Electronic Supplementary Information (ESI)

Hyperbranched PEG-based Multi-NHS Polymer and Bioconjugation with BSA

Sigen A †[a], Qian Xu †[a], Dezhong Zhou[a], Yongsheng Gao[a], Jeddah Marie Vasquez[a], Udo Greiser[a], Wei Wang[b, a]*, Wenguang Liu[b], Wenxin Wang[a,b]*

[a] *Charles Institute of Dermatology, School of Medicine, University College Dublin, Dublin 4, Ireland.*

[b] *School of Materials Science and Engineering, Tianjin Key Laboratory of Composite and Functional Materials, Tianjin University, Tianjin 300350, China.*

† *These authors contributed equally to this work.*

* *Corresponding authors.*

Email: wenxin.wang@ucd.ie
wwgfz@tju.edu.cn

Materials and Methods

Materials: Poly (ethylene glycol) diacrylate (PEGDA, Mn = 700 g/mol), ethyl α-bromoisobutyrate (EBriB, 98%), $CuCl_2$ (99%), N,N,N',N',N''-pentamethyldiethylenetriamine (PMDETA, 99%), L-ascorbic acid (AA, 99%), 3-mercaptopropionic acid (3-MPA, 99%), N-hydroxysuccinimide (NHS, 98%), bovine serum albumin (BSA, 96%), diethyl ether (99.5%), n-hexane (95%) and chloroform-d (99.8 atom % D) were purchased from Sigma-Aldrich and used without purification. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) was purchased from Thermo Scientific. Methyl ethyl ketone (MEK, 99%), tetrahydrofuran (THF, 99.9%), methanol (99.99%) and acetone (99.97%) were purchased from Fisher and used as received. Aluminium oxide (basic, 50-200 μm, 60A) was purchased from Acros Organics. Dialysis tubing (MWCO = 2 kDa) was purchased from SpectrumLab.

Instruments: ^{1}H-NMR spectra were collected on a Varian 400 MHz NMR spectrometer using chloroform-d as deuterated solvent. Tetramethylsilane (TMS, 0 ppm) was used as the internal standard with a concentration of 0.03% (v/v). Number-average molecular weight (Mn), weight-average molecular weight (Mw), polydispersity index (PDI) and the Mark−Houwink exponent were determined by gel permeation chromatography (Agilent 1260 infinity triple-detector GPC).

DMF containing 0.1% LiBr was used as the mobile phase at the flow rate of 1 ml/min with the column temperature of 60 °C. Rheological assessments were conducted on HR-2 Rheometer equipped with a 20 mm steel parallel-plate geometry.

Preparation of Hyperbranched PolyMultivinyl Polymer (HB-PMVP) via *in situ* DE-ATRP: $PEGDA_{700}$ (30 mmol), EBriB (10 mmol), $CuCl_2$ (0.25 mmol), PMDETA (0.25 mmol) and MEK (60 ml) in a two-necked round bottom flask were bubbled with argon for 0.5 h to remove the oxygen and then AA (0.125 mmol) solution was added. The system was sealed after bubbling with argon for another 5 min and the polymerisation was conducted at 50 °C. The molecular weights (Mn, Mw) were monitored by GPC until the target Mn/Mw was obtained and then the reaction was exposed to air under room temperature. The polymer was purified twice by precipitating the solution into a large excess of n-hexane and diethyl ether with the volume ratio of 1:2. After the monomer was fully removed, the polymer was dissolved in acetone and the solution was eluted through a short Al_2O_3 column to remove the Copper. Pure polymer was achieved as a viscous liquid after removing the acetone utilizing a rotary evaporator.

Endcapping HB-PMVP with 3-MPA: $NaHCO_3$ (30 mmol), 3-MPA (30 mmol) and methanol (30 ml) were added into a round bottom flask and stirred at 60 °C until the solid was fully dissolved then cooled to room temperature (RT). HB-PMVP was dissolved into methanol (10 ml) and the solution was added dropwise into the flask. The reaction was stirred at room temperature overnight and then the methanol was removed with a rotary evaporator. Deionized H_2O (5 ml) was added and the pH of the solution was adjusted to 1 with 1 M HCl. The water phase was extracted by DCM (3×100 ml), dried with Na_2SO_4 and then the DCM was removed to generate the crude polymer. Further dialysis against acetone was conducted to obtain the pure HB-PEG-COOH polymer.

Modification of HB-PEG-COOH with NHS: HB-PEG-COOH (4 g) and EDCI (8 mmol) were dissolved in THF (40 ml). Then, NHS (8 mmol) was added and the mixture was vortexed to dissolve the solid particles. The reaction was stirred overnight at RT. After the white precipitate was filtered, the THF was removed by rotary evaporation and the crude polymer was dialyzed for

3 days against an acetone solution that was changed twice a day. Final polymer was achieved by removing the acetone utilizing a rotary evaporator.

Ellman assay:

NHS content in HB-PEG-NHS

2-Mercaptoethanol was used as the standard substance and the standard solutions were prepared at 0 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM in 0.1 M sodium phosphate buffer (pH=8.5). The HB-PEG-COOH and HB-PEG-NHS were dissolved in sodium phosphate buffer at 1 mg/ml, respectively. 240 μl of standard solutions were mixed with 1 ml sodium phosphate buffer and 50 μl (10 mM) Ellman's reagent. 240 μl of sample solutions were mixed with 1 ml sodium phosphate buffer containing 0.48 μmol 2-mercaptoethanol, the mixtures were vortexed for 10 min and then 50 μl (10 mM) Ellman's reagent was added. 15 min later, the absorbance in the range from 350 nm to 600 nm was recorded on a SpectraMax M3 plate reader to determine the wavelength of maximum adsorption. Then, the standard curve was obtained based on the maximum adsorption at 408 nm. The reduction of the thiol groups is equal to the amount of the NHS groups in HB-PEG-NHS.

Free NHS group after gelation

2-Mercaptoethanol was also used as the standard substance and the standard solutions were prepared at 0 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM in 0.1 M sodium phosphate buffer (pH=8.5). 200 μl of hydrogel-L (composed of 5% (w/v) HB-PEG-NHS and 15% (w/v) BSA) and hydrogel-H (composed of 10% (w/v) HB-PEG-NHS and 15% (w/v) BSA) were fabricated and cultured at room temperature for 0.5 h. Then, the hydrogels were cut into pieces (1 mm × 1 mm × 1mm) and then hydrogel samples were diluted 10-fold (1.8 ml buffer was added to each sample) and filtered to afford the sample solutions. 240 μl of sample solutions or buffer (control group) were mixed with 1 ml buffer containing 0.48 μmol 2-Mercaptoethanol and the mixtures were vortexed for 10 min followed by the addition of 50 μl of a 10 mM Ellman's reagent. 15 min later, the absorbance at 408 nm was recorded on a SpectraMax M3 plate reader and the standard curve was obtained based on the absorbance at this wavelength. The measurements of each group were repeated 3 times. The reduction of thiol groups was converted into free NHS groups and percentage in the hydrogel.

BSA hydrogel fabrication: A 30% (w/v) BSA solution was prepared by dissolving BSA in 1X PBS solution. HB-PEG-NHS was suspended in 1X PBS solution at selected concentrations prior to use. Hydrogel fabrication was achieved by mixing equal amount of BSA solution with HB-PEG-NHS solution followed by pipetting onto a Teflon plate (or a glass vial) and incubation in a plastic petri dish at RT.

***In vitro* cell cytotoxicity assessment of HB-PEG-NHS polymer:** MTT assay was conducted to evaluate the cytotoxicity of HB-PEG-NHS using rat adipose-derived stem cells (rADSCs) and the 3T3 fibroblast cell line. In the current article, the rADSCs we used were the ones stored in our lab. The rADSCs were extracted from rat adipose tissue using previously reported method and the stem cells below passage 4 were used for this study.[1] According to the reference, all animal experimental protocols were approved by the Animal Care and Research Ethics Committee of the National University of Ireland, Galway (No.009/10(B)) and were conducted under an animal license (No. B100/4342) authorized by the Irish Department of Health and Children. Animal care was in compliance with the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science, NUIG. Murine 3T3 fibroblast cell line was purchased from Sigma-Aldrich. Briefly, the cells were cultured in DMEM at 37 °C, 5% CO_2 and 95% humidity. The cells were seeded in 96 well plates at the density of 6×10^3 cells per well for 24 h, followed by a change of the medium with HB-PolyPEG-NHS solutions in DMEM containing 10% v/v FBS and 1% v/v P/S at the concentration of 0 μg/ml (control), 50 μg/ml, 100 μg/ml, 250 μg/ml, 500 μg/ml and 1000 μg/ml, respectively (n = 4, each concentration repeated in quadruplicates). After 24 h, the polymer solution was changed with 200 μl MTT solution (0.2 mg/ml), the plates were cultured at standard condition for 4 h and then the MTT solution was removed. 100 μl DMSO was added into each well and the plates were placed on a shaker for 15 min to fully dissolve the purple crystal. The absorbance was measured at 570 nm and 630 nm on a plate reader.

Cell viability in the HB-PEG-NHS-BSA hydrogel: 3T3 fibroblasts and rat adipose-derived stem cells (rADSCs) at the concentration of 5×10^5 cells per ml were mixed with sterilized BSA solution (30%, w/v) which was dissolved in cell culture media (Dulbecco's modified Eagle's

medium (DMEM), containing fetal bovine serum (FBS, 10% v/v) and penicillin/streptomycin (P/S, 1% v/v). The cell-containing solution was mixed with a sterilized solution of HB-PEG-NHS in DMEM and the mixture was cultured at 37 °C for 0.5 h to achieve complete crosslinking. The hydrogel was placed into a 24 well plate, the cells were cultured into an incubator at 37 °C, 5% CO_2 replenishing the nutrients by fresh media every 2 days. LIVE/DEAD assays were employed to visualize the living cells and dead cells after a 48 h culture period.

Statistics analysis: Cell viability data were presented as means ± standard deviations, with sample number of 4. Statistical differences between two groups were analyzed by one way ANOVA and a value of $p < 0.05$ (*) was considered as statistically significant.

Reference

1. Y. Dong, W. U. Hassan, R. Kennedy, U. Greiser, A. Pandit, Y. Garcia, W. Wang, *Acta Biomater.,* 2014, **10**, 2076.

Table S1 GPC results of the kinetic experiment

Time point	M_n (Da)	M_w (Da)	PDI	Conv. %
1 h	3193	4038	1.3	15
2 h	3765	5414	1.4	32
2.5 h	4117	6217	1.5	38
3 h	4425	7305	1.7	44
3.5 h	4890	8664	1.8	48
4 h	5142	10312	2.0	52
4.5 h	5682	12701	2.2	56

Table S2 GPC results of the polymer pre and post functionalisation.

Time point	M_n (Da)	M_w (Da)	PDI	α	Branching degree
HB-PMVP (Purifed)	6196	13295	2.1	0.32	38%
HB-PEG-NHS	5052	15484	3.0	0.34	40%

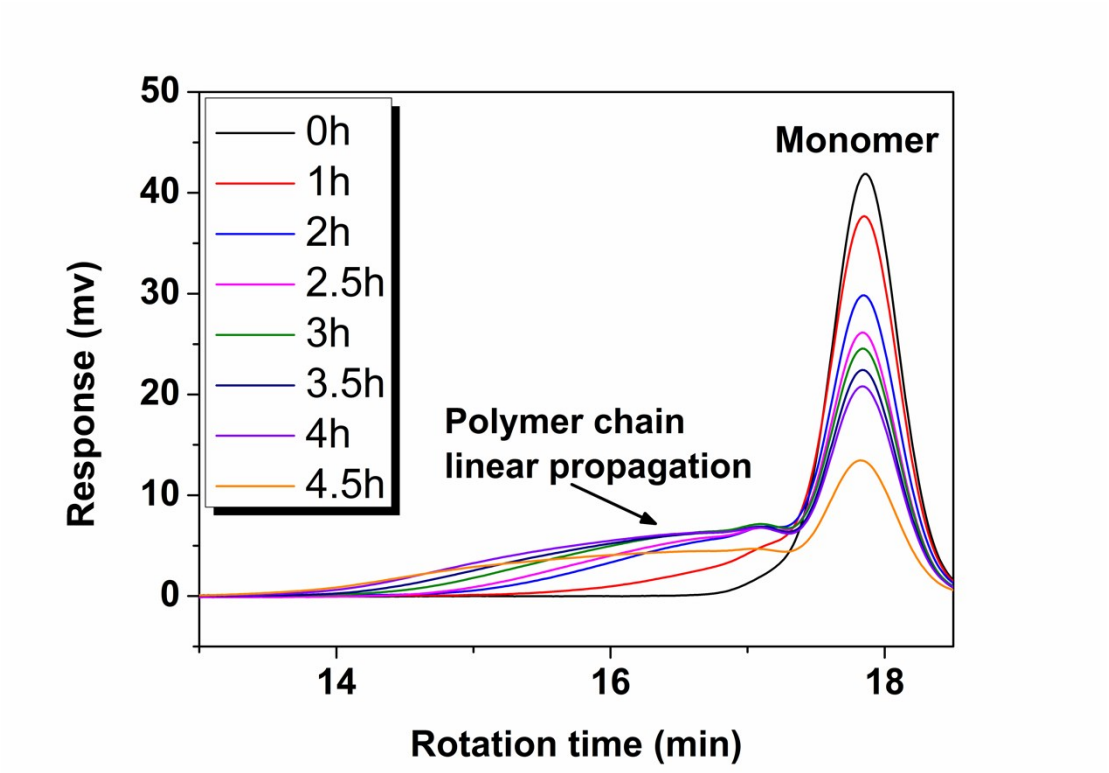

Figure S1 GPC traces of *in situ* DE-ATRP of $PEGDA_{700}$.

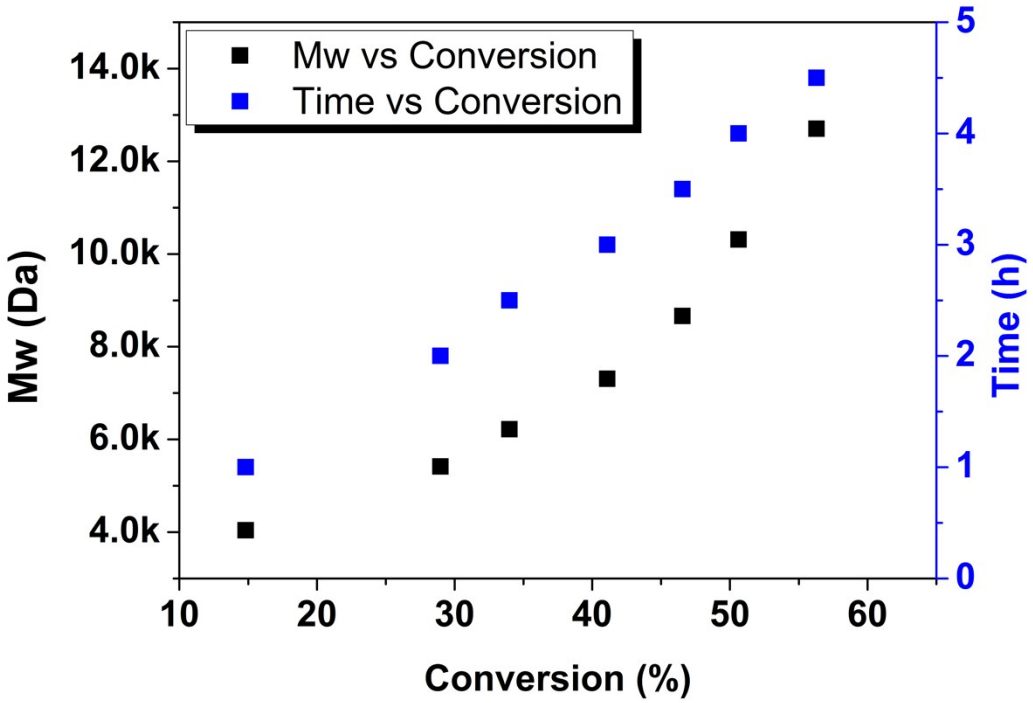

Figure S2 Kinetic plot of *in situ* DE-ATRP $PEGDA_{700}$ (Mw versus conversion and time versus conversion).

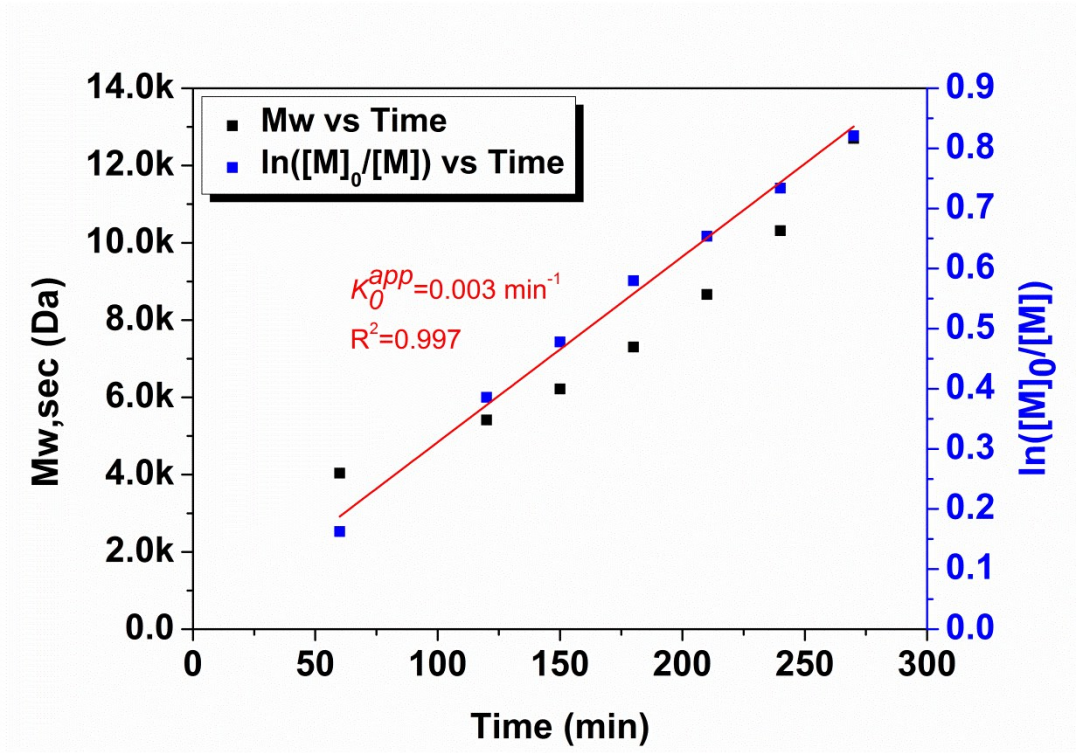

Figure S3 Kinetic plot of *in situ* DE-ATRP $PEGDA_{700}$ (Mw versus time and $\ln([M]_0/[M])$ versus time).

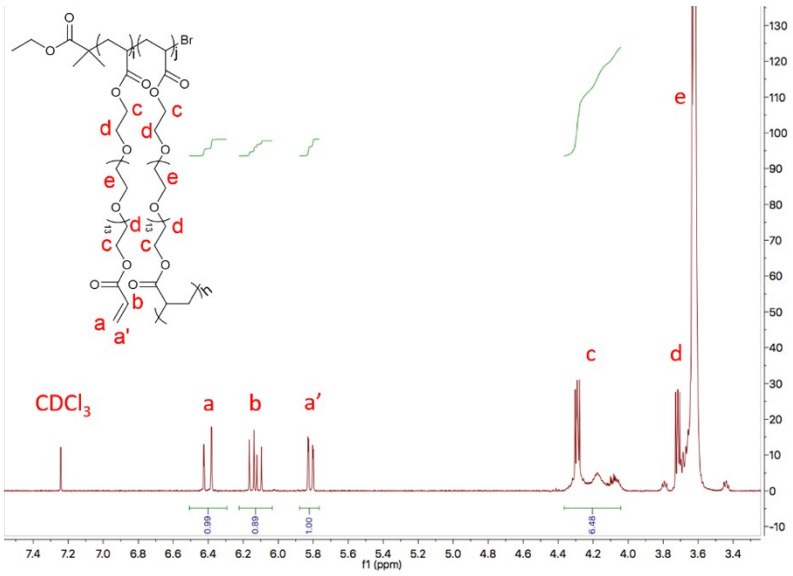

Figure S4 ^{1}H-NMR spectrum of HB-PMVP

Calculation of HB-PMVP composition based on Figure S4:

$$\text{Vinyl ratio (mol\%)} = \frac{(a + a')/2}{c/4} \qquad \text{E1}$$

$$\text{Branch ratio (mol\%)} = 1 - \frac{(a + a')/2}{c/4} \qquad \text{E2}$$

$$\text{Vinyl content (mmol/g)} = \frac{\frac{62\% \times Mw}{Mn\ of\ PEGDA700}}{Mw} \times 1000 \qquad \text{E3}$$

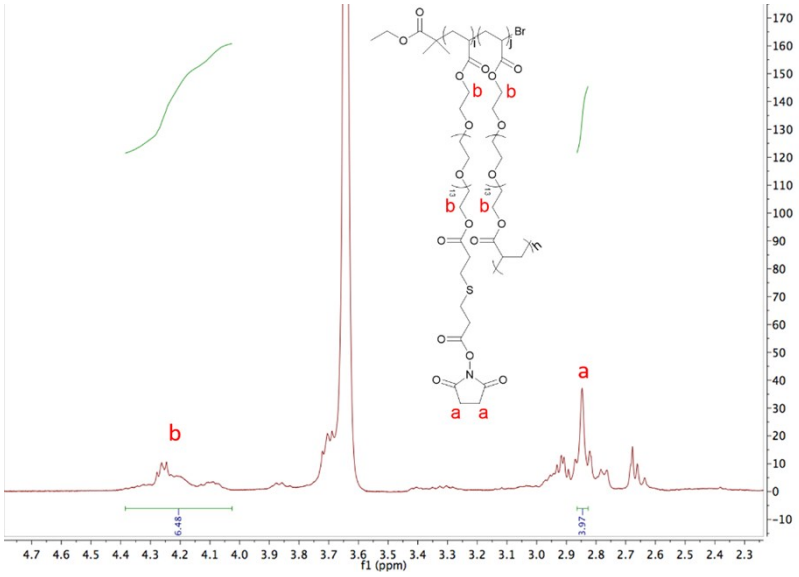

Figure S5 ^{1}H-NMR spectrum of HB-PEG-NHS

Calculation of HB-PEG-NHS composition based on Figure S5:

$$\text{NHS group per chain} = \frac{(Mw\ of\ HB-PEG-NHS)-(Mw\ of\ HB-PMVP)}{Mw\ of\ NHS\ unit}$$

$$\text{NHS content (mmol/g)} = \frac{NHS\ groups\ per\ chain}{Mw\ of\ HB-PEG-NHS} \times 1000$$

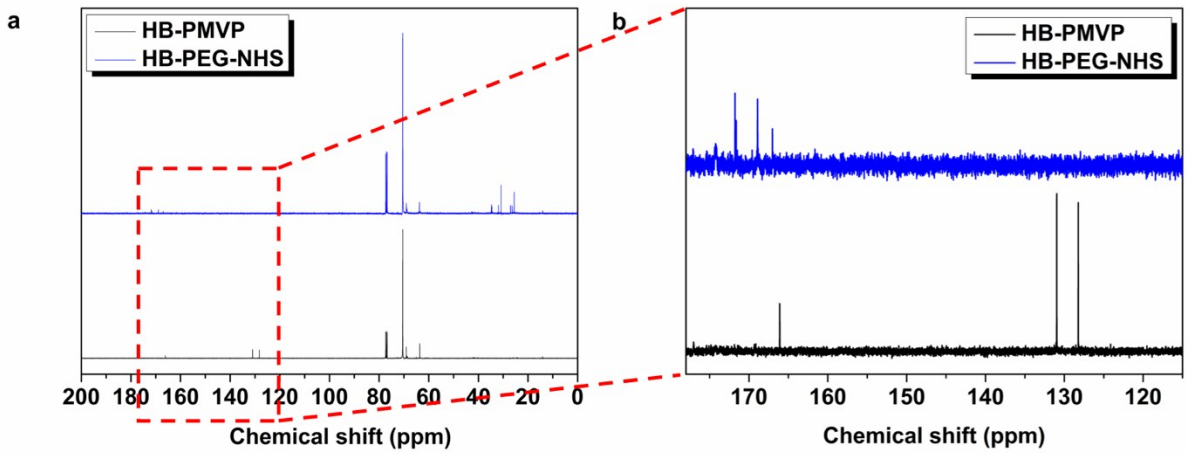

Figure S6 ^{13}C-NMR results of HB-PMVP (black) and HB-PEG-NHS (blue). (a) Full spectrum of ^{13}C-NMR results. (b) Zoom in area of (a) from 115 ppm to 178 ppm.

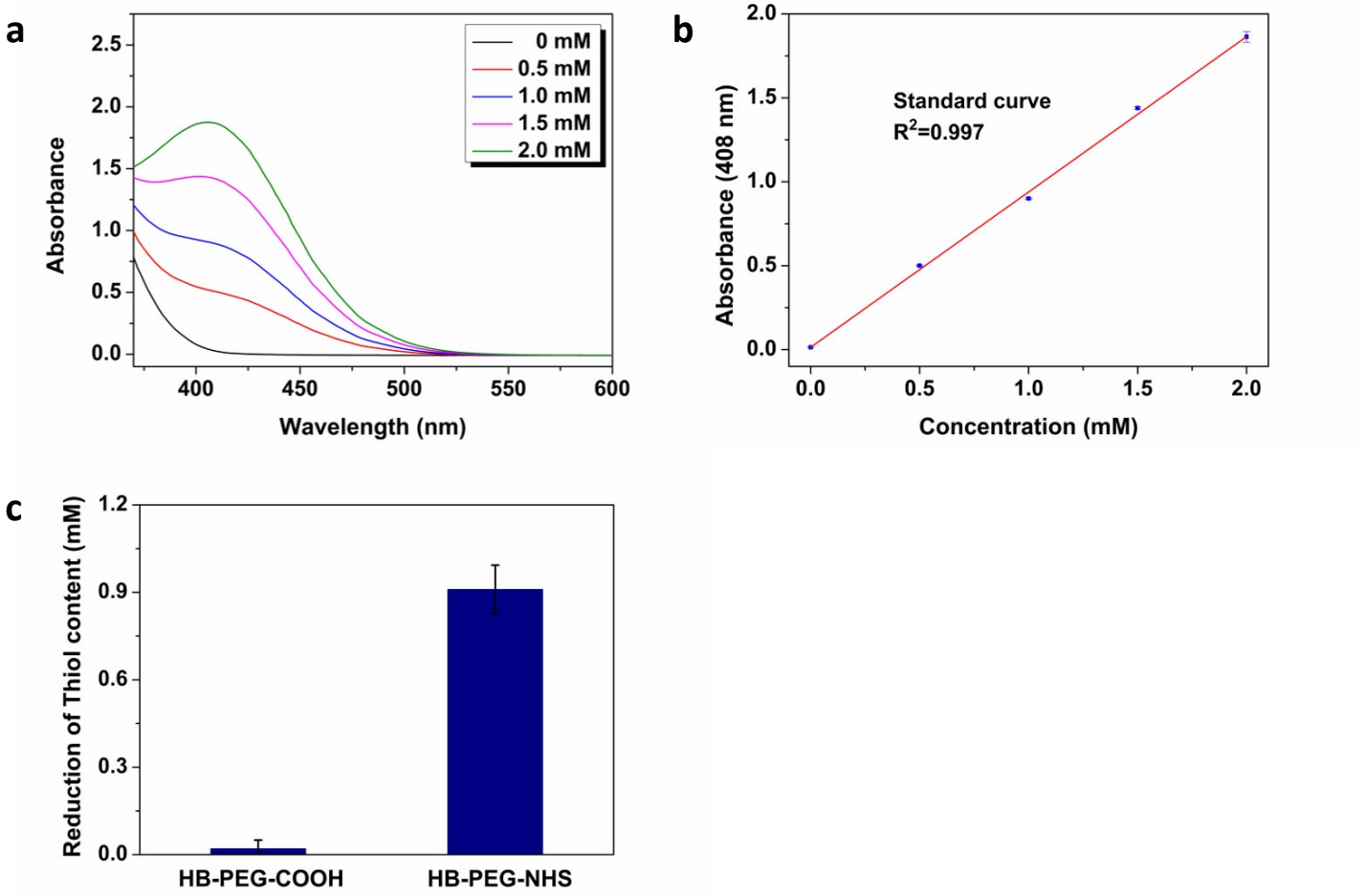

Figure S7 (a) Absorbance of the standard samples. (b) Fitted standard curve of absorbance at 408 nm. (c) NHS content determination by Ellman method.

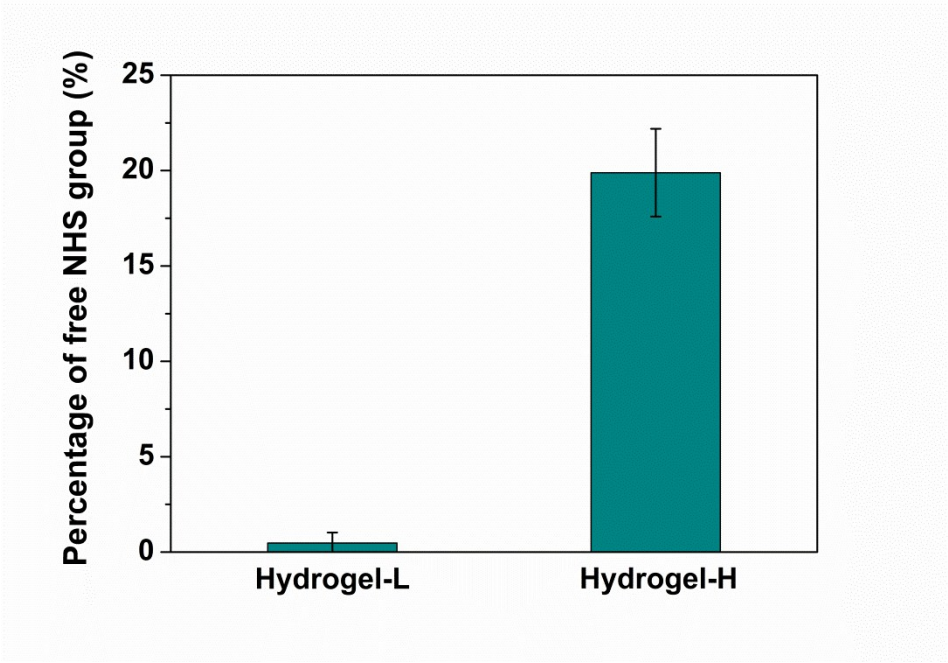

Figure S8 Percentage of free NHS groups in crosslinked hydrogel.

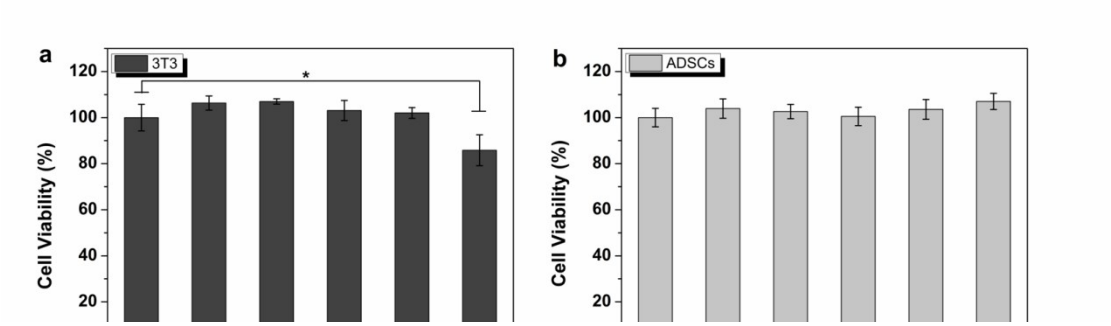

Figure S9 Cytotoxicity assessment of HB-PEG-NHS using the MTT method. (a) 3T3 fibroblast and (b) rADSCs cell lines were cultured with HB-PEG-NHS at the concentration of 50, 100, 250, 500 and 1000 μg/ml in media at standard condition for 24 h (n = 4, mean ± SD).