Supporting Information

Adaptable to Mechanically Stable Hydrogels Based on the Dynamic Covalent Cross-Linking of Thiol-Aldehyde Addition

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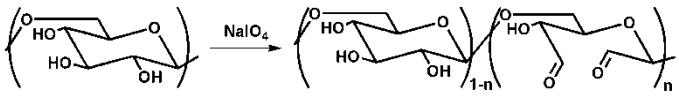
Experimental section

1. Materials, hydrogel precursors synthesis and characterization

1.1 Materials

Dextran (Mw: 70 kDa), sodium periodate ($NaIO_4$), acryloyl chloride, and 6-maleimidocaproic acid were purchased from Sigma-Aldrich. The thiolated polyethylene glycol polymer of 4-arm-PEG-SH (**TP**, 10 kDa) was purchased from JenKem company. Dialysis bags (MWCO 7 kDa) were purchased from Aladdin. The syringe filter unit (PES, 0.22 μm pore size) was purchased from Millex. Fetal bovine serum (FBS) was acquired from Sigma-Aldrich and penicillin-streptomycin (Pen-Strep) solution was from HyClone. DMEM (1:1, 1×) cell culture media were purchased from HyClone. CCK-8 assay kit was purchased from DOJINDO. All other chemicals were reagent grade and deionized water was used.

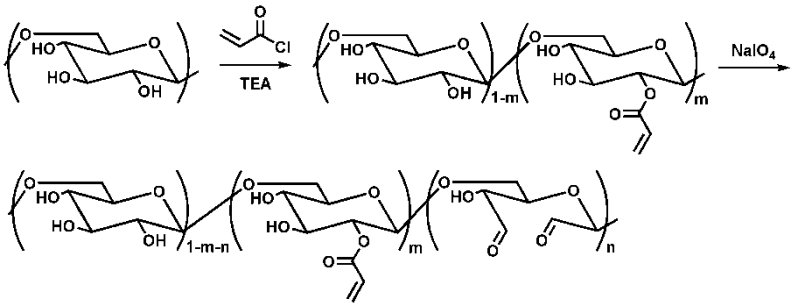
1.2 Hydrogel precursors synthesis



Scheme S1. Synthesis route of oxidized dextran (**OD**).

Dextran (70 kDa, 2 g) was dissolved in deionized water (10% w/v), then sodium periodate ($NaIO_4$) dissolved in deionized water at a certain molar ratio of sugar units of dextran/$NaIO_4$ (1:0.4) was added to the above solution and stirred for 8 hrs at room temperature. The reaction was quenched by adding excess amount of ethylene glycol with a molar ratio of 2:1 (OH/$NaIO_4$). Then the solution was dialyzed against deionized water with dialysis bags (MWCO 7 kDa) every 5 hrs for 3 days, and followed by freezing and lyophilizing. The oxidation degree was quantified using a hydroxylamine hydrochloride assay as previously described.[1] The oxidized dextran was also

characterized by ^{1}H NMR (400 MHz, D_2O). The signal at 5.7 ppm was the characteristic peak of CH_2 nearby the aldehyde groups.



Scheme S2. Synthesis route of acrylated dextran (**AD**) and oxidized and acrylated dextran (**OAD**).

OAD was synthesized by two steps. First, 2 g dextran (70 kDa) and 1 mL trimethylamine were mixed in 20 mL dimethyl sulfoxide (DMSO). And then acryloyl chloride was dissolved in 5 mL dichloromethane (DCM) at a certain molar ratio of sugar units of dextran/acryloyl chloride (1:0.16) and added to the above solution for 5 hrs at room temperature. After the reaction, the crude product was precipitated in ethyl alcohol and dissolved in deionized water again to dialyze against deionized water with dialysis bags (MWCO 7 kDa) every 5 hrs for 3 days, and followed by freezing and lyophilizing to obtain acrylated dextran (**AD**). The substitution of acrylate groups was verified by ^{1}H NMR (400 MHz, D_2O). The signal peaks from 6.0 to 6.6 ppm (the characteristic peak of double bond) indicated the successful graft of acrylate groups. Next, the above **AD** (2 g) was dissolved in deionized water (10% w/v), then sodium periodate ($NaIO_4$) dissolved in deionized water at a certain molar ratio of sugar units of dextran/$NaIO_4$ (1:0.2) was added to the above solution and stirred for 8 hrs at room temperature. The reaction was quenched by adding excess amount of ethylene glycol with a molar ratio of 2:1 (OH/$NaIO_4$). Then the solution was dialyzed against deionized water with dialysis bags (MWCO 7 kDa) every 5 hrs for 3 days and followed by freezing and lyophilizing to obtain the final **OAD**. The oxidation degree was quantified using a hydroxylamine hydrochloride assay as mentioned above.[1] The substitution of acrylate groups was quantified by ^{1}H NMR (400 MHz, D_2O). The signal peaks from 6.0 to 6.6 ppm (the characteristic peak of double bond) indicated the successful graft of acrylate groups. The signal at 5.7 ppm was the characteristic peak of CH_2 nearby the aldehyde groups.

1.3 Hydrogel preparation

Hydrogel precursors of **OD**, **AD** or **OAD** were mixed with 4-arm-PEG-SH (**TP**) (10% w/v, 1:1) in Phosphate Buffer Saline (PBS, pH 7.4) or other solvents. Then the above specimens with different curing time were subjected to different measurements.

2. Mechanism, rheological and mechanical properties characterization

2.1 Kinetic and equilibrium constants experiments

Stock solutions of the oxidized dextran (**OD**) and PEG-thiols (**TP**) were prepared in PBS (pH 7.4). First, the relationship between the concentration of **OD** and UV absorption intensity at 250 nm (the absorption of carbonyl groups) was established to make a standard curve. Then, the variable value of the concentration of **OD** after mixing the solution of **OD** and **TP** (2% w/v, 1:1, pH 7.4) was traced by UV-vis spectroscopy as the decrease of carbonyl absorption at 250 nm. The data collection were conducted at the time points of 0, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 270, 300 s. The kinetic and equilibrium constants parameters were calculated using standard kinetic models[2] as follows:

The reaction between oxidized dextran (**OD**) and PEG-thiols (**TP**) is a bimolecular reversible reaction following 2nd order reaction kinetics. The reaction rates, k_1 and k_{-1}, were calculated from the decreasing concentration of **OD** in time, by fitting x (t) against t (s) to the solution of the rate equation (1).

$$x(t) = \frac{a_+(x_0 - a_-) - a_-(x_0 - a_+)e^{-k_1(a_+ - a_-)t}}{(x_0 - a_-) - (x_0 - a_+)e^{-k_1(a_+ - a_-)t}} \quad (1)$$

In which,

$$a_+ = \frac{-k_{-1} + \sqrt{k_{-1}^2 + 4k_1k_{-1}x_0}}{2k_1} \qquad a_- = \frac{-k_{-1} - \sqrt{k_{-1}^2 + 4k_1k_{-1}x_0}}{2k_1}$$

x (t) is the variable value of the concentration of oxidized dextran as the progress of reaction, x_0 is the initial concentration of oxidized dextran. Equation (1) was programmed into Origin version 9.0 (OriginLab®), which was used as the software for the data analysis to get the value of k_1 and k_{-1}.

Then, the equilibrium constants (K_{eq}) of the thiol-aldehyde addition reaction was calculated from the equation (2).

$$K_{eq} = \frac{k_1}{k_{-1}} \quad (2)$$

In which, k_1 is forward rate constant, and k_{-1} is backward rate constant.

2.2 ^{1}H NMR experiments

Hydrogel precursors of **OD**, **AD** or **OAD** were mixed with 4-arm-PEG-SH (**TP**) (2% w/v, 1:1, pH 7.4) and PBS powder (1% w/v) in D_2O. Additionally, hydroxyethyl acrylate (5 eq.) was added into the **OD/TP** mixture to trace the thiol-acrylate Michael

addition. ^{1}H NMR spectra were recorded at different curing time on a Bruker 500 MHz NMR spectrometer. Chemical shifts were reported in parts per million (ppm).

2.3 ATR-FTIR and XPS experiments

The **OAD/TP** hydrogels (10% w/v, 1:1, pH 7.4) were prepared in H_2O following reaction time of 15 min or 60 min and then dried instantly to make film-like samples. The unreacted powders of **OAD** and **TP** were mixed for the control. All of the samples were dried at 40 °C for 12 h. Then, ATR-FTIR spectra were recorded on a Nicolet 6700 FTIR spectrometer. The ATR-FTIR spectrum of 1650 cm^{-1} is the typical peaks of the C=O stretching vibration, 1480 cm^{-1} is the typical peaks of the C=C stretching vibration, and 1010 cm^{-1} is the typical peaks of the C-S-C stretching vibration. According to the intensity change of the characteristic peaks, the occurrence of crosslinking reaction can be analyzed. X-ray photoelectron spectroscopy was carried out in an ultrahigh vacuum chamber by a ESCALAB 250Xi XPS system. Then, XPS spectra were analyzed by XPSPEAK software to conduct peak separation. The binding energy of 287.3 ev is attributed to the hemithioacetal bonds (OH-C-S), 286.0 ev is attributed to the double bonds of carbon (C=C), and 284.5 ev is attributed to the single bonds of carbon (C-C). According to the intensity change of the characteristic peaks, the occurrence of crosslinking reaction can be analyzed.

2.4 Rheological analysis

Dynamic rheology experiments were performed on HAAKE MARS Rotational Rheometer with parallel-plate (P20 TiL, 20-mm diameter) geometry at 25 °C. Time sweep oscillatory tests were performed at a 10% strain (CD mode), 1 Hz frequency and a 0.5 mm gap for 3600 s (**OD/TP** hydrogels and **OAD/TP** hydrogels) (excluding the mixing time of 60 s). The gel point was determined as the time when the storage modulus (G') surpassed the loss modulus (G''). The final shear modules was evaluated as 90% of G_∞ occurring at 3600 s (**OD/TP** hydrogels and **OAD/TP** hydrogels). Frequency-dependent oscillatory tests were performed at 10% strain (CD mode) and a 0.5 mm gap from 0.5 rad/s to 100 rad/s. Strain-dependent and step-strain tests were performed at 10 Hz frequency and a 0.5 mm gap. The range of strains used for strain test was from 0.05% to 500% strain. The step-strain measurements were conducted at γ = 1% or 500% for multiple cycles. Shear-thinning behavior was performed at a 0.5 mm gap from 0 to 100 1/s.

2.5 Mechanical properties tests

Mechanical tests were carried out on as-prepared hydrogels using GT-TCS-2000 universal material testing machine with the capacity of 100 N. For compression tests,

hydrogel samples were prepared to have cylindrical shape with 10-mm diameter and 3-mm length, and the speed was set at 1 mm/min. The **OD/TP** or **OAD/TP** (10% w/v, 1:1) hydrogels after complete gelation (12 hrs) were subjected to compression tests.

2.6 Dissolution tests

For characterization of the gel stability, the **OD/TP** (10% w/v, 1:1, pH 7.4) hydrogels after complete gelation (12 hrs) were instantly immersed in PBS (pH 7.4) only or with 0.1 M 6-maleimidocaproic acid (Mal). The dissolution rate of **OD/TP** (10% w/v, 1:1) hydrogels was calculated as the mass ratio of immersed weight ($W_{immersed}$) to previous weight ($W_{previous}$) using wet mass at different time points.

$$Mass\ \ Ratio(\%) = \frac{W_{immersed}}{W_{previous}} \times 100\% \qquad (3)$$

2.7 Degradation tests

For characterization of degradation, the **OD/TP** or **OAD/TP** (10% w/v, 1:1, pH 7.4) hydrogels after complete gelation (12 hrs) were immersed in PBS (pH 7.4). The degradation rate of **OAD/TP** (10% w/v, 1:1) hydrogels was calculated as the mass ratio of immersed weight ($W_{immersed}$) to previous weight ($W_{previous}$) using dry mass at different time points (1, 3, 5, 7 days).

$$Mass\ \ Ratio(\%) = \frac{W_{immersed}}{W_{previous}} \times 100\% \qquad (3)$$

3 Evaluation of bone repair

3.1 Release kinetics of the BMP-2

The BMP-2 loaded hydrogels were prepared by mixing **OAD** and **TP** precursors (10% w/v, 1:1) and BMP-2 (40 ng/mL). The release kinetics of the BMP-2 were determined *in vitro* as previously described.[3] Briefly, the BMP-2-loaded hydrogels were suspended with 3mL of the PBS. 500 μL of supernatant was collected and stored at –20 ℃ on the predetermined time points (1, 2, 4, 8, 16, 24, 36, 48, 72, 144 and 288 hrs) after centrifugation. Then, 500 μL of the fresh PBS was added. The released quantity of the BMP-2 was determined by the ELISA kit (Sigma Aldrich) (n = 5).

3.2 Critical-sized radius defect and implantation of the OAD/TP hydrogel

Twenty-Seven New Zealand male rabbits were randomly divided into three groups implanted with: (1) hydrogel only (n = 9); (2) BMP-2 loaded hydrogel (40 ng/mL, n =

9) and (3) negative control (n = 9). The negative control is the bone defects without any implanted materials. All rabbits were anesthetized by intravenous injection of 3% pentobarbital sodium (30 mg/kg), and a unilateral segment of the periosteum and radius with a critical-sized length of 2.5 cm was excised using a circular saw. Next, the hydrogel was prepared by mixing **OAD** and **TP** precursors (10% w/v, 1:1) and BMP-2 (40 ng/mL) or not. After the gel was in the semisoild-like state, the defects were filled with the pre-molded hydrogel, BMP-2 loaded hydrogel or blank as described above. After the gel was harden, the incision was sutured.

3.3 μ-CT examination

All the radius and ulnas were harvested of 2, 4 and 8 weeks post-implantation for μ-CT scanning (Skyscan1272, Bruker, USA) with the following parameters: the resolution of the scans was 20 μm, the exposure time was 2100 ms, the source voltage was 100 kV, the thresholding value was set at 155. 3D reconstructions were generated by the NRecon Reconstruction software (Bruker) in the region of interest (ROI) that was set as a cylinder at the center of each radius defect. The morphometric indices including bone mineral density, bone volume, and bone volume fraction (bone volume/tissue volume ratio, BV/TV) and trabecular thickness (Tb.Th) were assessed.

3.4 Histological evaluation

At 2, 4 and 8 weeks post-implantation, radius with treated defects (n = 9/group) were fixed in 4% paraformaldehyde for 10 days and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 30 days. Then the samples were embedded in paraffin and sectioned at 5 μm. H&E and Masson’s trichrome staining were performed to evaluate the tissue morphology.

4 Statistical Analysis

All data were presented as mean ± standard deviation (s. d.). Differences between the values were evaluated using One-Way analysis of variance (ANOVA). In the analyses, $p < 0.05$ was considered statistically significant.

Supplementary Figures

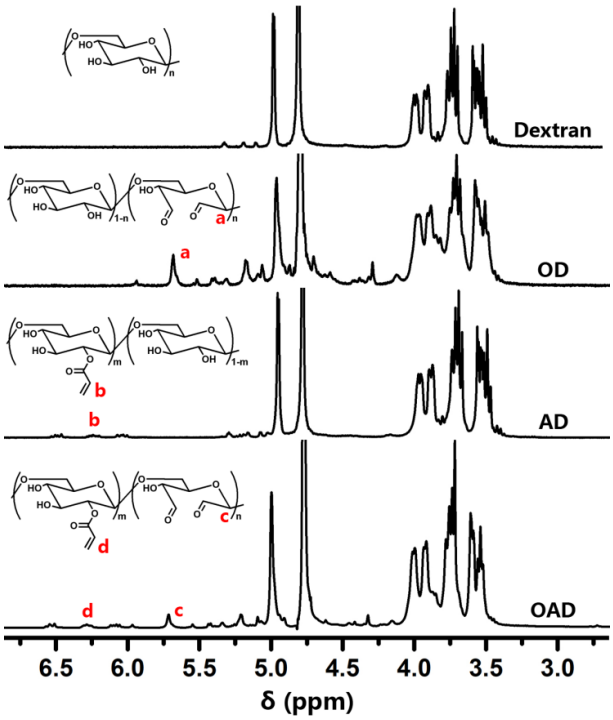


Figure S1. ^{1}HNMR spectra of **OD** (oxidized dextran), **AD** (acrylated dextran) and **OAD** (oxidized and acrylated dextran) compared with dextran. The signal peaks from 6.0 to 6.6 ppm (the characteristic peak of double bond) indicated the successful graft of acrylate groups. The signal at 5.7 ppm was the characteristic peak of CH_2 nearby the aldehyde groups.

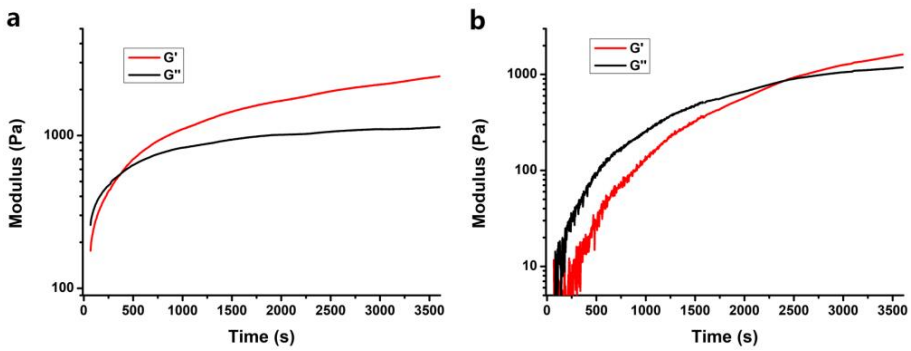


Figure S2. Time sweep rheological plots of the **OD/TP** hydrogels (10% w/v, 1:1) were conducted in pH 6.5 (a) or pH 8.0 (b), which indicated the adaptable character in physiological-pH environment.

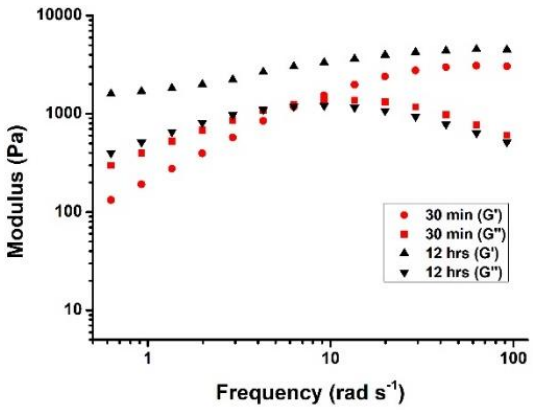


Figure S3. A frequency sweep plot at curing time of 30 min of the **OD/TP** hydrogel (10% w/v, 1:1, pH 7.4) indicated a typical characteristic of dynamic covalent network.

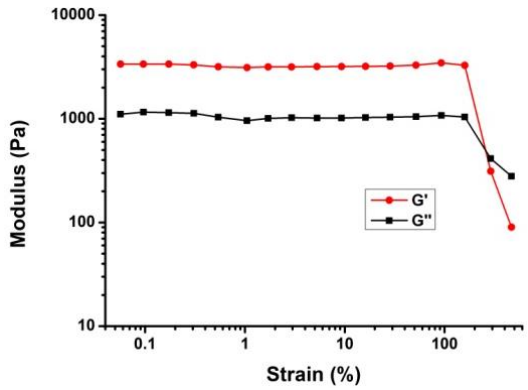


Figure S4. Strain amplitude sweep plot of the **OD/TP** hydrogel (10% w/v, 1:1, pH 7.4).

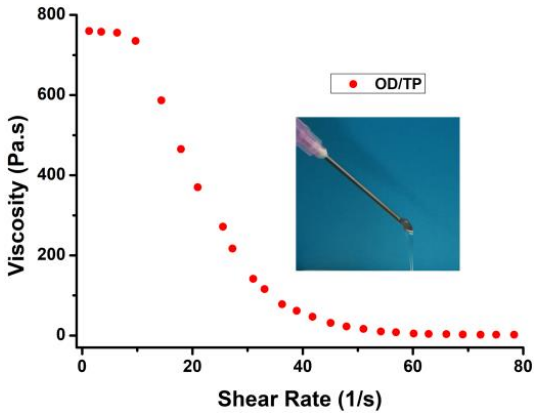


Figure S5. Viscosity and shear-thinning behavior of the **OD/TP** hydrogel (10% w/v, 1:1, pH 7.4) with curing time of 10 min. The inset showed the hydrogel was injectable through a conventional syringe using 18 G needle.

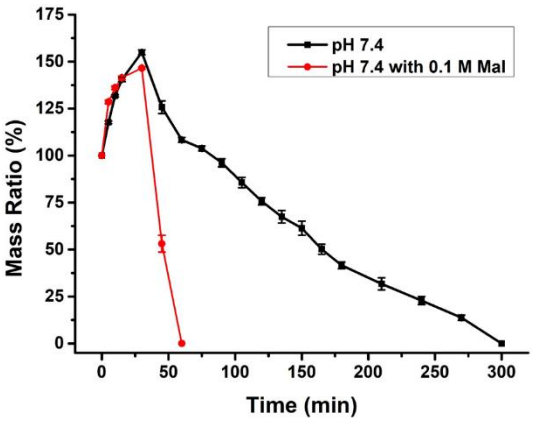


Figure S6. The dissolvable curves of the **OD/TP** hydrogels (10% w/v, 1:1, pH 7.4) immersed in PBS (pH 7.4) only and with 0.1 M of 6-maleimidocaproic acid (Mal).

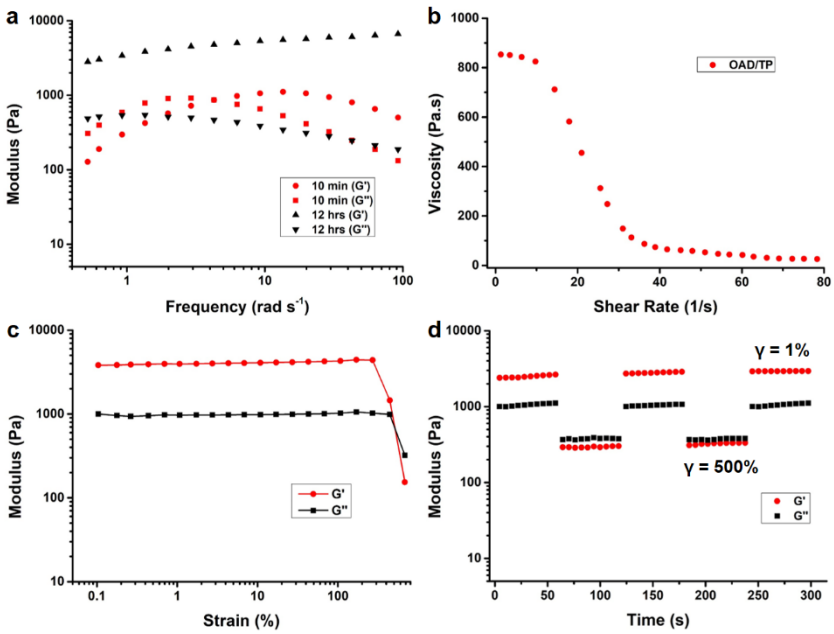


Figure S7. a) A frequency sweep plot at curing time of 10 min or 12 hrs of the **OAD/TP** hydrogel (10% w/v, 1:1, pH 7.4). b) Viscosity and shear-thinning behavior of the **OAD/TP** hydrogel (10% w/v, 1:1, pH 7.4) with curing time of 10 min. c) Strain amplitude sweep plot of the **OAD/TP** hydrogel (10% w/v, 1:1, pH 7.4). d) Step-strain measurements of the **OAD/TP** hydrogel (10% w/v, 1:1, pH 7.4) were conducted at γ = 1% or 500% for multiple cycles with curing time of 10 min.

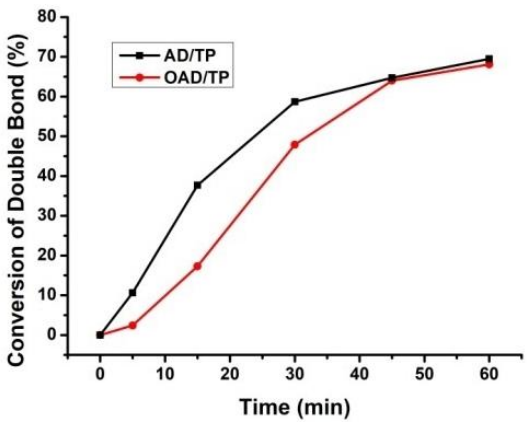


Figure S8. The slower initial conversion rate of the acrylate groups for **OAD/TP** compared to that of the **AD/TP** mixture demonstrated that the primary capture of thiols by the rapid thiol-aldehyde addition retarded the thiol-acrylate Michael addition.

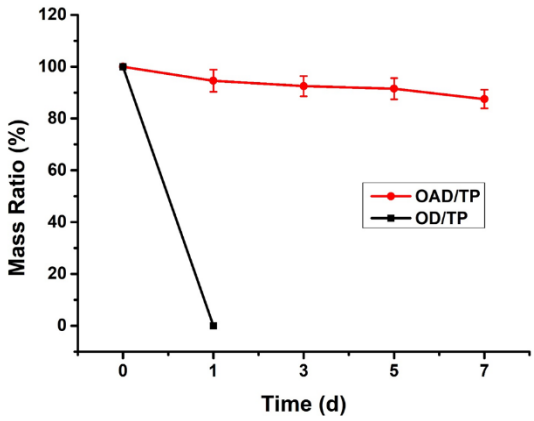


Figure S9. The degradation tests of the **OD/TP** or **OAD/TP** hydrogels (10% w/v, 1:1, pH 7.4).

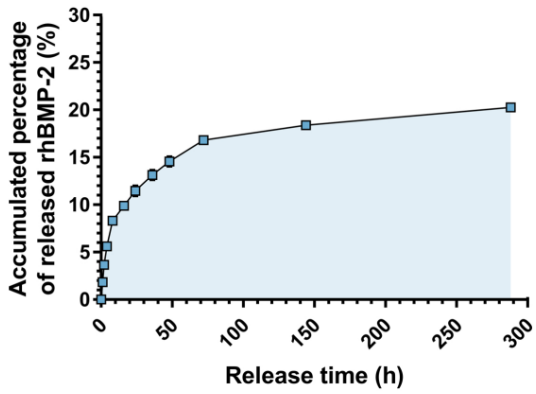


Figure S10. Release kinetics of the BMP-2 from the **OAD/TP** hydrogel. Data were expressed as means ± SD (n = 5).

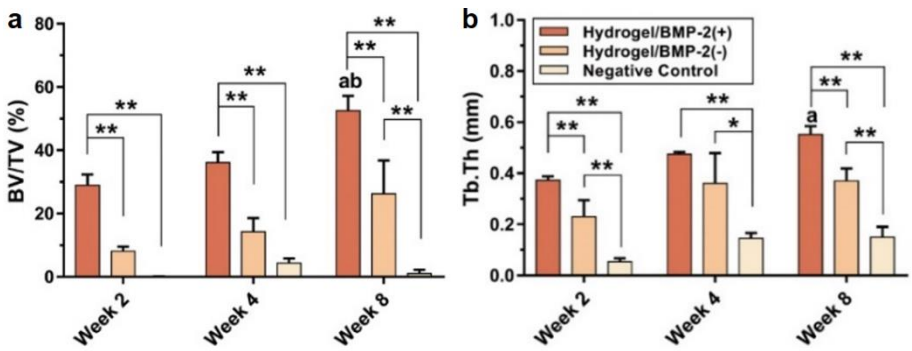


Figure S11. Quantitative analysis of morphometric indices, including bone volume fraction (BV/TV) (a) and trabecular thickness (Tb.Th) (b) of regenerated tissues in the defect area. Data are expressed as the means ± SD. *$p < 0.05$; **$p < 0.005$; ($n = 5$). $^{a}p < 0.05$ for comparison with the same group in week 2, $^{b}p < 0.05$ for comparison with the same group on in week 4.

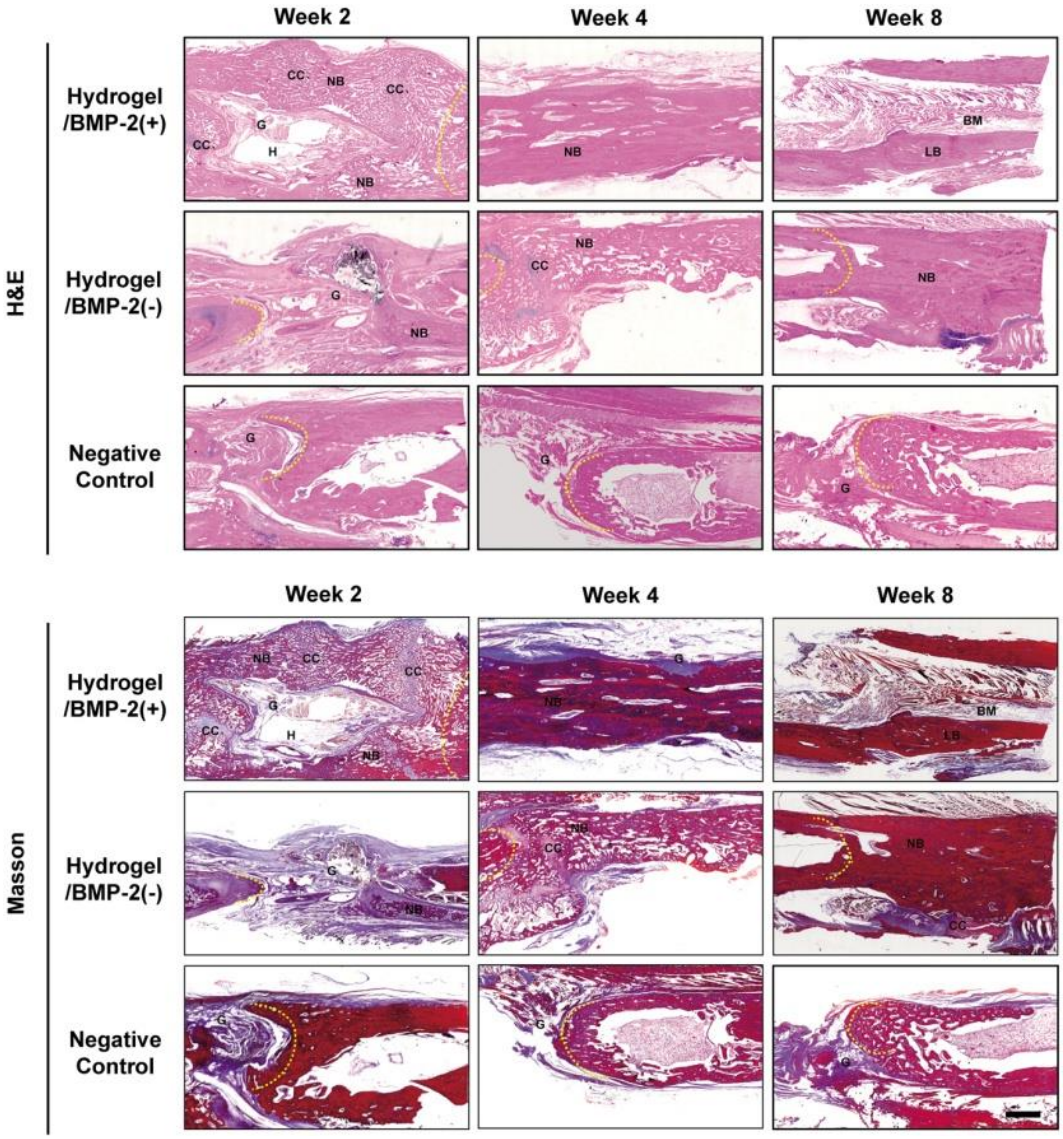


Figure S12. Histological evaluation of longitudinal sections (parallel to the long axis of bone) of bone formation treated by the BMP-2 loaded hydrogel (hydrogel/BMP-2(+)), hydrogel alone (hydrogel/BMP-2(-)) and the untreated defect (negative control) at 2, 4 and 8 weeks after implantation (H&E and Masson trichrome staining). H: hydrogel; NB: new bone; G: granulation tissue; CC: chondrocytes; LB: lamellar bone; O: osteon; BM: bone marrow. Black bar = 1 mm. White bar = 200 μm. (n = 3).

Reference

[1] Zhao, H.; Heindel, N. D. Determination of Degree of Substitution of Formyl Groups in Polyaldehyde Dextran by the Hydroxylamine Hydrochloride Method. *Pharm. Res.* **1991**, *8*, 400-402.

[2] Dirksen, A.; Dirksen, S.; Hackeng, T. M.; Dawson, P. E. Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. *J. Am. Chem. Soc.* **2006**, *128*, 15602-15603.

[3] Cao, L.; Wang, J.; Hou, J.; Xing, W.; Liu, C. Vascularization and bone regeneration in a critical sized defect using 2-N,6-O-sulfated chitosan nanoparticles incorporating BMP-2. *Biomaterials* **2014**, *35*, 684-698.