# Supporting Information

## A High Stiffness Bio-inspired Hydrogel from the Combination of Poly (amido amine) Dendrimer with DOPA

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# Experiments

## Materials

8-arm-PEG-SH (O-PEG) (20KDa) was purchased from JenKem technology (Beijing, China). Methoxy PEG succinimidyl carbonate ester (mPEG-NHS, Mw = 1000) was purchased from Biomatrik Inc. (Jiaxing, China). Maleimide-PEG-carbonate-NHS (MAL-PEG-NHS, Mw = 5000) was purchased from NOF Corporation (Japan). 1, 2-di(pyridin-2-yl)disulfane, 3-mercaptopropan-1-ol and 4-nitrophenyl carbonochloridate were purchased from J&K Scientific Ltd. (Beijing, China). Dopamine hydrochloride and glutathione (GSH) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). H-arg-gly-asp-D-tyr-cys-$NH_2$ (RGDyC) and Ac-arg-ala-ala-asp-D-tyr-cys-$NH_2$ (RAADyC) were purchased from CL Biochem (Shanghai, China). Other reagents were purchased from Beijing Chemical Reagents (Beijing, China). All the Reagents were used as received and the solvents were purified according to the general procedures before used.

$^1H$ and $^{13}C$ NMR data were collected on Bruker 400 MHz spectrometers operated at room temperature. Chemical shifts (δ) are reported in ppm with $(CH_3)_4Si$ and the residual solvent peak as the reference, respectively. UV-Vis spectra were recorded on the Perkin-Elmer Instruments Lambda spectrometer. Resolution mass spectra (RMS) were measured by Bruker APEX IV Fourier Transform Ion Cyclotron Resonance Mass Spectrometer. Differential Scanning Calorimetry (DSC) measurements were tested on Micro DSC III (Setaram, France).

## Synthesis

### 3-(pyridin-2-yldisulfanyl)propan-1-ol

2, 2'-Dipyridyldisulfide (5.0 g, 22.7 mmol) was dissolved in 20 mL anhydrous methanol. Acetic acid (2.5 mL) and 3-mercaptopropan-1-ol (1.05 g, 11.4 mmol) were dropwise added and the solution was stirring at room temperature for 8 h. The flash chromatography was performed using dichloromethane/hexane = 1/1. The product was yellow oil. $^1H$ NMR (400 MHz, $CD_3Cl$): δ 8.47 (d, $J$ = 4.8 Hz, 1H), 7.72 – 7.57 (m, 2H), 7.10 (ddd, $J$ = 6.2, 4.9, 2.4 Hz,

1H), 4.31 (s, 1H), 3.80 (s, 2H), 2.97 (s, 2H), 1.95 (s, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$): δ 159.93, 149.60, 137.09, 120.84, 120.26, 60.74, 35.79, 31.36. MS (ESI, positive mode): calcd for $C_8H_{11}NOS_2$: 201.0; found: 202.0 $[M + H]^+$.

**4-nitrophenyl (3-(pyridin-2-yldisulfanyl)propyl) carbonate (Linker)**

3-(pyridin-2-yldisulfanyl)propan-1-ol (1.84 g, 9.14 mmol) and 4-nitrophenyl carbonochloridate (2.44 g, 12.1 mmol) was dissolved in 20 mL dry tetrahydrofuran. Triethylamine (1 mL) was added and the reaction mixture was stirred at room temperature overnight under the nitrogen atmosphere. The reaction was checked for completion by TLC ($CH_2Cl_2$/hexane = 10/1) and the mixture was evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a step gradient of $CH_2Cl_2$, giving the product as a pale yellow oil. $^1$H NMR (400 MHz, $CDCl_3$): δ 8.56 – 8.40 (m, 1H), 8.35 – 8.19 (m, 2H), 7.75 – 7.54 (m, 2H), 7.46 – 7.28 (m, 2H), 7.19 – 7.01 (m, 1H), 4.42 (t, *J* = 6.1 Hz, 2H), 2.94 (t, *J* = 7.1 Hz, 2H), 2.38 – 2.06 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$): δ 159.70, 155.46, 152.38, 149.77, 145.45, 137.08, 125.76, 121.61, 120.05, 119.99, 67.48, 34.71, 27.80. MS (ESI, positive mode): calcd for $C_{15}H_{14}N_2O_5S_2$: 366.03441; found: 367.04092 $[M + H]^+$.

**O-PEG-DOPA (OPD)**

Dopamine hydrochloride (170.7 mg, 0.9 mmol) and 4-nitrophenyl (3-(pyridin-2-yldisulfanyl)propyl) carbonate (219.6 mg, 0.6 mmol) were dissolved in dimethyl sulfoxide (DMSO) and mixed together with triethylamine (500 μL) firstly in 25 mL round-bottom flask at room temperature. The reaction was stirred in dark place for 48 h. Then, 8-arm-PEG-SH (O-PEG) (2.0 g, 0.1 mmol) were distributed in the system for another 48 h. Finally, MWCO = 2000 dialyzed bag was used to purify the final product with the dialysis fluid from DMSO to deionized water for 4 d. $^1$H NMR (400 MHz, $D_2O$): δ 6.75 (s, 8H), 6.70 (s, 8H), 6.56 (d, J = 26.5 Hz, 8H), 4.02 (s, 15H), 3.86 – 3.31 (m, 880H), 3.24 (s, 22H).

**Synthesis of G4.0-RGDyC (1), G4.0-RAADyC (2)**

The PEGylated PAMAM was prepared by reacting the surface amino groups of G4.0 PAMAM with mPEGs bearing NHS end groups. Then the sulfydryl groups on cysteine were conjugated to the PEGylated PAMAM by the Michael addition reaction with MAL groups of PEGs. Take **1** as the example. Specifically, G4.0 PAMAM (25.03 mg, 1.76 μmol) was stirred with MAL-PEG-NHS (176.00 mg, 35.20 μmol) for 30 min, then mPEG-NHS (79.20 mg, 79.20 μmol) was added and kept for another 60 min. Finally, RGDyC (27.46 mg, 35.22 μmol) was added dropwise into the system, and the mixture was stirred for 2 h in water. The pure product was collected through freeze-drying after dialysis at MWCO = 8000 dialyzed bag for 48 h.

## Methods

### Preparation of Hydrogels.

Hydrogels with different components of **1**/**2** and **OPD** were prepared by a similar method. Taking the hydrogel from **1/OPD** (1/2) as an example, 15 mg **OPD** and 7.5 mg **1** were mixed together into 50 μL phosphate buffered saline (PBS, Invitrogen) in an EP tube. Then, 50 μL 1% w/v $NaIO_4$ in PBS buffer was sterilized by filtering through a 0.2 μm nylon syringe filter and was added to the tube. The hydrogel disks were obtained for less than 5 min, transferred to a 24-well culture plate and immersed in PBS at 37$^{o}$C for further swelling.

### Hydrogel Swelling and Degradation

The swelling characteristic of hydrogel scaffolds were evaluated as below. The crosslinked hydrogel pellets were subsequently stored at 37°C in phosphate buffered saline (PBS) for 48 h. The hydrogels were lyophilized and dry weights ($W_d$) as the original mass of the xerogel was measured. The resulting hydrogel was placed in a vial containing 10 mL of PBS and incubated at 37°C for 48 h. Excess fluid was removed to obtain the weight of the wet hydrogel ($W_s$). The hydrogel was then freeze-dried to obtain the weight of the dried hydrogel ($W_i$). Gel swelling ratio and mass loss were defined as follows:

Swelling ratio (%) = $[(W_s-W_i)/W_i]\times100$

Mass loss (%) = $[(W_d-W_i)/W_i]\times100$

We tracked the mass every 3 days of the dried hydrogel and fully swelled hydrogel to calculate the mass loss. The process was measured over a period of 50 days to detect the degradation of hydrogels.

**Scanning Electron Microscopy (SEM)**

The samples were prepared by a freeze-drying method without coating a thin layer of platinum (50 Å) prior to SEM imaging. Images were captured on a scanning electron microscope (SEM, Hitachi S4800, 5 kV).

**Mechanical Testing**

Cylinder-shaped hydrogel samples for dynamic mechanical testing were prepared by mixing $NaIO_4$ and the components in a 24-well culture plate mold within 5 min. The samples were removed from the culture plate and subsequently allowed to equilibrate in PBS solution at 37$^o$C for 48 h. Then, the samples were measured using a rheometer MCR 301, Anton Paar with parallel-plate geometry. The hydrogel was cut into a disk with a thickness of 2 mm and a diameter of 25 mm. The gap between the two plates was accurately set by controlling the normal force. Oscillatory rheological measurements were performed in the linear viscoelastic regime at 37$^o$C. The strain was kept at 10% and the frequency was changed from 0.1 to 100 rad $s^{-1}$. The shear storage modulus (G') and the shear loss modulus (G'') were measured. The complex shear modulus (G* = (G'+ G'')1/2) and the phase angle (δ with tanδ = G'/G'') were calculated accordingly. A hydrogel should show a value of G' independent of frequency and considerably larger than G''. Compressive stress-relaxation experiments were performed on similar hydrogels in strain increments from 5% to 20% maximum compressive strain for investigating the equilibrium compressive modulus. After each increment, the compressive stress would be recorded and determined until the deviation of the data was less than 1% for

30 s. The compressive moduli were subsequently determined by linear regression of the equilibrium stress versus strain data; errors were estimated from the standard deviation.

**Cell Culture**

Mouse bone marrow mesenchymal stem cells (mMSCs) were isolated from 8 week old C57BL/6 mice [1]. All mMSCs used for experiment were passage 10 to 15 and cultured in α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (regular medium). Osteogenic medium (OM) for differentiation consisted of regular medium supplemented with ascorbic acid (50 ng/mL) and β-glycerophosphate (20 mM).

To prepare cell-hydrogel constructs, PBS (50 μL) dilution of 15% **OPD**, 7.5% **1** and 1% $NaIO_4$ was mixed with mMSCs at a density of 10 million per milliliter in a 1CC syringe and then waited for 5 min. Cell-hydrogel constructs were transferred into individual wells of a 24-well plate with regular medium (1 mL) per well and incubated at 37°C. The medium was replaced by OM after 24 h and changed every 3 d. The hydrogels from **OPD** and **2/OPD** were served as the control. Constructs were harvested and processed for XTT analysis.

**XTT Test**

The cytotoxicity of the hydrogel frameworks was evaluated using the XTT Cell Proliferation Assay Kit (ATCC). To fabricate a standard curve, cells were loaded into a 96-well plate at densities of 0/mL, $1\times10^5$/mL, $2\times10^5$/mL, $5\times10^5$/mL, and $1\times10^6$/mL. After 24 h culturing in regular medium at 37°C with 5% $CO_2$, the samples were incubated with 50 μL per well activated-XTT reagent for 4 h and the absorbance was measured at wavelengths of 475 nm and 660 nm. For testing, cells of all experimental and control groups were encapsulated into gels at the density of $1\times10^7$/mL and cultured in a 24-well plate for 24, 48 and 72 h. At each time point, activated-XTT reagent was added to the wells, followed by gentle shaking to mix the reagent with the culture media. The OD values were read at the same wavelength and calculated according to following formula:

Specific Absorbance = $A_{475nm}$(Test) - $A_{475nm}$(Blank) - $A_{660nm}$(Test)

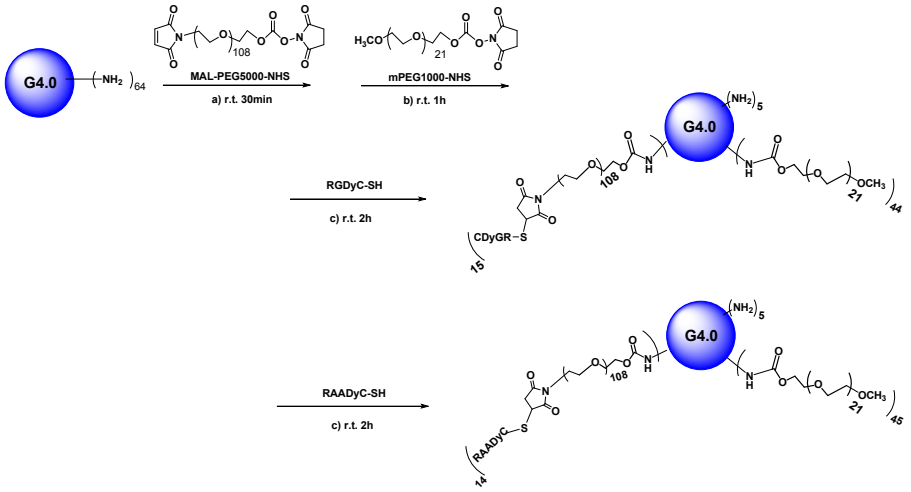
**Mouse calvarial critical size defect model**

Mouse calvarial critical size defect (CSD) model was used to test the ability of from **OPD**, **1/OPD** at a ratio of 1/2 to heal the bony wound (**OPD**: 15% w/v). 8-12 weeks old C57black/6 mice (female) were used to construct CSD models. The animals were anesthetized through intra-peritoneal injection with 124/8.8mg/kg ketamine/xylazine. After the hair was shaved, the incision site was sterilized with betadine before surgery. The parietal bones were exposed along with the sagittal suture by using a scalpel. Then, a 4 mm diameter of defect (CSD) was created by utilizing a trephine bur attached to a dental hand piece at 800 rpm with water cooling (saline). To stop bleeding, the wound was pressed gently with saline wet gauze. Around 15 μL preminxed gelator mixed with 10 μL 1% w/v $NaIO_4$ were placed over the defect (5 μL 0.5% nano-hydroxyapatite (Sigma Aldrich, USA) saline suspension was also added in followed experiment). After 2 min gelation, the hydrogel sealed the defect and the skin was sutured by using absorbable 4-0 chromic gut sutures (Ethicon, Somerville, NJ). The mice were allowed to recover on a heating pad (35$^{o}$C-37$^{o}$C) before transferred to recovery cages with soft bedding. All procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committees of the Chongqing Medical University and Peking University.

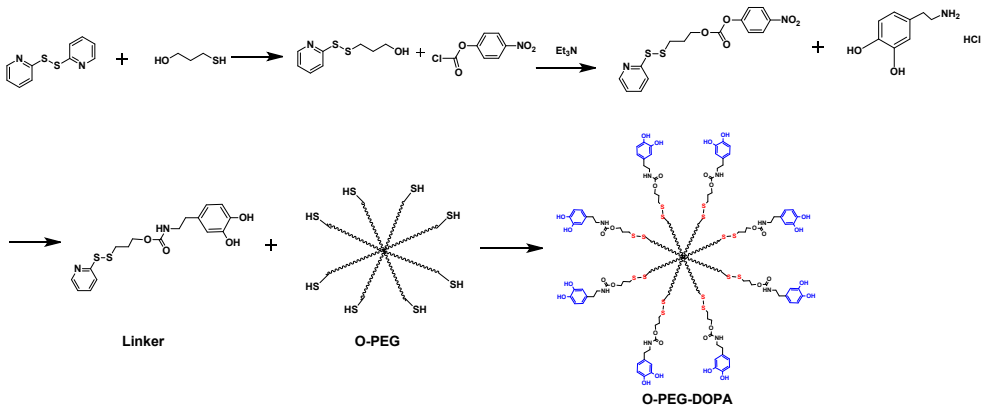
**Micro-CT imaging and analysis**

After 4 and 6 weeks of surgery, studied mice were euthanized using $CO_2$ asphyxiation. The heads were harvested and immediately soaked in pH 7.1-7.4 4% paraformaldehyde for 3 d for fixation. Cranial defects of studied animal were scanned after fixation, using a μCT imaging system (Skyscan 1074HR, Aartselaar, Belgium) at a resolution of 20.5 μm/pixel. The following settings were used for the scan: X-ray voltage of 40 kV, anode current of 1000 μA for the 360 rotational steps. Scanned images were reconstructed using NRecon software (Skyscan, Aartselaar, Belgium). Representative 3-Dementional images were created using CTAn and CTVol software provided by Skyscan.

## Schemes




**Scheme S1.** The synthetic route of G4.0-RGDyC (**1**) and G4.0-RAADyC (**2**). r.t. = room temperature.




**Scheme S2.** The synthetic route of **OPD**

## Figures

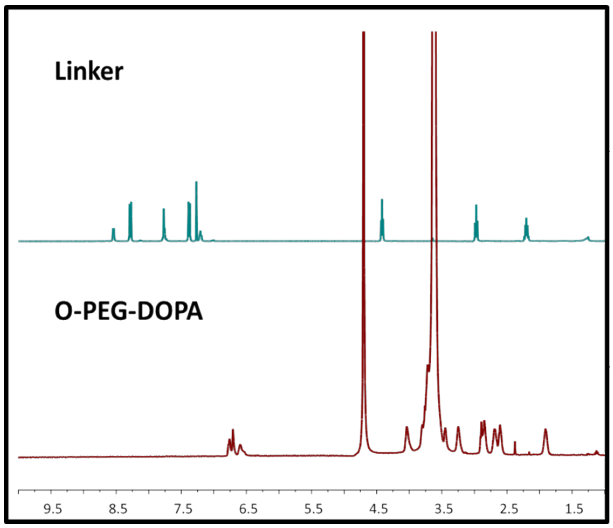



**Fig. S1.** $^{1}$H NMR spectra of **Linker** and **O-PEG-DOPA** (**OPD**). The appearance of the peaks at 6.76, 6.71, 6.60 ppm for benzene of **O-PEG-DOPA** (**OPD**) and the disappearance of the peaks at 8.49, 8.29, 7.65, 7.38, 7.13 ppm for benzene and pyridine of **Linker** indicated the formation of **O-PEG-DOPA** (**OPD**). Solvent: $D_2O$.

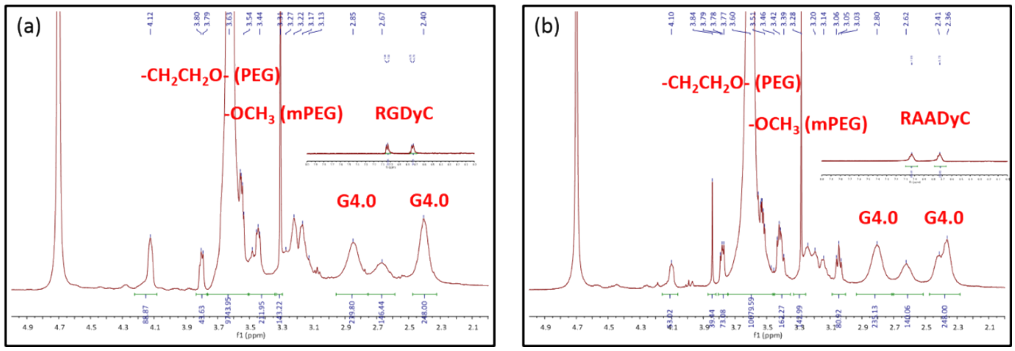



**Fig. S2.** $^{1}$H NMR spectra of (a) G4.0-RGDyC (**1**). $^{1}$H NMR (400 MHz, D2O): δ 7.03 (d, 30H), 6.75 (d, 30H), 4.12 (s, 89H), 3.80 (d, J = 4.3 Hz, 44H), 3.77 – 3.50 (m, 9744H), 3.45 (dd, J = 11.6, 7.3 Hz, 212H), 3.31 (s, 143H), 2.85 (s, 240H), 2.67 (s, 146H), 2.40 (s, 248H). (b) G4.0-RAADyC (**2**). $^{1}$H NMR (400 MHz, D2O): δ 7.03(s, 30H), 6.75 (s, 30H), 4.10 (s, 53H), 3.84 (s, 39H), 3.81 – 3.74 (m, 73H), 3.53 (d, J = 59.7 Hz, 10080H), 3.46 – 3.35 (m, 162H), 3.27 (d, J = 11.2 Hz, 143H), 3.05 (t, J = 5.9 Hz, 81H), 2.80 (s, 235H), 2.62 (s, 140H), 2.39 (d, J = 20.2 Hz, 248H). Solvent: $D_2O$.

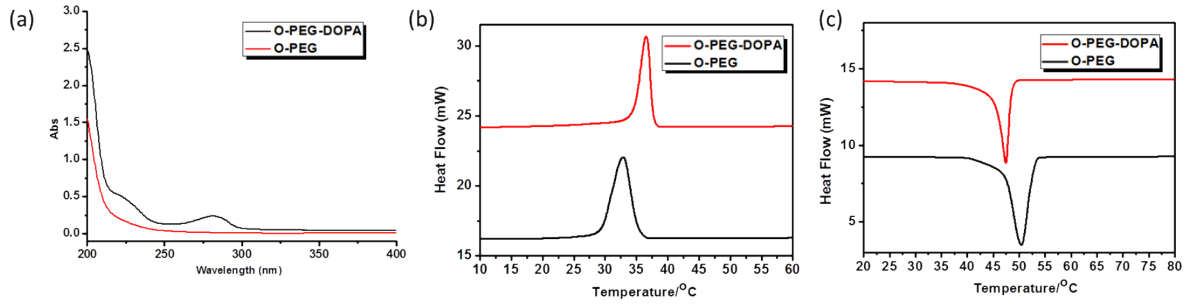



**Fig. S3.** (a) UV-Vis spectra of O-PEG and **O-PEG-DOPA** (**OPD**); DSC profiles of O-PEG and **O-PEG-DOPA** (**OPD**) at (b) the first cooling cycle revealed crystallization temperatures ($T_c$) of 32.88°C and 36.54°C for O-PEG and **O-PEG-DOPA** (**OPD**); and (c) the second heating cycle revealed melting points ($T_m$) of 50.41°C and 47.42°C for the samples of O-PEG and **O-PEG-DOPA** (**OPD**) respectively.

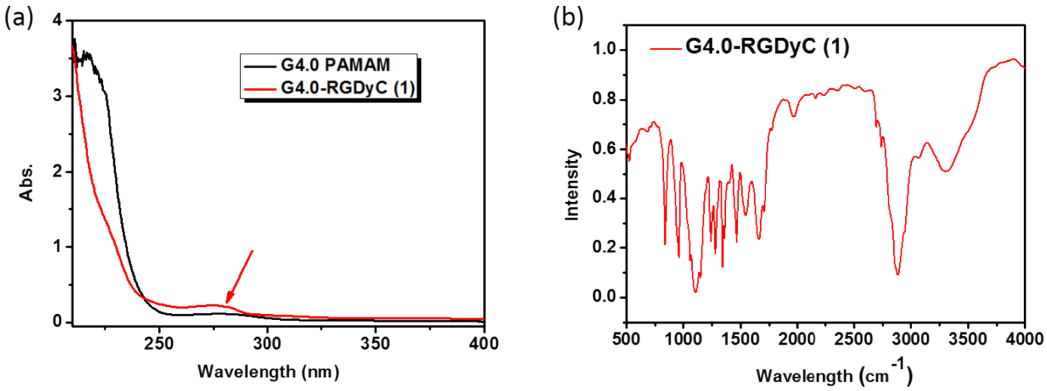



**Fig. S4.** (a) UV-Vis spectra G4.0 PAMAM and G4.0-RGDyC (**1**); and (b) FT-IR spectrum of G4.0-RGDyC (**1**).

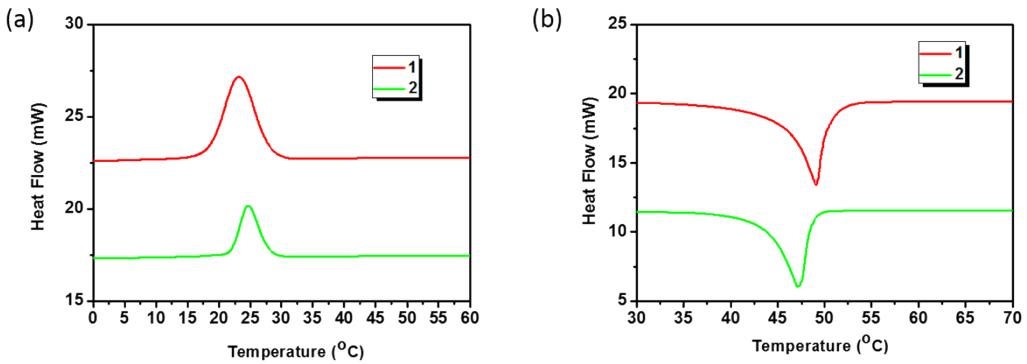


**Fig. S5.** DSC profiles of the modified dendrimers of G4.0-RGDyC (**1**) and G4.0-RAADyC (**2**). (a) The cooling cycle revealed crystallization temperatures ($T_c$) of 23.20 and 24.66°C, respectively; and (b) the second heating cycle revealed melting points ($T_m$) of 49.09 and 47.19°C, respectively.

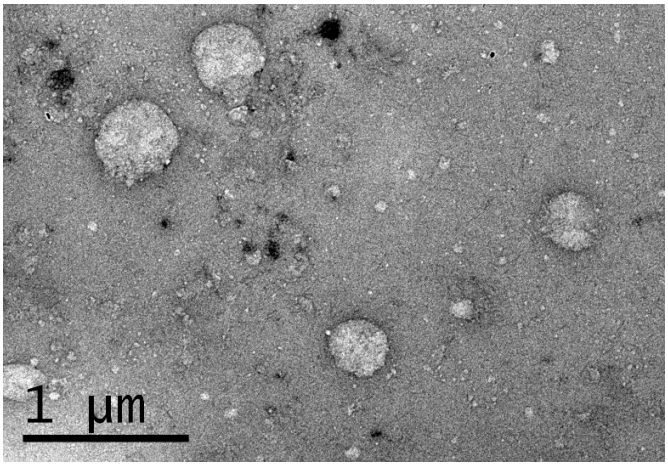



**Fig. S6.** TEM images of **1** (15% w/v) with $NaIO_4$ in deionized water (scale bar: 1 μm).

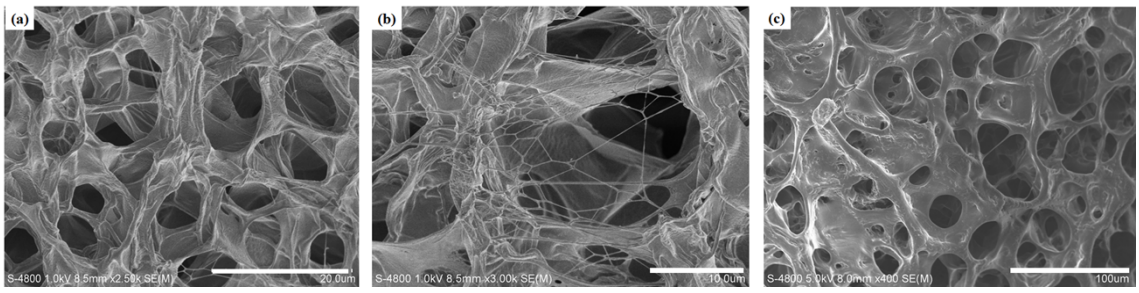



**Fig. S7**. SEM images of **1/OPD** hydrogel with scale bar of (a) 20 and (b) 10 μm, and **2/OPD** hydrogel with scale bar of (c) 100 μm. The hydrogels were prepared from **1/OPD** and **2/OPD** (at a 1/2 ratio, **OPD**: 15% (w/v)) and fully swelled at 37°C after 48 h.

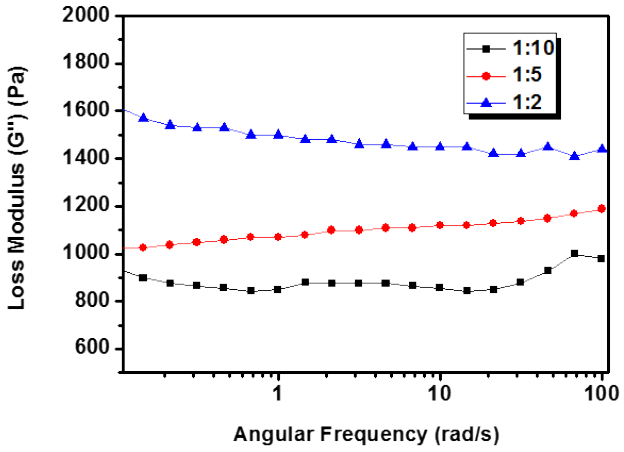



**Fig. S8**. Plots of the angular frequency (**ω**) versus loss modulus (G'') of **1/OPD** hydrogels with different ratios. **OPD**: 15% (w/v). (measured at a strain of 10%)

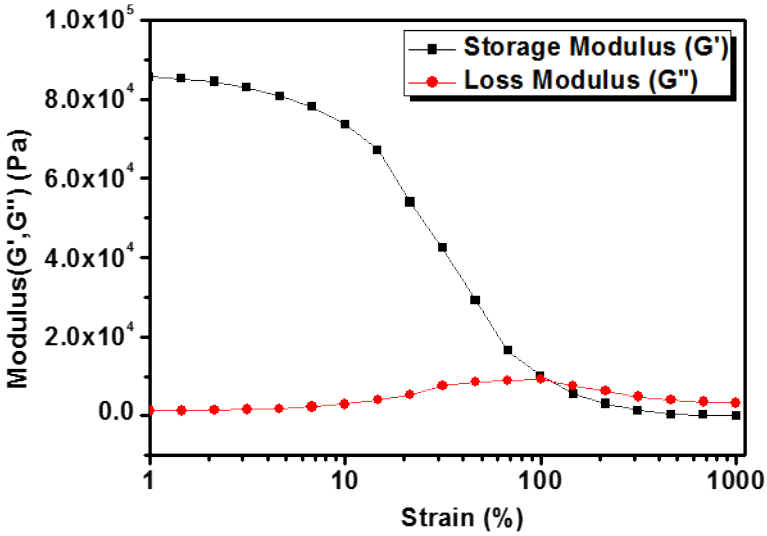



**Fig. S9.** Oscillatory rheological measurements of the hydrogel from **1/OPD** at a 1/2 ratio. **OPD**: 15% (w/v).

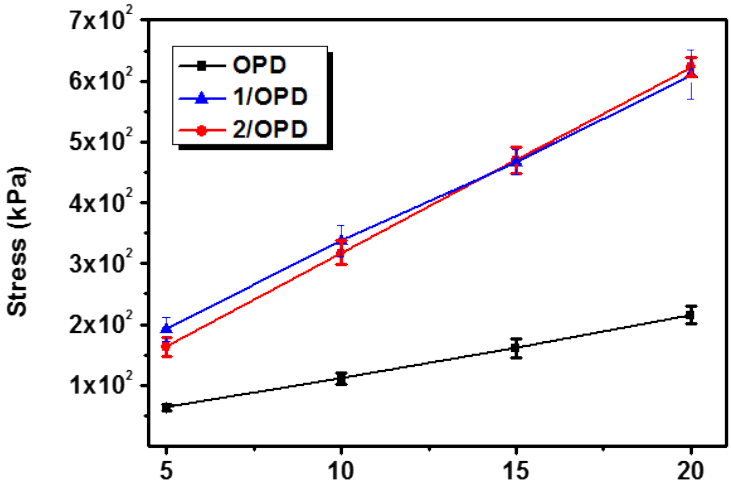



**Fig. S10.** Determination of the compressive modulus E of the hydrogels from **OPD**, **1/OPD** and **2/OPD** at the ratio of 1/2. **OPD**: 15% (w/v).

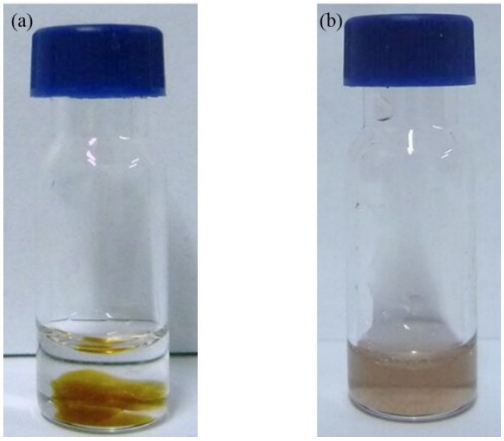



**Fig. S11.** Rapidly degraded phenomena of the **1/OPD** hydrogel with the ratio of 1/2 immersed into 10 mM GSH PBS buffer. (a) The fully swelled hydrogel was immersed into 10 mM GSH PBS buffer and then (b) the hydrogel was immediately degraded and turned to be a solution in 5 min. **OPD**: 15% (w/v).

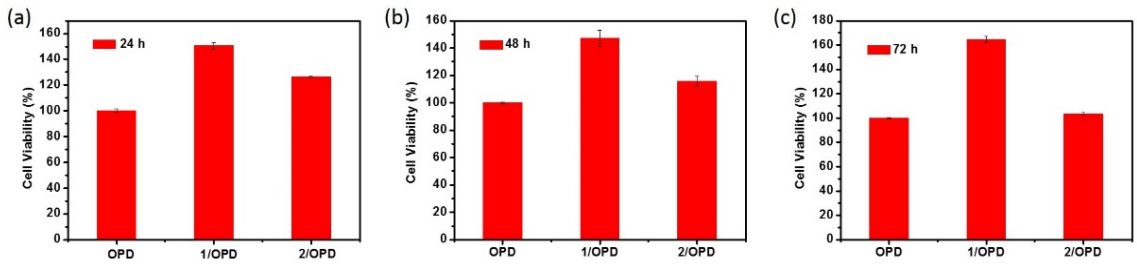



**Fig. S12.** Cell viability of mMSC-hydrogel constructs measured by the XTT assay after (a) 24 (b) 48 and (c) 72 h in culture comparing with hydrogels composed of **OPD**, **1/OPD** and **2/OPD** at the ratio of 1/2. **OPD**: 15% (w/v).

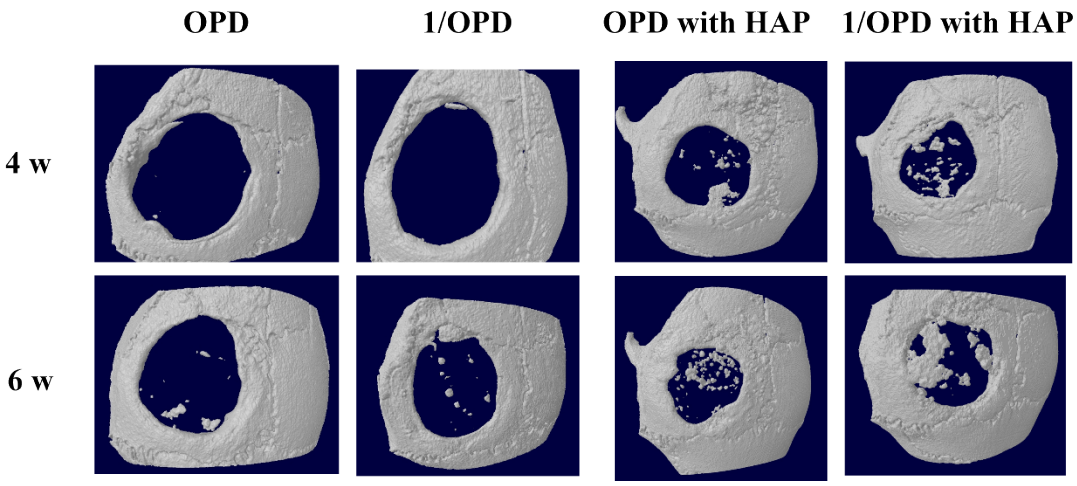



**Fig. S13.** μCT images of mouse cranial defect in 4 or 6 weeks. Hydrogels from **OPD** alone (15% w/v) and **1/OPD** at the ratio of 1/2 were used for repairing the defect. And HAP was also added for further observation of the defect repair. **OPD**: 15% (w/v).

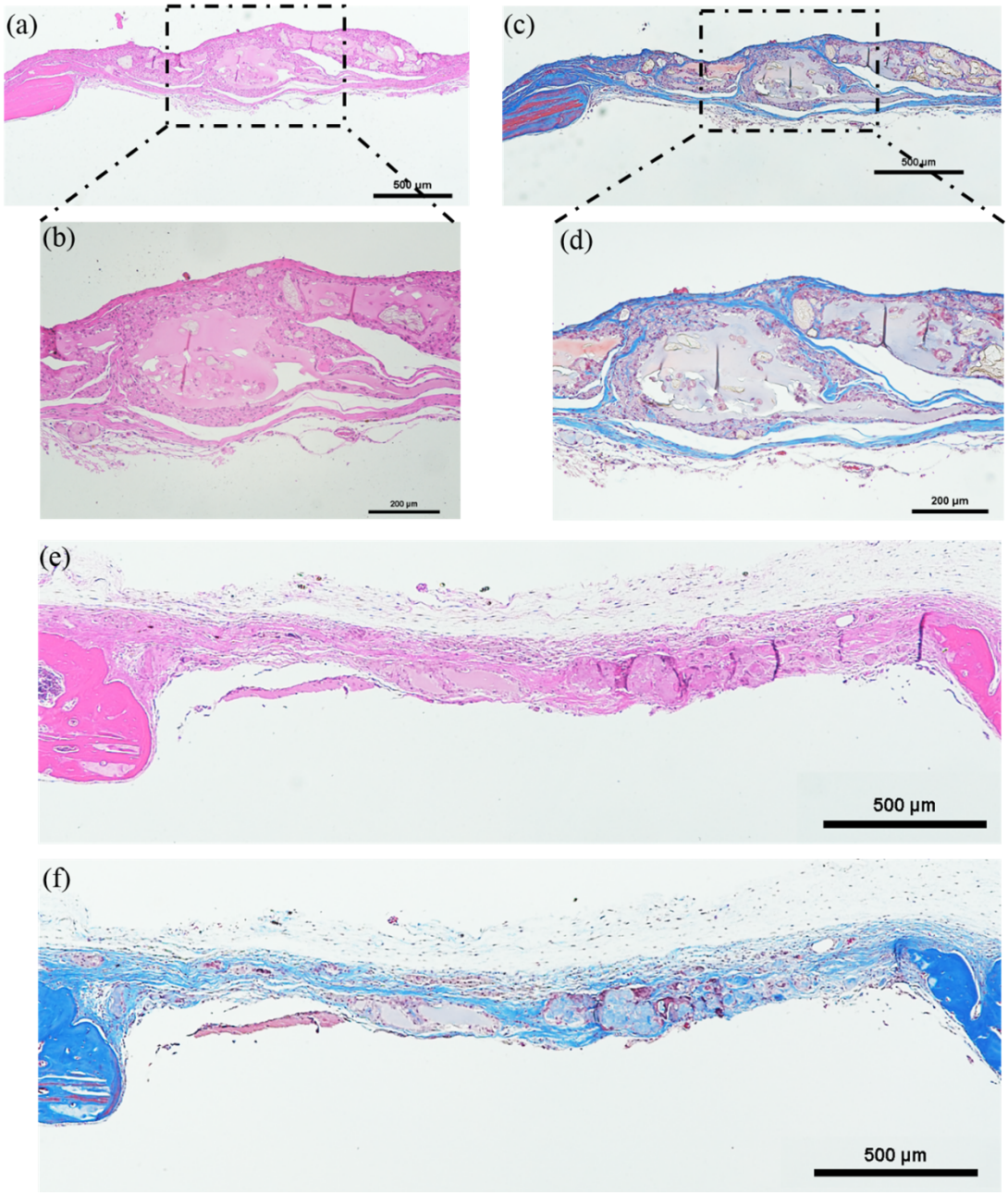



**Fig. S14.** Histology of treated bone defects with 15% (w/v) **OPD** hydrogel after 4 weeks (a-d) and 6 weeks (e, f). H&E (a, b, e) and Masson's trichrome staining (c, d, f) were used to show the defect repair.

## Tables

**Table S1.** The Gelation of **OPD**

| **OPD (w/v)** | **$NaIO_4$ (w/v)** | **Gelling Time** |
|---|---|---|
| 5% | 1% | >4h |

| | | |
|---|---|---|
| 10% | 1% | 30 min |
| 12.5% | 1% | 20 min |
| 15% | 0% | No gel |
| 15% | 0.1% | 30 min |
| 15% | 1% | 5 min |
| 20% | 1% | 5 min |

**Table S2.** The Gelation of **OPD** with 1/2 of Different Ratios

| With OPD | G4.0-RGDyC (1) | G4.0-RGDyC (2) |
|---|---|---|
| **10:1** | gel | gel |
| **5:1** | gel | gel |
| **2:1** | gel | gel |
| **Without OPD** | nanogel | nanogel |

NB: The concentration of **OPD** is 15% (w/v).

**Table S3.** The Swelling ratio of the Hydrogels

| Swelling Ratio (%) | OPD | 1/OPD | 2/OPD |
|---|---|---|---|
| **Average** | 1511.44 | 248.55 | 245.28 |
| **SD** | 355.21 | 13.92 | 8.92 |

NB: The concentration of **OPD** is 15% (w/v). The ratio of **1(2)/OPD** = 1/2.

**Table S4.** Storage and Loss Modulus of the Hydrogels

| | OPD | 1/OPD | 2/OPD |
|---|---|---|---|
| **G' (mean±SD)(Pa)** | 36220±1075 | 83720±2123 | 77410±2761 |
| **G'' (mean±SD) (Pa)** | 997±54 | 1441±40 | 1234±28 |

NB: The concentration of **OPD** is 15% (w/v). The ratio of **1**(**2**)**/OPD** = 1/2.

**Table S5.** Compressive Modulus of the Hydrogels

| | OPD | 1/OPD | 2/OPD |
|---|---|---|---|

| | | | |
|---|---|---|---|
| **Compressive Modulus/E (KPa)** | 10 | 28 | 31 |

NB: The concentration of **OPD** is 15% (w/v). The ratio of **1**(**2**)**/OPD** = 1/2.

## Reference

[1] M. Soleimani, S. Nadri, *Nat. Protocols* **2009**, *4*, 102.