay-sous-Bois, France), respectively. Dulbecco's modified Eagle medium (DMEM, Cat. No.: 12800017), fetal bovine serum (FBS, Cat. No.: 26140079), penicillin/streptomycin (P/S, Cat. No.: 15140122), amphotericin B (Cat. No.: 15290026), trypsin (Cat. No.: 15090046), trypsin inhibitor (Cat. No.: R007100), tetramethylrhodamine isothiocyanate-labeled phalloidin (phalloidin-TRITC, Cat. No.: R415), RNA purification kit (PureLink®, Cat. No: 12183018A) and one-step qRT-PCR kit (SuperScript® III Platinum® SYBR® Green, Cat. No.: 11736-051) were acquired from Thermo Fisher Scientific. Peptide RGDC (purity>95%) and primers for qRT-PCR were synthesized by ChinaPeptides (Suzhou, China) and Beijing Genomics Institute (Shenzhen, China), respectively. Oil Red O (Cat. No.: O0625) was supplied by Sigma-Aldrich. Rabbit polyclonal anti-collagen I antibody (Cat. No.: ab34710) and Alexa Fluor® 647 donkey polyclonal anti-rabbit IgG H&L secondary antibody (Cat. No.: ab150075) were purchased from Abcam (Hong Kong, China). Unless stated otherwise, the rest chemicals were bought from Sigma-Aldrich and used as received.

1.2. Modification of chitosan

1.2.1. Labeling of chitosan with fluorescent tag

Chitosan (2.1 g) was dissolved in hot PBS solution (50 mL) with agitation. The solution was rapidly cooled down in an ice bath and processed by centrifugation (7,000 g, 10 min), after which the supernatant was collected and passed through 0.22 μm filters (Minisart®, Cat. No.: 16532, Sartorius). Subsequently, the pH of it was adjusted to about 7.0 with NaOH solution (1 M) and chitosan was labeled with fluorescent tag by reaction with FTIC (4 mg in 1 mL ethanol) at ambient temperature for 24 h.

1.2.2. Coupling of chitosan with maleimide

BMPS (164.3, 328.6 or 657.2 mg; 0.726, 1.453 or 2.905 mmol) was added into the chitosan solution (either with or without fluorescent tag) as prepared above and the reaction was performed at ambient temperature for 6 to 8 h. After that, the mixture was transferred into a dialysis bag (Spectra/Por® 3, MWCO: 3.5 kDa, Spectrum Laboratories Inc.) and dialyzed against ultrapure water (NANOpure® Water Purification System, Thermo Scientific Barnstead, 2 L × 10) for 3 days. The newly purified chitosan solution was concentrated by serial centrifugal filtrations with two types of centrifugal filters (preliminary enrichment: Ultracel®, Cat. No.: 4302, 3kDa NMWL, Millipore; advanced enrichment: Amicon® Ultra, Cat. No.: UFC500396, 3 kDa MWCO, Millipore) to a final concentration slightly larger than 5.0 w/v%. Subsequently, it was passed through 0.22 μm filters again, flash frozen in liquid nitrogen and then immediately stored in a -80 °C freezer (MDF-U76VC, SANYO, Osaka, Japan) for later use. The accurate concentration of it was determined by weighing the chitosan solid in 1 mL solution in a 1.5 mL plastic tube (Axygen®, Cat. No.: MCT-150-C, Corning Life Sciences) after lyophilization. As a control, pristine chitosan stock solution was prepared, flash frozen and stored in the same way.

1.3. Formation of microporous chitosan hydrogels

Pristine and the modified chitosan solutions were supplemented with PBS powder (1×) and diluted to varying mass concentrations in PBS solution. Then the solutions were added with 0.5 mM RGDC and sterilized by being passed through 0.22 μm filters. Afterwards, they were loaded into 1.5 mL plastic tubes (80 μL for each, hydrogel columns for morphological study), transferred onto Lab-Tek® II chamber glass slides (Cat. No.: 154526, Thermo Fisher Scientific, 350 μL for each, hydrogel bricks for cell behavior study), or casted into 8 mm (D) × 1.2 mm (h) wells (60 μL for each, hydrogel discs for mechanical test) formed by tightly laying a perforated plastic plate on a piece of thick polydimethylsiloxane (PDMS, Cat No.: RTV615, Momentive Performance Materials, Waterford, USA) membrane. At the very beginning, hydrogel formation was attempted in a -24 °C freezer (LSC263EU, Lec Medical, Merseyside, UK). However, the whole gelation process takes almost one month to complete at this temperature. In order to accelerate the gelation kinetics, specimens were then cured at -8 °C in the chamber of a centrifuge (Avanti JE, Beckman Coulter, Brea, USA) after freeze for 2 h in the -24 °C freezer. The effect of pH on gelation rate was evaluated after supplementing the chitosan precursor solutions with 10 mM, 20 mM or 50 mM NaOH. To investigate the influence of freeze temperature on the inner structure of hydrogel, some specimens were frozen in the -80 °C

freezer (2h) or liquid nitrogen (30 min) ahead of cure in the -8 °C chamber. All the specimens were maintained at frozen until the scheduled time points.

1.4. Characterization of chitosan

1.4.1. Measurement on the melting point of chitosan solution

The melting points of chitosan solutions (in PBS solution) with varying mass concentrations (2 to 5 w/v%) of pristine chitosan were measured by differential scanning calorimetry (DSC, Q1000, TA Instruments, New Castle, USA) during heat-up process. Briefly, a specimen was rapidly cooled down to -40 °C in the closed chamber of the instrument. After freeze for 10 min, the temperature was gradually raised up to 10 °C at a ramp of 2 °C min^{-1}. The generated data were analyzed by TA Universal Analysis 2000 (TA instruments). As controls, the heat-flow curves of ultrapure water and PBS solution were also acquired. Three specimens were tested for each group.

1.4.2. Determination on the degree of modification (DM) of chitosan

The ultrapure water in a chitosan solution was firstly replaced by D_2O with repeated cycles of centrifugal filtration and replenishment of it. Then the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the modified chitosan (around 20 mg mL^{-1} in D_2O) was immediately acquired on a 400 MHz NMR spectrometer (Avance II, Bruker) after 128 scans to obtain a high signal-to-noise (SNR) ratio. The spectrum was further calibrated based on the chemical shift (δ) of HOD (4.8 ppm). As the control, the ^{1}H-NMR spectrum of pristine chitosan in D_2O was also attained. The data were analyzed by MestReNova 6.1.1 (Mestrelab Research).

1.5. Characterization of the hydrogels

1.5.1. Dynamic mechanical analysis (DMA)

A hydrogel disc (prepared in Section 1.3) was placed onto a parallel plate fixture (8 mm in diameter) mounted on a rheometer (ARES, TA Instruments, New Castle, USA). The upper plate was slowly lowered until close contact with the sample. DMA for the hydrogel was then carried out at strain sweep mode (1% to 100% strain at 1 rad s^{-1} frequency) and frequency sweep mode (1 to 100 rad s^{-1} frequency at 5% strain) successively. More than six specimens for each group were tested.

1.5.2. Swelling behaviors of the microporous hydrogels

Chitosan hydrogel precursor solutions (150 μL) were loaded into 2 mL plastic tubes (Axygen®, Cat. No.: MCT-200-C, Corning Life Sciences), which were frozen at -24 °C for 2h and then cured at -8 °C to achieve their full mechanical strength. After that, the specimens were thawed and their weights were determined by subtracting the weights of empty tubes from those of loaded ones. Subsequently, PBS solution (1.5 mL) was added into each tube and the microporous hydrogels were incubated at 37 °C with 5% CO_2. At the scheduled time points, PBS solution was carefully removed and the weights of hydrogels were measured again, after which fresh PBS solution (1.5 mL) was refilled in the tubes until the last measurement. The relative weight of a hydrogel at time *t* is defined as the ratio of its weight at *t* to its initial weight. More than five specimens for each group were tested.

1.5.3. Molecular mechanism on the formation of hydrogel

A chitosan solution or hydrogel was ground with potassium bromide (KBr) powder and then desiccated in vacuum over night. The dried solid was further crumbled into fine particles, after which the sample was pressed into a compact translucent disc (D=13 mm) in a mold under high pressure. The as-prepared disc (2 wt% chitosan) was further dehydrated in vacuum over night and then fixed on a commercial sample holder. Fourier transform infrared (FTIR) spectra for the specimens (16 scans for each) were generated on a Tensor 27 spectrometer (Bruker, Billerica, USA) in the range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}.

1.5.4. Morphological study on the microporous hydrogels

Each hydrogel column (prepared in Section 1.3) at its full mechanical strength was carefully rinsed with PBS for three times, cut into halves and then laid onto a glass coverslip. Afterwards, the fluorescence images of it were taken with a confocal laser scanning microscope (CLSM, TCS SP5 II, Leica). Imaris (Bitplane, Northern Ireland) was used to present 3D visualization of the hydrogels. The images were also analyzed by ImageJ (NIH, USA) to measure the pore size, pore roundness and porosity for the microporous hydrogels. More than three hundred pores from three specimens for each group were analyzed.

1.6. Biological study on the chitosan hydrogels

1.6.1. Differentiation of hESCs into CMs

hESCs (H7, WiCell Research Institute, Madison, USA) were maintained in E8 medium (Cat. No.: A1517001, Thermo Fisher Scientific) in a tissue culture dish (Cat. No.: 93100, TPP). At confluence, the cell monolayer was dispersed with trypsin (0.05 w/v%)/EDTA (0.53 μM). After

neutralization with trypsin inhibitor, the cells were deposited by centrifugation and resuspended in their growth medium. Subsequently, they were seeded onto a 48-well plate (Cat. No.: 92148, TPP) at a density about 5,000 cells/well and cultured until confluence again. Differentiation of hESCs into CMs[1, 2] was induced in RPMI 1640 medium (Cat. No.: 11875093, Thermo Fisher Scientific) supplemented with B27 (Cat. No.: 17504044, Thermo Fisher Scientific) and CHIR-99021 (6 μM, Cat. No.: S2924, Selleckchem) for days 0 to 2, followed by the addition of IWR-1 (10 μM, Cat. No.: BML-WN103-0005, Enzo Life Sciences) for days 3 to 5. Beating was observed on day 7 or 8, and the derived CMs were maintained in RPMI 1640 medium with B27 containing insulin (Cat. No.: A1895601, Thermo Fisher Scientific).

1.6.2. Proliferation of hAMSCs

hAMSCs (Cat. No.: PCS-500-011, ATCC) were raised in their basal growth medium (Cat. No.: PCS-500-030, ATCC) supplemented with the growth kit (Cat. No.: PCS-500-040, ATCC). They were subcultured at confluence until passage 8. For the biocompatibility test of the maleimide-modified chitosan, some of the cells were seeded onto a 48-well plate at the density about 5,000 cells/well as well and cultured until confluence.

1.6.3. Biocompatibility of the maleimide-modified chitosan

Both hESC-CMs and hAMSCs were untreated or incubated with the addition of 1 μg mL^{-1} to 10 mg mL^{-1} chitosan-BMPS(8.7) for 24 h, after which their media were renewed. Before live/dead staining, the beating of hESC-CMs was recorded on a live cell observation station (LCOS, consisted of a Nikon TiE-PFS microscope, a Chamlide TC chamber, and an Andor Zyla sCMOS camera).[3] For live/dead staining, the cell media were added with fluorescein diacetate and propidium iodide (10 μg mL^{-1} for both). After incubation for 30 min, the old media were removed and the specimens were gently washed with fresh media for three times. Fluorescence images were immediately taken using an upright epifluorescence microscope (BX41, Olympus) equipped with a computerized charge-coupled device (CCD) camera (12.0 Monochrome w/o IR-18, Diagnostic Instruments). ImageJ was used to count the number of cells in each image and the cell viability was defined as the proportion of live cells. Five specimens for each treatment were examined.

1.6.4. Culture of hAMSCs on the hydrogels

At passage 8, some hAMSCs were dispersed with trypsin (0.05 w/v%)/EDTA (0.53 μM). After neutralization with trypsin inhibitor, the cells were deposited by centrifugation and resuspended

in an osteogenic induction medium (DMEM supplemented with 10 v/v% FBS, 100 U mL^{-1} P/S, 1 μg mL^{-1} amphotericin B, 10 mM β-glycerol phosphate disodium salt, 50 μg mL^{-1} L-ascorbic acid and 100 nM dexamethasone 21-acetate).[4, 5] Cells were seeded onto the hydrogel bricks (prepared in Section 1.3) at a density about 10,000 cells/cm^2 and cultured in the induction medium for up to 7 days with the medium refreshed every other day.

1.6.5. Morphological study of hAMSCs on the hydrogels

On day 3 or day 7, hAMSCs grown on some hydrogels were fixed with paraformaldehyde (4 wt% in PBS, 2 h), permeabilized with Triton X-100 (0.2 v/v% in PBS, 2 h), and then blocked with bovine serum albumin (5 wt% in PBS, overnight). Subsequently, they were gently washed with PBS and then stained with the mixed solution of DAPI (10 μg mL^{-1} in PBS) and phalloidin-TRITC (1 μg mL^{-1} in PBS) for a day. The specimens were extensively washed and immersed in PBS for another day before being placed onto glass coverslips. The fluorescence images of them were also taken with CLSM and presented in 3D using Imaris.

1.6.6. Real-time qRT-PCR analysis on the differentiation of hAMSCs

Also on day 3 or 7, the total RNA of hAMSCs grown on some hydrogels was extracted using the RNA purification kit following the manufacturer's protocol. To quantify the expression level of a gene, real-time qRT-PCR was run on a PCR system (LightCycler® 480 Instrument II, Roche, Basel, Switzerland) with the one-step qRT-PCR kit. The procedures for running qRT-PCR are: cDNA synthesis (50 °C for 3 min), denaturation (95 °C for 5 min), amplification (45 cycles of 95 °C for 15 s and 60 °C for 30 s), melting (95 °C for 5 s, 65 °C for 1 min and 0.11 °C s^{-1} to 97 °C), and cooling (40°C for 30 s). As the external reference, some hAMSCs right before seeding (day 0) were also analyzed by real-time qRT-PCR. The relative expression level of a target gene (y) was calculated using double normalizations, i.e. y = ([Target gene]$_{\text{group X}}$/[ACTB]$_{\text{group X}}$) : ([Target gene]$_{\text{day 0}}$/[ACTB]$_{\text{day 0}}$), where housing-keeping gene β-actin was used as the internal reference. The genes assessed and the sequences for their primers are listed in Table S2.[6-12]

1.6.7. Oil Red O staining of hAMSCs

On day 7, hAMSCs grown on some hydrogels were fixed with paraformaldehyde (4 wt% in PBS, 2 h). Subsequently, they were gently washed with PBS, incubated with 60% isopropanol solution for 5 min and then stained with Oil Red O working solution (1 mg mL^{-1} in 60% isopropanol) for 20 min. Afterwards, the solution was discarded and the samples were

extensively rinsed with ultrapure water. Bright-field images of them were taken with an optical microscope (AZ100, Nikon).

1.6.8. Immunocytochemical staining of type I collagen protein

Also on day 7, hAMSCs grown on the remaining hydrogels were fixed with paraformaldehyde (4 wt% in PBS, 2 h), permeabilized with Triton X-100 (0.2 v/v% in PBS, 2 h), and then blocked with bovine serum albumin (5 wt% in PBS, overnight). Subsequently, they were gently washed with PBS and then incubated with rabbit polyclonal anti-collagen I antibody (1/100 dilution in PBS, overnight). Afterwards, the specimens were gently washed with PBS again and stained with Alexa Fluor® 647 donkey polyclonal anti-rabbit IgG H&L secondary antibody (1/100 dilution in PBS) and DAPI (10 μg mL^{-1} in PBS) for 2 h. The fluorescence images of them were also taken with CLSM after extensive rinsing.

1.6. Statistical Analysis

All the experimental data were presented as mean ± standard deviation. Statistical significance was assessed by Welch's t-test with a homemade program in Matlab R2013b (The MathWorks)

2. Supplementary results

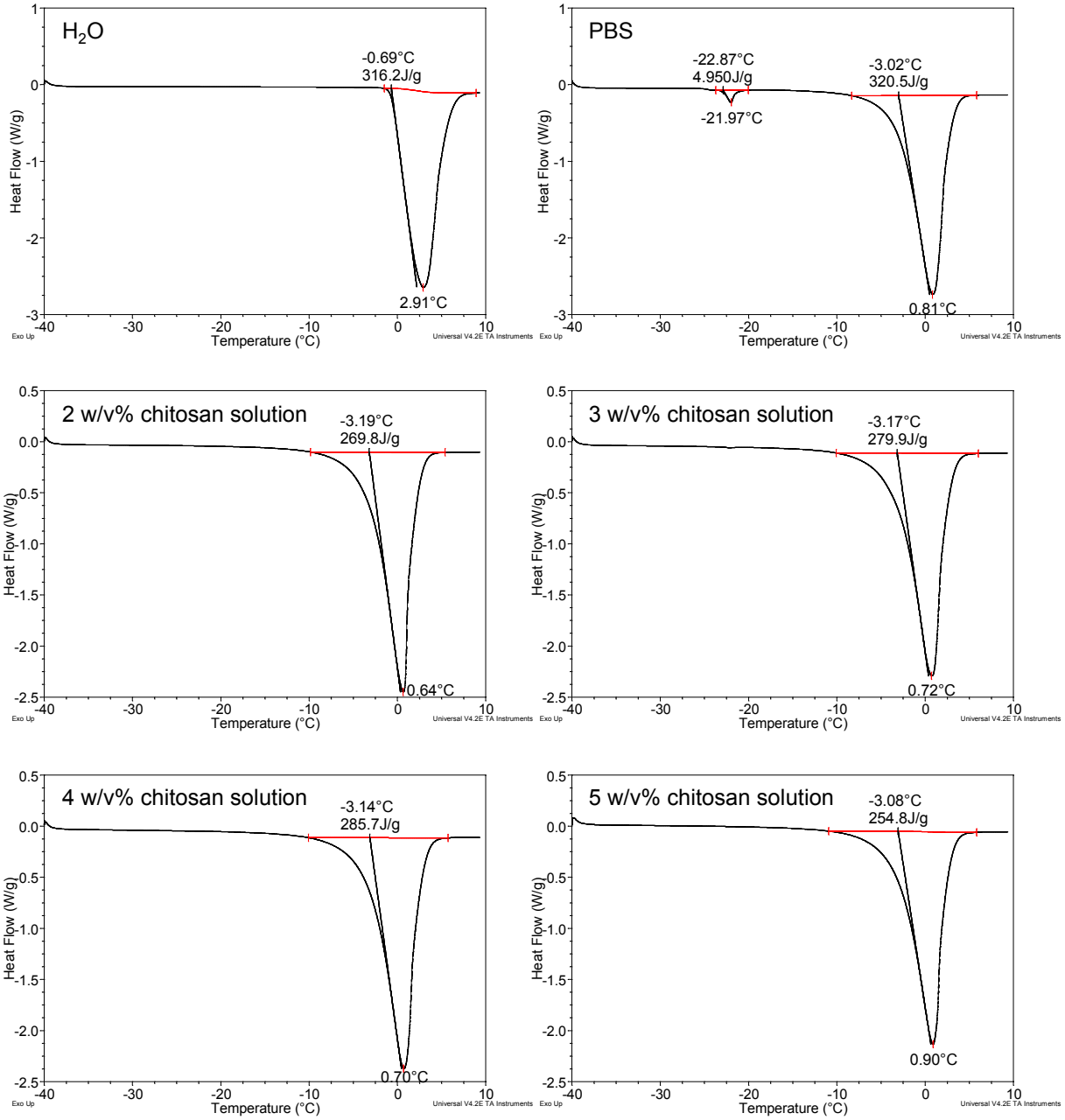

Fig. S1 Representative heat-flow curves during heat-up process presented by differential scanning calorimetry (DSC), showing the melting points of frozen ultrapure water, PBS, and chitosan solutions (2 to 5 w/v%).

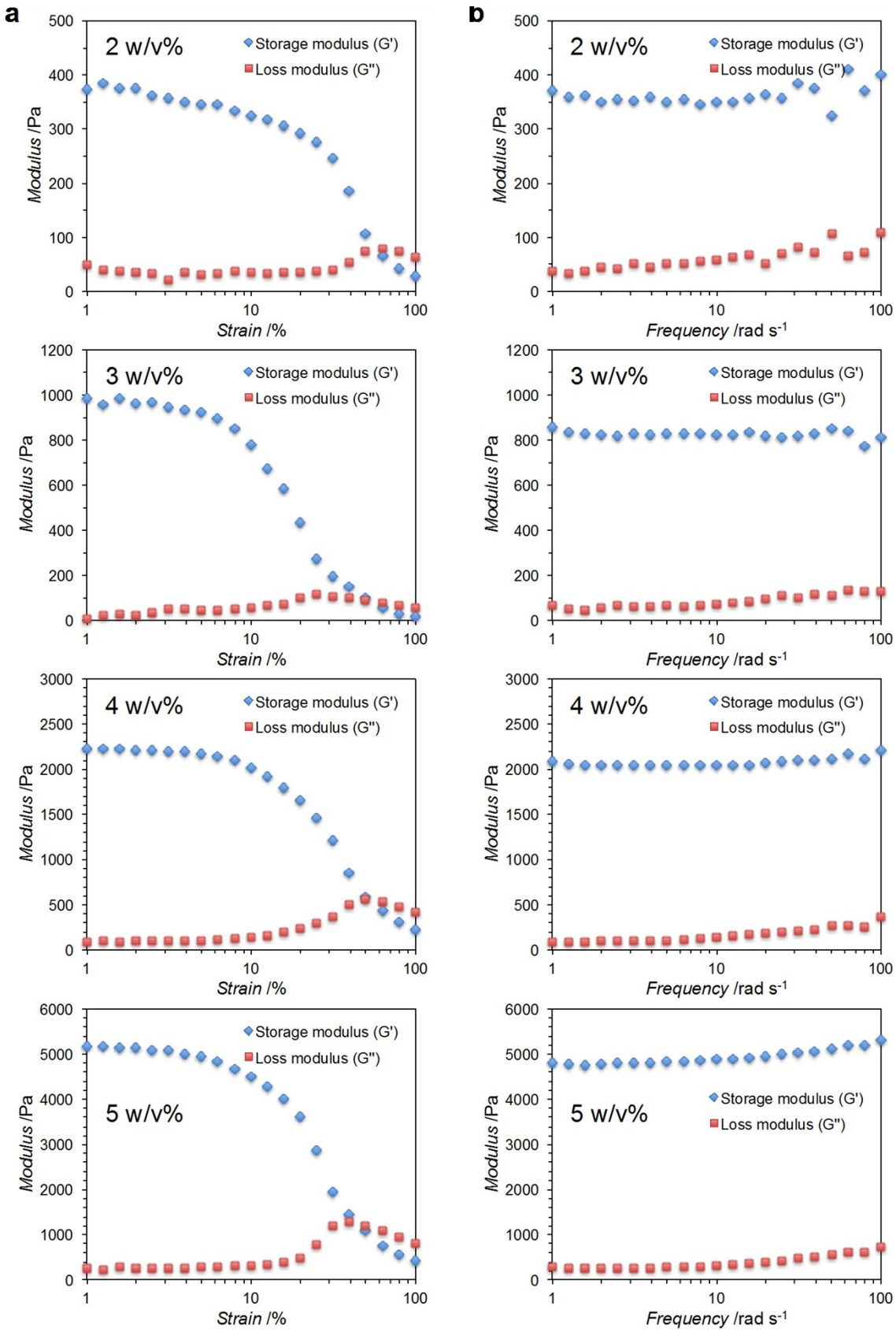

Fig. S2 Dynamic mechanical properties of the microporous hydrogels with varying mass concentrations of chitosan-BMPS(8.7), which were prepared after freeze at -24 °C for 2 h and then cure at -8 °C until their full mechanical strength. The measurements were performed at (**a**) strain sweep mode (1% to 100% strain at 1 rad s^{-1} frequency) or (**b**) frequency sweep mode (1 to 100 rad s^{-1} frequency at 5% strain).

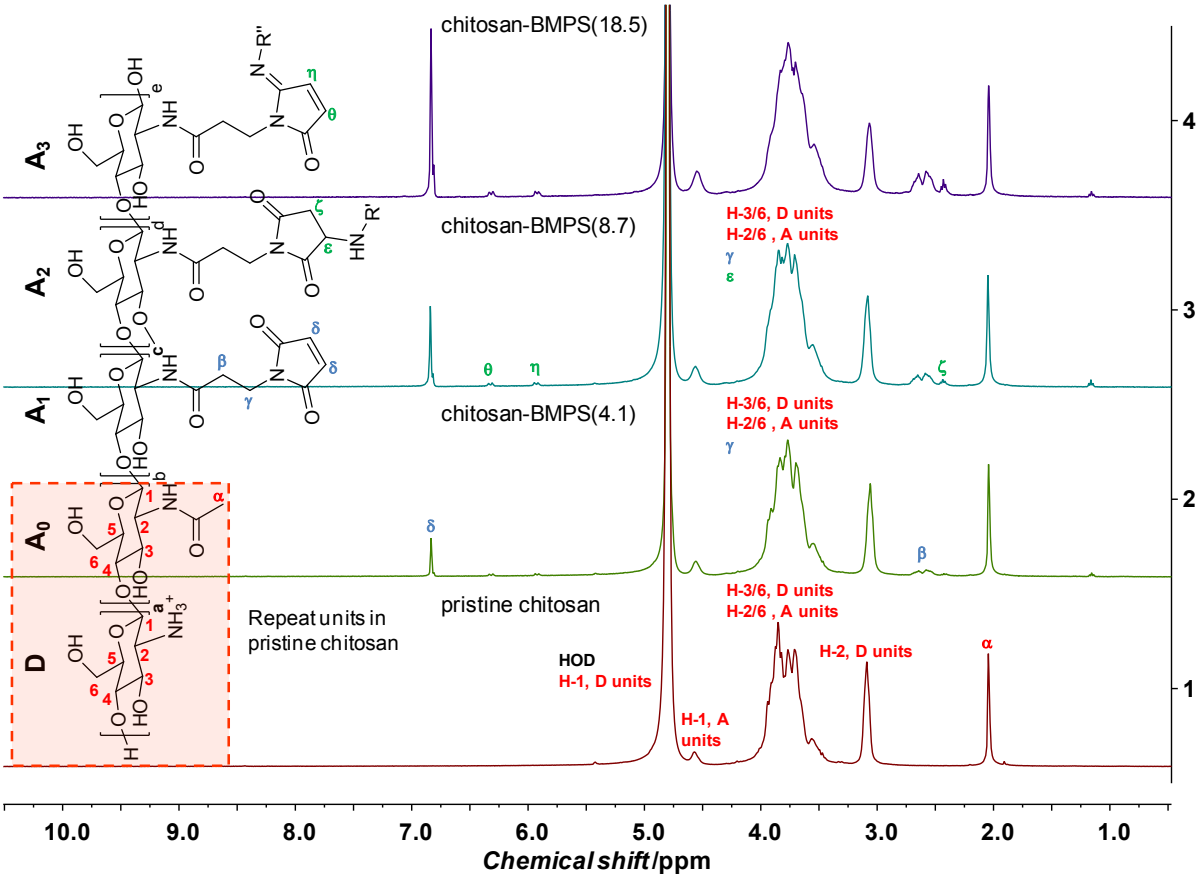

Fig. S3 Proton nuclear magnetic resonance (^{1}H-NMR) spectra of pristine and the modified chitosan at varying degrees of modification (DM). The assignments of peaks to the corresponding protons were designated.

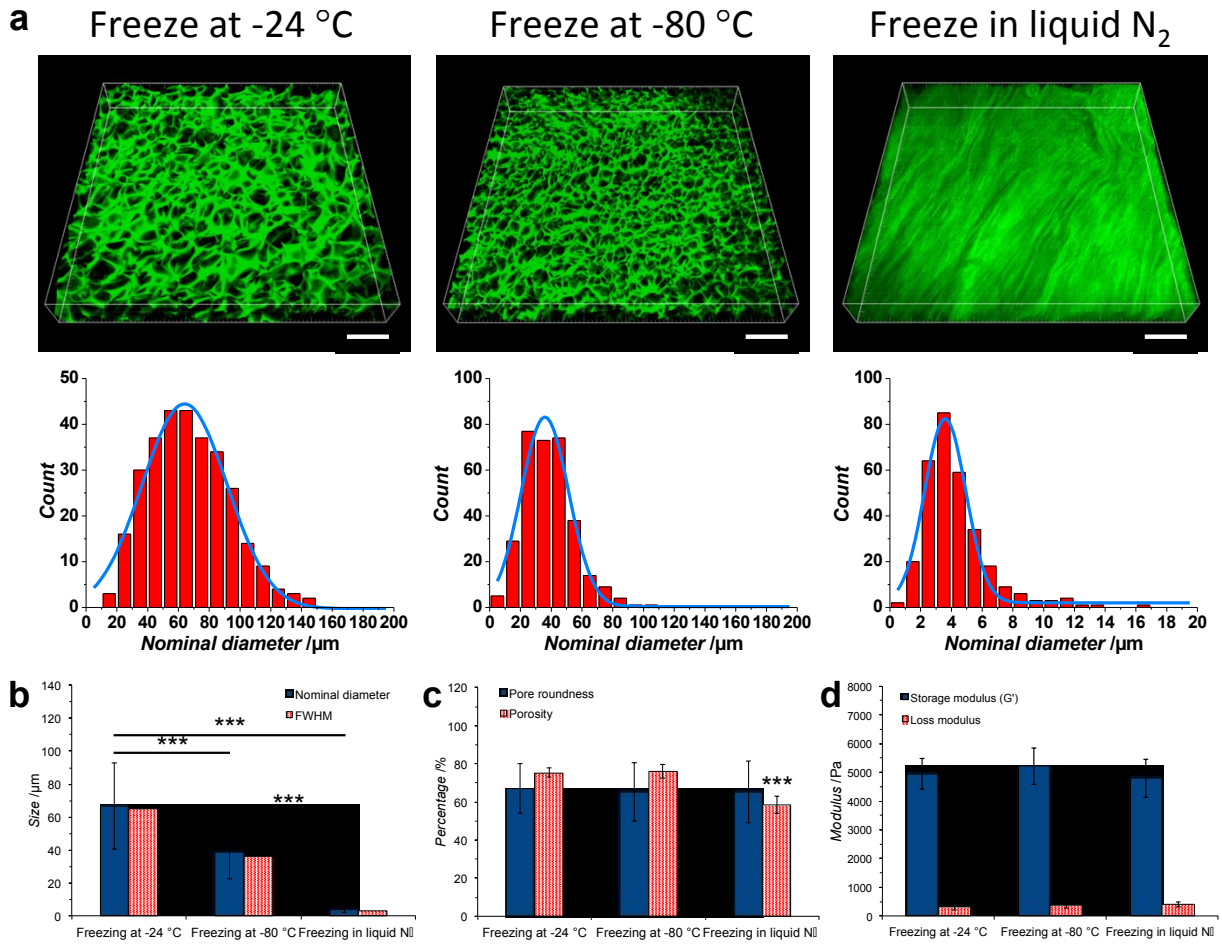

Fig. S4 Morphological and rheological studies on the hydrogels with 5 w/v% chitosan-BMPS(8.7), which were prepared after freeze at varying temperatures and then cure at -8 °C until their full mechanical strength. (**a**) CLSM images (top) displaying their inner structures and histograms (bottom) revealing their pore size distributions. (Scale bar: 100 μm) (**b**) Quantification on pore size and its distribution for the hydrogels as functions of freeze temperature. (**c**) Quantification on pore roundness and porosity for the hydrogels as functions of the freeze temperature. (**d**) Rheological properties of the hydrogels at 5 % shear strain and 1 rad s^{-1} shear frequency as functions of the freeze temperature. (***P<0.001, compared between the two designated groups or to other groups.)

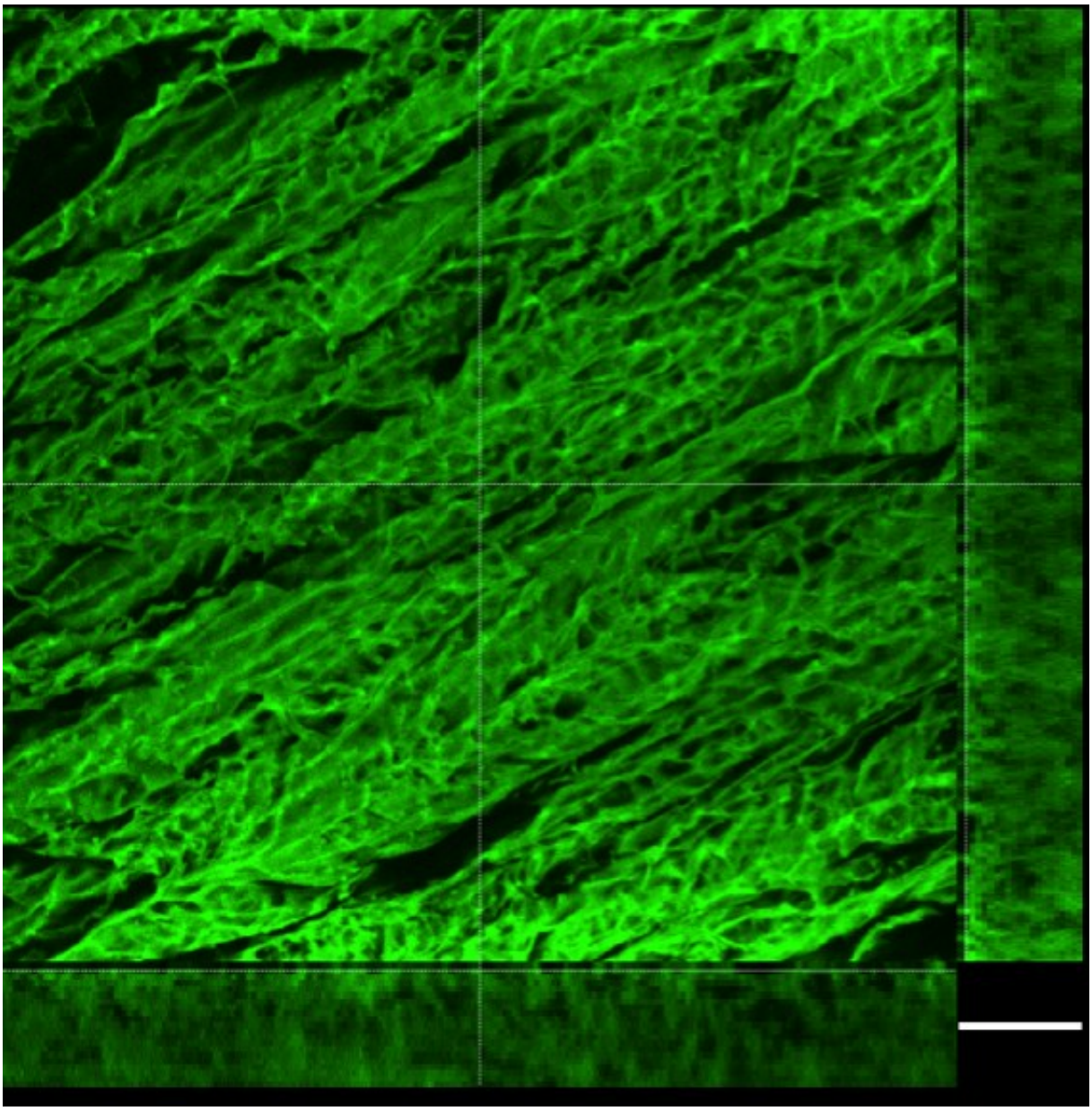

Fig. S5 CLSM image at a higher magnification showing the inner structure of a hydrogel with 5 w/v% chitosan-BMPS(8.7), which was prepared after freeze in liquid N_2 and then cure at -8 °C until its full mechanical strength. (Scale bar: 20 μm)

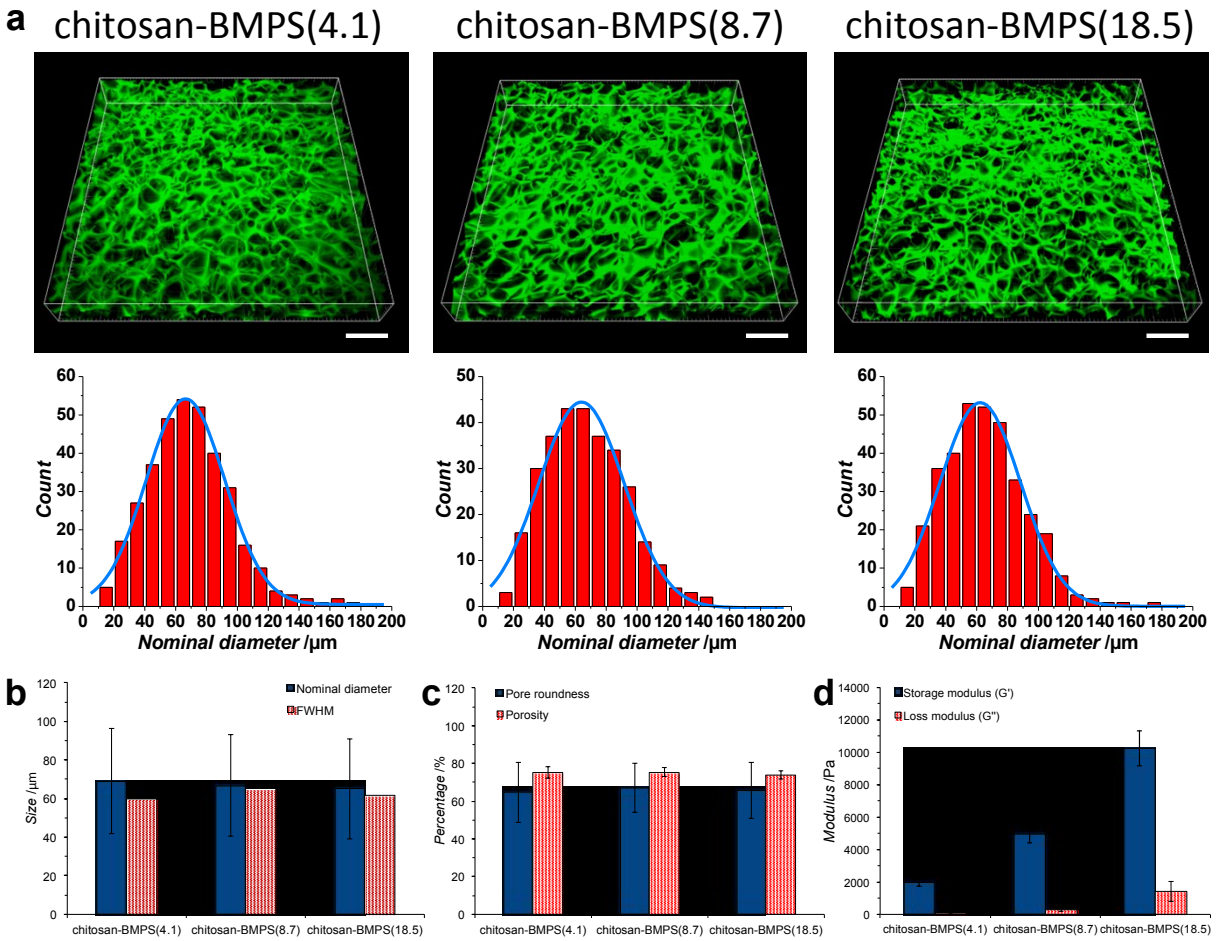

Fig. S6 Morphological and rheological studies on the hydrogels with 5 w/v% modified chitosan at varying DM, which were prepared after freeze at -24 °C for 2 h and then cure at -8 °C until their full mechanical strength. (**a**) CLSM images (top) displaying their inner structures and histograms (bottom) revealing their pore size distributions. (Scale bar: 100 μm) (**b**) Quantification on pore size and its distribution for the hydrogels as functions of DM. (**c**) Quantification on pore roundness and porosity for the hydrogels as functions of DM. (**d**) Rheological properties of the hydrogels at 5% shear strain and 1 rad s^{-1} shear frequency as functions of DM.

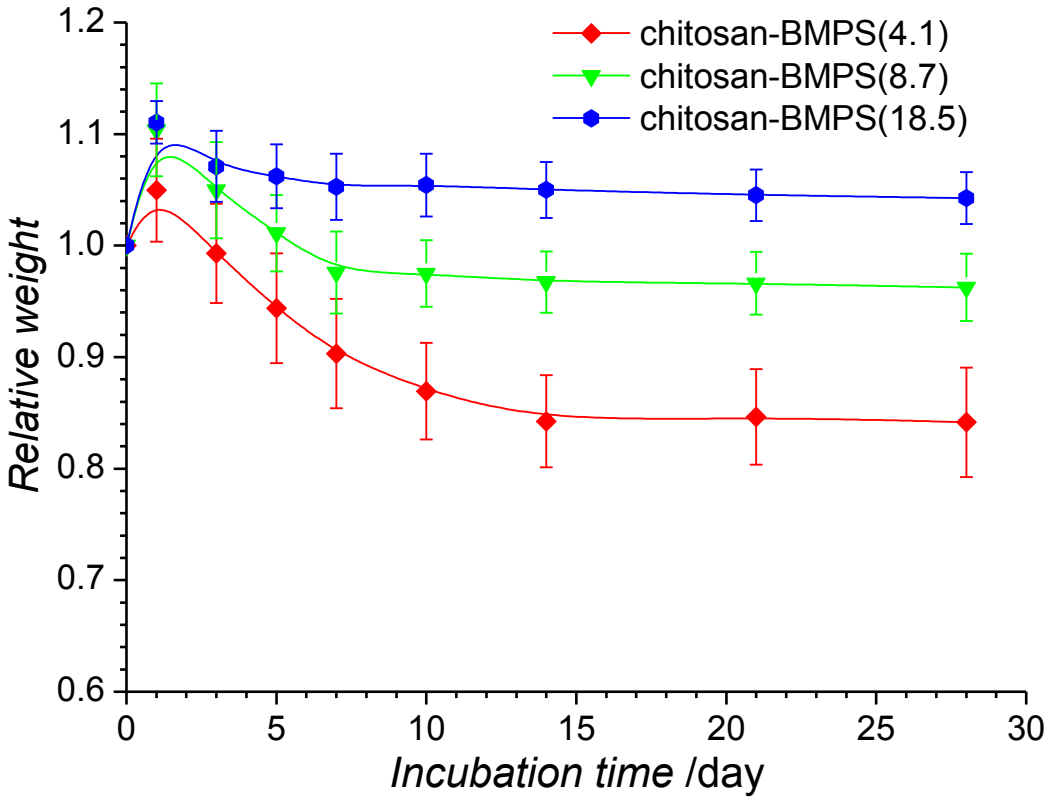

Fig. S7 Swelling behaviors of the microporous hydrogels with 5 w/v% modified chitosan at varying DM, which were prepared after freeze at -24 °C for 2 h and then cure at -8 °C until their full mechanical strength.

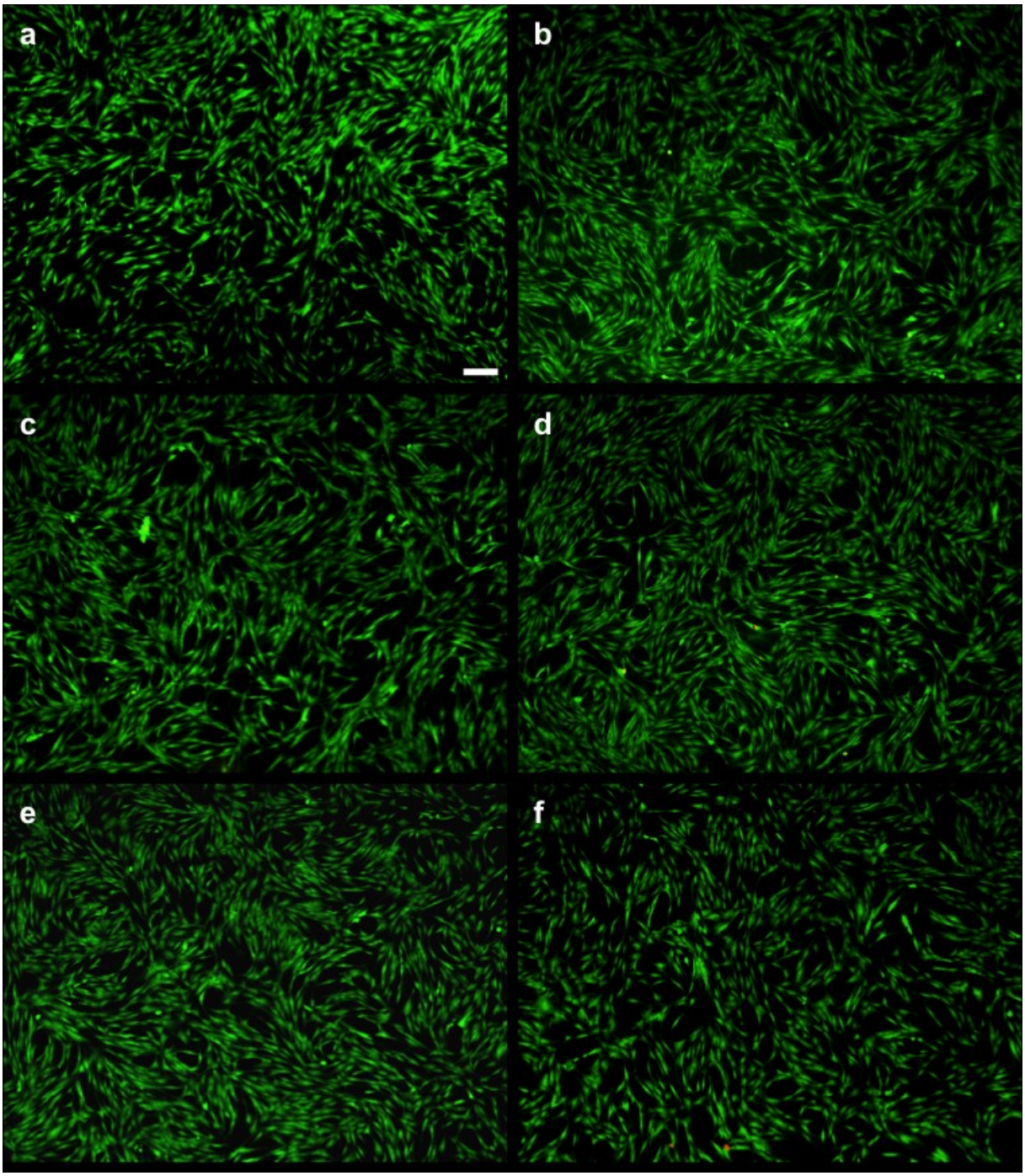

Fig. S8 Live/dead staining of untreated hAMSCs (**a**) and those after 24 h incubation with 1 μg mL^{-1} (**b**), 10 μg mL^{-1} (**c**), 100 μg mL^{-1} (**d**), 1 mg mL^{-1} (**e**), or 10 mg mL^{-1} (**f**) chitosan-BMPS(8.7). Live cells were stained in green (FDA) while dead ones in red (PI). The cell viabilities for groups (**a**) to (**f**) were 99.5±0.3%, 99.5±0.4%, 99.8±0.3%, 99.5±0.5%, 99.7±0.4%, and 99.8±0.2%, respectively. (Scale bar: 200 μm)

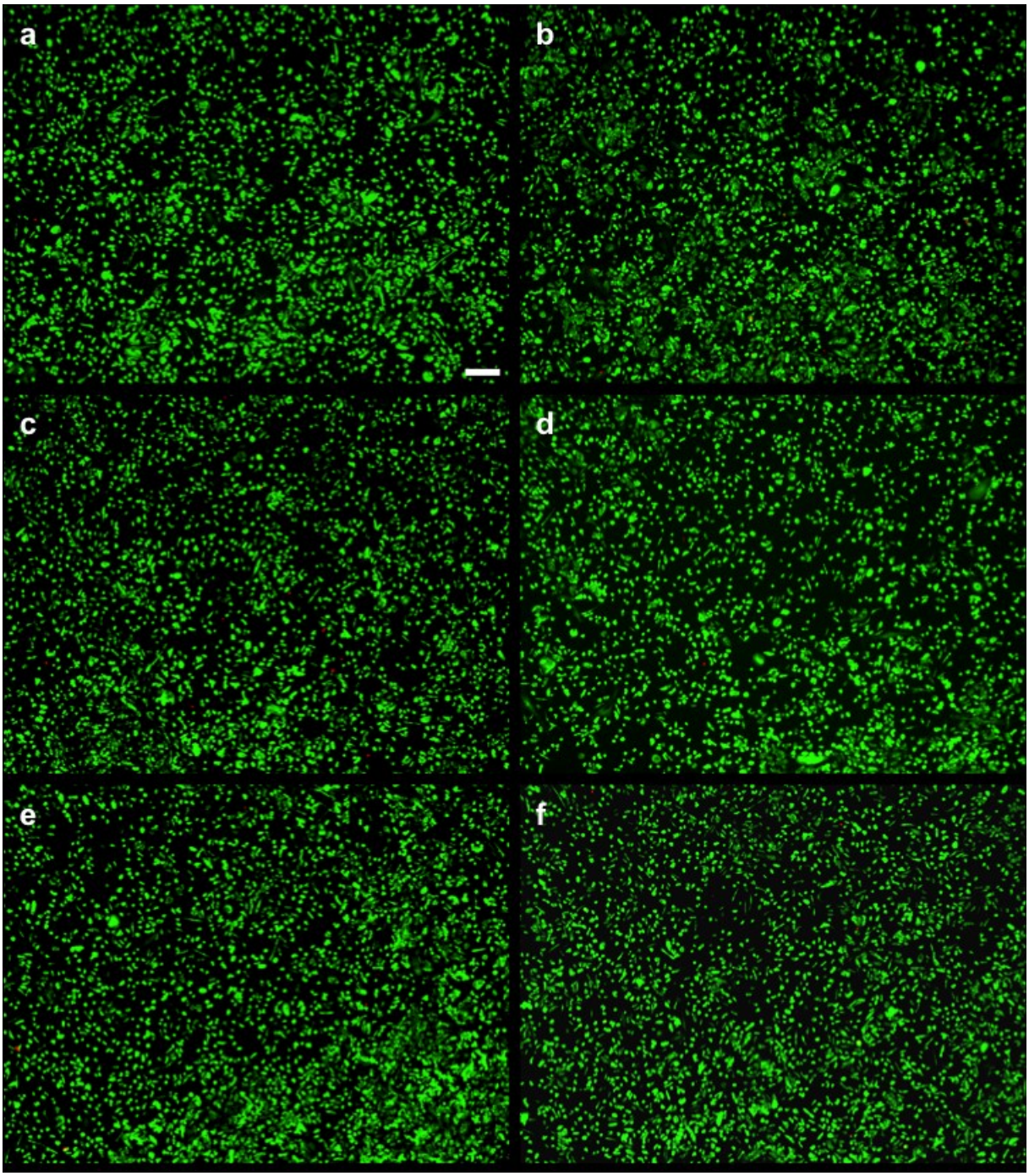

Fig. S9 Live/dead staining of untreated hESC-CMs (**a**) and those after 24 h incubation with 1 μg mL^{-1} (**b**), 10 μg mL^{-1} (**c**), 100 μg mL^{-1} (**d**), 1 mg mL^{-1} (**e**), or 10 mg mL^{-1} (**f**) chitosan-BMPS(8.7). Live cells were stained in green (FDA) while dead ones in red (PI). The cell viabilities for groups (**a**) to (**f**) were 99.5±0.3%, 99.5±0.3%, 99.4±0.3%, 99.4±0.2%, 99.7±0.1%, and 99.1±0.2%, respectively. (Scale bar: 200 μm)

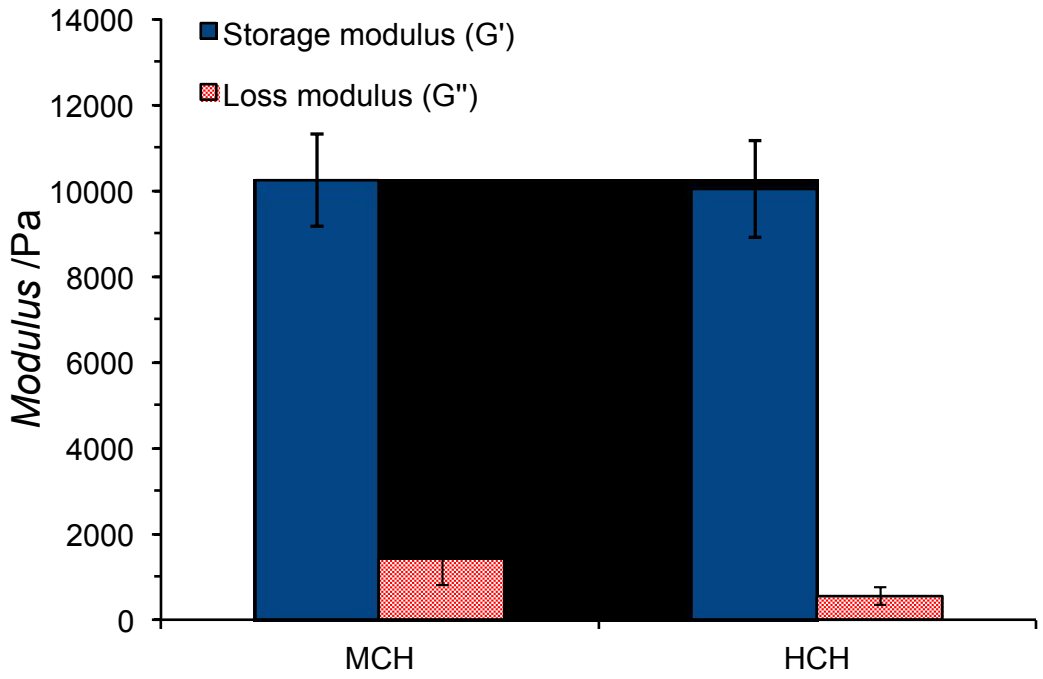

Fig. S10 Comparison in shear moduli between the microporous chitosan hydrogel (MCH) and homogenous chitosan hydrogel (HCH) made of 5 w/v% chitosan-BMPS(18.5). The measurements were performed at 5% shear strain and 1 rad s^{-1} shear frequency.

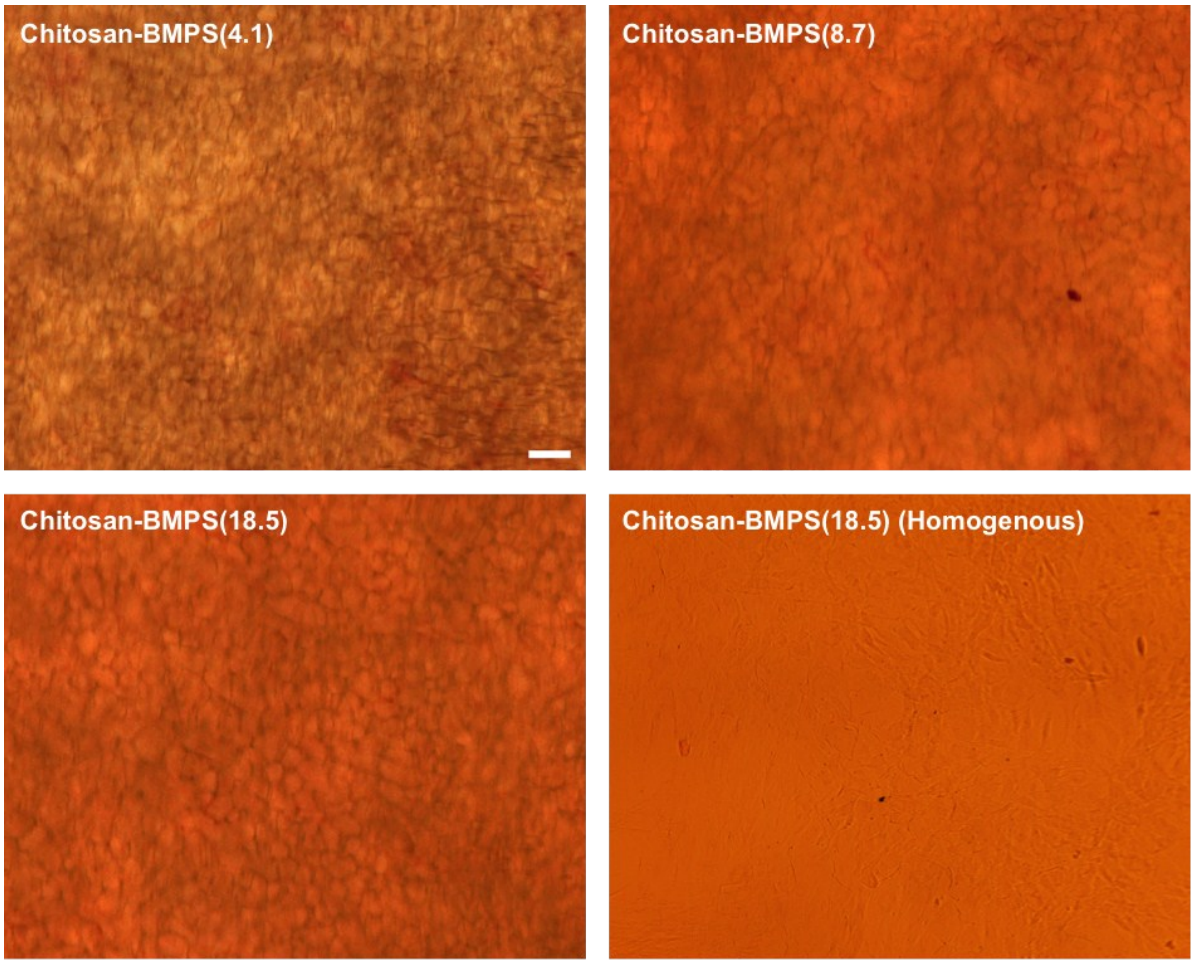

Fig. S11 Oil Red O staining of hAMSCs on various chitosan hydrogels after 7 days in the osteogenic induction medium. (Scale bar: 100 μm)

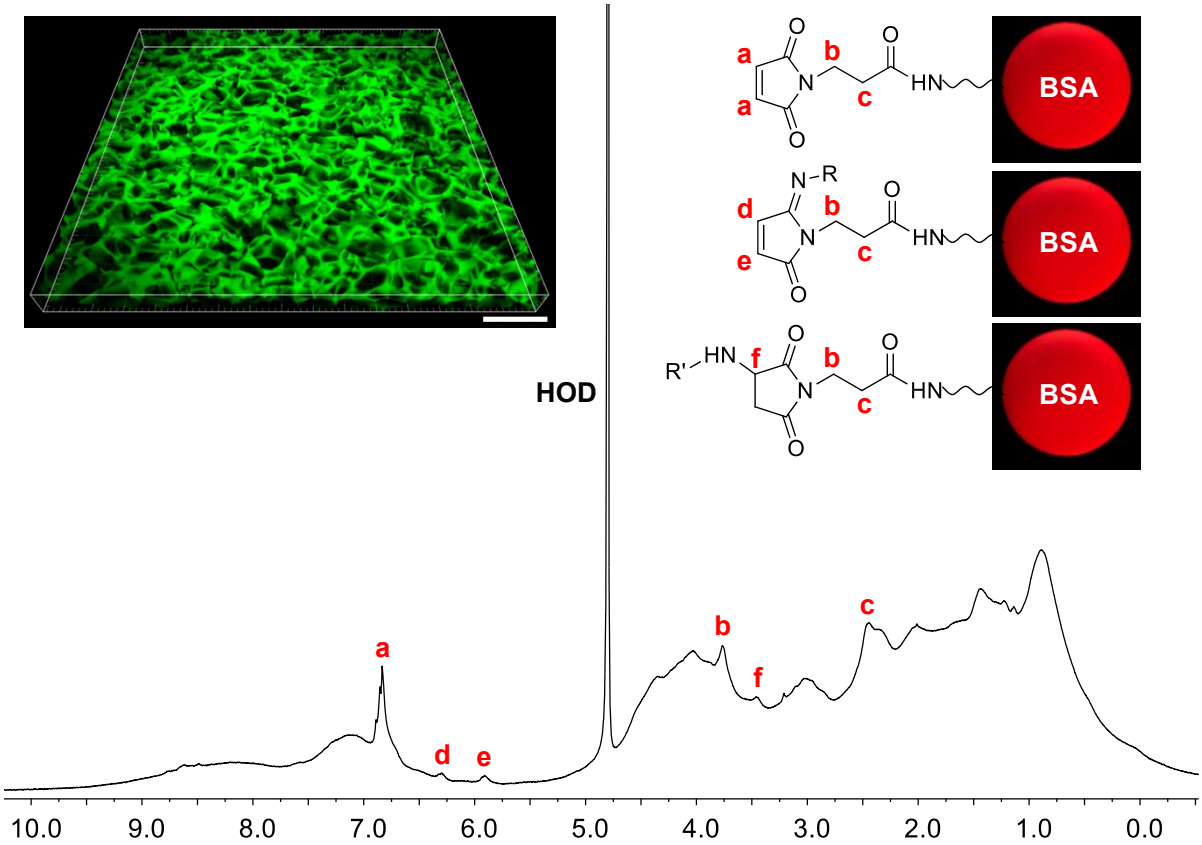

Fig. S12 ^{1}H-NMR spectrum of BSA-BMPS and CLSM image (inset) of a microporous hydrogel (10 w/v%) made of it. (Scale bar: 100 μm)

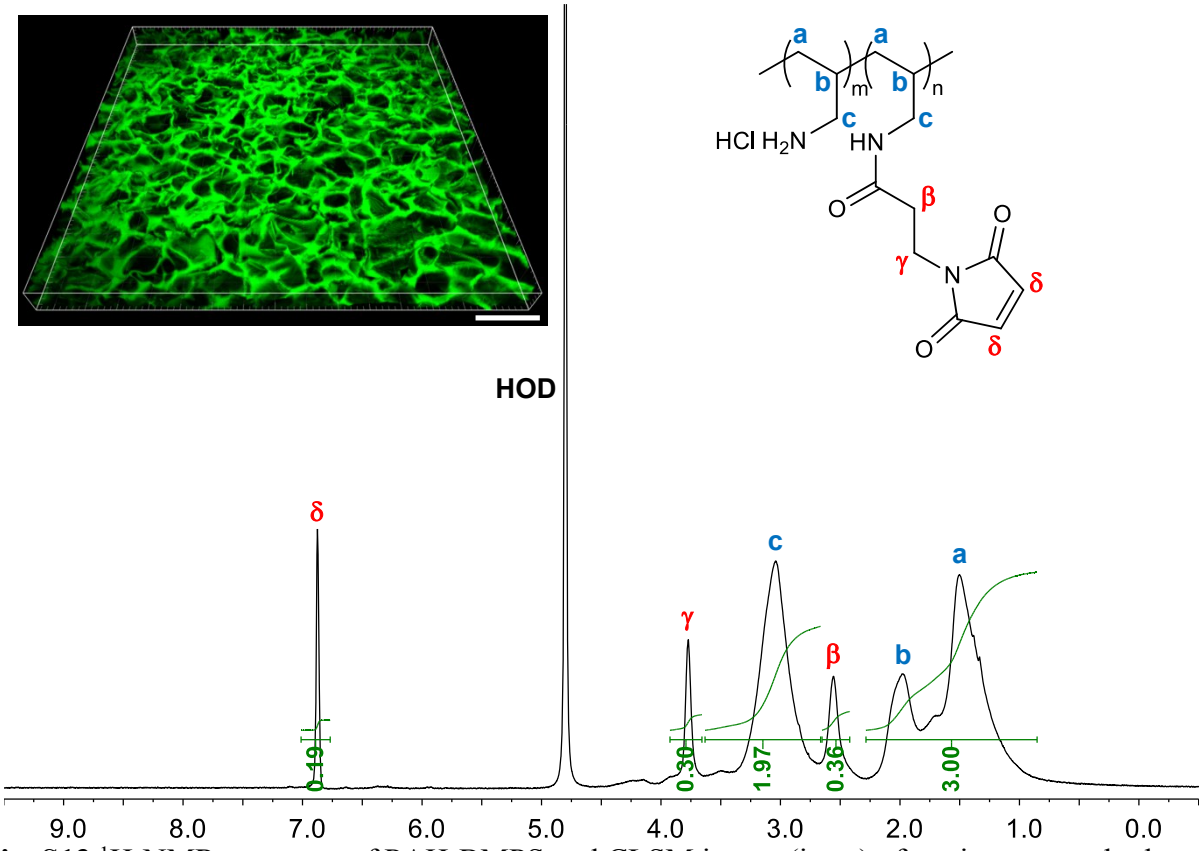

Fig. S13 ^{1}H-NMR spectrum of PAH-BMPS and CLSM image (inset) of a microporous hydrogel (10 w/v%) made of it. (Scale bar: 100 μm)

Table S1 Melting points for H_2O, PBS and chitosan solutions determined by DSC

Group	Measured melting point [°C]	Calibrated melting point [°C]
H_2O	-0.65±0.12	0
PBS	-3.11±0.15	-2.46±0.19
2 w/v% chitosan solution	-3.22±0.05	-2.57±0.13
3 w/v% chitosan solution	-3.19±0.06	-2.54±0.13
4 w/v% chitosan solution	-3.19±0.11	-2.54±0.16
5 w/v% chitosan solution	-3.13±0.07	-2.48±0.14

Table S2 Genes assessed and the sequences for their primers used in qRT-PCR[6-12]

Gene	Alias	Direction	Sequence (5'-3')
β-actin	ATCB	Forward	TGGCACCCAGCACAATGAAG
		Reverse	AGCATTTGCGGTGGACGATG
Fatty acid binding protein 4	FABP4	Forward	AACCTTAGATGGGGGTGTCC
		Reverse	TCGTGGAAGTGACGCCTTTC
Fatty acid transport protein 1	FATP1	Forward	CCACTTGGATGTCACCACTG
		Reverse	TGGGACCCTCCAGTAGACAC
Desmin	DES	Forward	GGCAGCCAACAAGAACAACG
		Reverse	ATTCCCGCATCTGCCTCATC
Myogenic differentiation	MyoD	Forward	GAACCCCAACCCGATATACC
		Reverse	CTCCTACCTCAAGAGATGGC
Alkaline phosphatase	ALPL	Forward	CTCCCACTTCATCTGGAACC
		Reverse	TCAGCTCGTACTGCATGTCC
Bone morphogenetic protein-2	BMP-2	Forward	TGCTAGACCTGTATCGCAGG
		Reverse	TCCGGGTTGTTTTCCCACTC
Osteocalcin	OCN	Forward	TCACACTCCTCGCCCTATTG
		Reverse	CCTCCTGCTTGGACACAAAG
Collagen, type I, alpha 1	COL1A1	Forward	AACAGCCGCTTCACCTACAG
		Reverse	CAACGTCGAAGCCGAATTCC

References

1. K. R. Boheler, S. Bhattacharya, E. M. Kropp, S. Chuppa, D. R. Riordon, D. Bausch-Fluck, P. W. Burridge, J. C. Wu, R. P. Wersto, G. C. F. Chan, S. Rao, B. Wollscheid and R. L. Gundry, *Stem Cell Rep.*, 2014, **3**, 185.
2. Y. Wang, Z. C. Li, P. Zhang, E. Poon, C. W. Kong, K. R. Boheler, Y. Huang, R. A. Li and X. Yao, *Stem Cells*, 2015, **33**, 2973.
3. L. Huang, Y. Chen, Y. Chen and H. Wu, *Anal. Chem.*, 2015, **87**, 12169.
4. N. Huebsch, P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano and D. J. Mooney, *Nat. Mater.*, 2010, **9**, 518.
5. X. Tong and F. Yang, *Adv. Mater.*, 2016, **28**, 7257.
6. O. Frank, M. Heim, M. Jakob, A. Barbero, D. Schäfer, I. Bendik, W. Dick, M. Heberer and I. Martin, *J. Cell. Biochem.*, 2002, **85**, 737.
7. P.-C. Tseng, S.-M. Hou, R.-J. Chen, H.-W. Peng, C.-F. Hsieh, M.-L. Kuo and M.-L. Yen, *J. Bone Miner. Res.*, 2011, **26**, 2552.
8. S.-H. Park, E. S. Gil, H. Shi, H. J. Kim, K. Lee and D. L. Kaplan, *Biomaterials*, 2010, **31**, 6162.
9. S. Chen, S. Takanashi, Q. Zhang, W. Xiong, S. Zhu, E. C. Peters, S. Ding and P. G. Schultz, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 10482.
10. R. W. Mitchell, N. H. On, M. R. Del Bigio, D. W. Miller and G. M. Hatch, *J. Neurochem.*, 2011, **117**, 735.
11. R. Sarugaser, L. Hanoun, A. Keating, W. L. Stanford and J. E. Davies, *PloS One*, 2009, **4**, e6498.
12. R. Galli, U. Borello, A. Gritti, A. G. Minasi, C. Bjornson, M. Coletta, M. Mora, M. G. C. De Angelis, R. Fiocco, G. Cossu and A. L. Vescovi, *Nat. Neurosci.*, 2000, **3**, 986.