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A dual-enzymatically cross-linked injectable gelatin hydrogel loaded with BMSC improves neurological function recovery of traumatic brain injury in rats

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Supporting Information

1.1 Phenolic contents of the Gelatin-Hydroxyphenyl (GH) conjugates

The phenolic contents of the GH conjugates were measured quantitatively at 275 nm using a UV visible spectrophotometer (UV-vis-NIR SPECTROPHOTOMETER, UV-3600Plus, Japan). Briefly, the absorbance of a gradient concentration of HPA at 275 nm was measured and shown in Fig. S1a. A standard curve of HPA was made in according to these data (Fig. S1b). Then the absorbance of 1 mg/ml GH solution at 275 nm was also measured, and the graft ratio of GH was calculated from the standard curve.

(a)

HPA (μg/ml)	0	1	3	5	7	9
Absbance	0	0.0849	0.2914	0.4684	0.6543	0.812
GH (mg/ml)	1					
Absbance	0.6635					

(b)

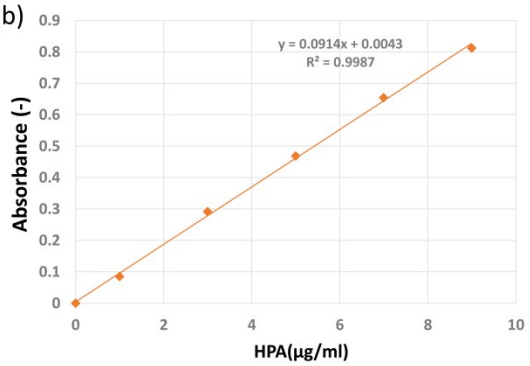

Fig. S1 (a) The absorbance of 0, 1, 3, 5, 7, 9 μg/ml HPA and 1mg/ml GH at 275 nm using a UV visible spectrophotometer, (b) standard curve of HPA.

1.2 Degradation of GH hydrogel in vitro

The degradation performance of the hydrogels was investigated via another formula: L (%) = $[(W_I-W_D)/W_I] \times 100$, where L denotes the mass retention rate after hydrogels were immersed into 0.01 M pH=7.4 PBS solution for 1, 3, 7, 9, 14, 21, 25, and 28 days. The initial mass of hydrogels on day0 before immersing into PBS was labeled as W_I, and the mass of hydrogels after immersion for 1, 3, 7, 9, 14, 21, 25, and 28 days was labeled as W_D.

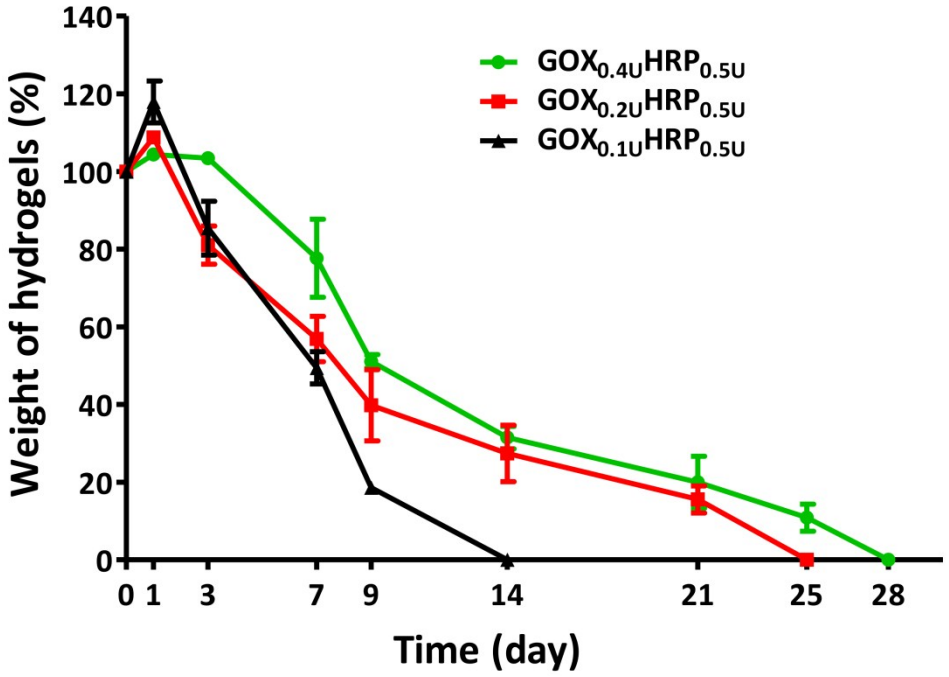

Fig. S2 Degradation of hydrogels of $GOX_{0.4U}HRP_{0.5U}$, $GOX_{0.2U}HRP_{0.5U}$, $GOX_{0.1U}HRP_{0.5U}$ within 0.01M pH=7.4 PBS solution.

1.3 Effect of GOX content on the hydrogel morphology and cellular activity

GH hydrogel formed by different GOX displays a different morphology after soaking in the culture solution. As GOX content gradually increases, the color of GH hydrogel darkens, and the transparency decreases (Fig. S3a). When GOX content exceeds 0.25U, an obviously shrinkage occurs, which may affect the activity of cells within hydrogel. This phenomenon may attribute to the different degree of crosslinking of GH hydrogel under different GOX condition. 3D cellular culture study shows that lots of cells died within over crosslinking or shrinking hydrogel (exceeding 0.5 U GOX), while cells present a high activity encapsulated in low crosslinking or shrinking hydrogel (< 0.25 U GOX) (Fig. S3b).

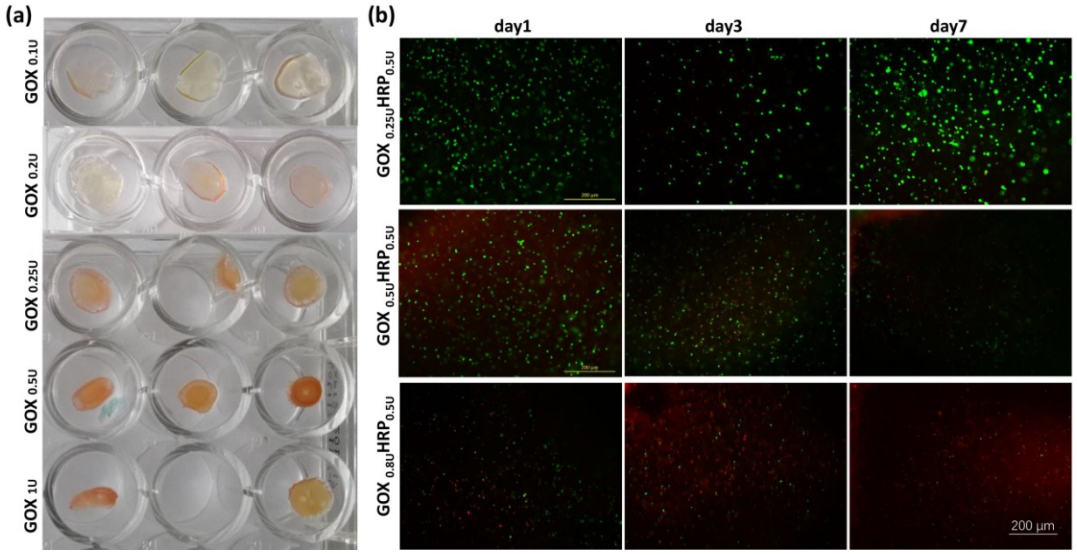

Fig. S3 (a) Morphology of 100 μL GH hydrogel formed by 0.1, 0.2, 0.25, 0.5, and 1U GOX and 0.5U HRP after soaking in the culture solution. (b) Fluorescent images of BMSC encapsulated within 8%

GH hydrogels of $GOX_{0.25U}HRP_{0.5U}$, $GOX_{0.5U}HRP_{0.5U}$, and $GOX_{0.8U}HRP_{0.5U}$ on day1, day3 and day7. Green labels the living cells, red labels the dead cells. Scale bar is 200 μm.

1.4 Quantization and cytotoxicity of the generated H_2O_2 from GH hydrogel to BMSC

The generated H_2O_2 during the gelation was quantify by Micro Hydrogen Peroxide (H_2O_2) Assay Kit, and was shown in the Fig. S4. Briefly, 100 μl of a GH hydrogel was prepared in a 24-well plate and incubated with 900 μl of DMEM/F12 medium. At predetermined time points, each medium (including H_2O_2 released from the hydrogel matrices) was collected and tested by Micro Hydrogen Peroxide Assay Kit. In addition, CCK-8 was used to evaluate the cytotoxicity of the accumulated generated H_2O_2 from GH hydrogel to BMSC and the result was shown in Fig. S5. All results indicated that the accumulated generated H_2O_2 from GH hydrogel of $GOX_{0.1U}HRP_{0.5U}$ or $GOX_{0.2U}HRP_{0.5U}$ has little cytotoxicity to BMSC, while the accumulated generated H_2O_2 from GH hydrogel of $GOX_{0.4U}HRP_{0.5U}$ has a great cytotoxicity to BMSC. And this result is consistent with the result of 3D cellular culture.

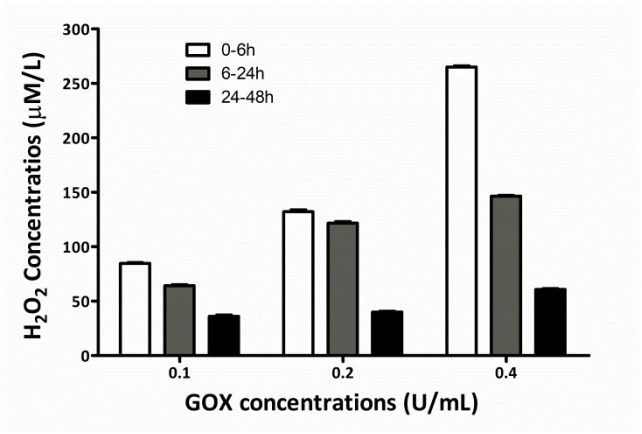

Fig. S4 Quantization of the generated H_2O_2 from GH hydrogels of $GOX_{0.1U}HRP_{0.5U}$, $GOX_{0.2U}HRP_{0.5U}$, and $GOX_{0.4U}HRP_{0.5U}$ in 0-6, 6-24, and 24-48 h by Micro Hydrogen Peroxide (H_2O_2) Assay Kit.

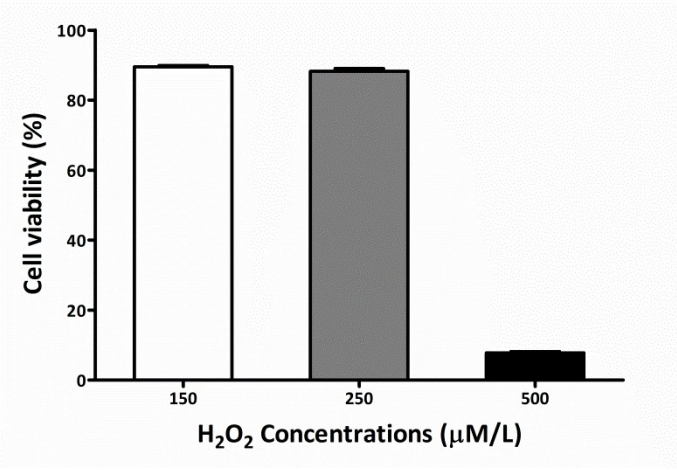

Fig. S5 Evaluate the cytotoxicity of the generated H_2O_2 from GH hydrogels of $GOX_{0.1U}HRP_{0.5U}$, $GOX_{0.2U}HRP_{0.5U}$, and $GOX_{0.4U}HRP_{0.5U}$ to BMSC by CCK-8 on 24 h.

1.5 Biodegradability of GH hydrogel in vivo

To evaluate the biodegradability of GH hydrogel, GH/HRP/GOX/D-Glucose (8% GH, 1U HRP, 0.1U GOX, 50 mM D-Glucose) mixed solution was subcutaneously injected into SD rats. The solution formed hydrogel quickly *in situ*. On day3 and day7, the SD rats were sacrificed and the

biodegradability of GH hydrogels was observed.

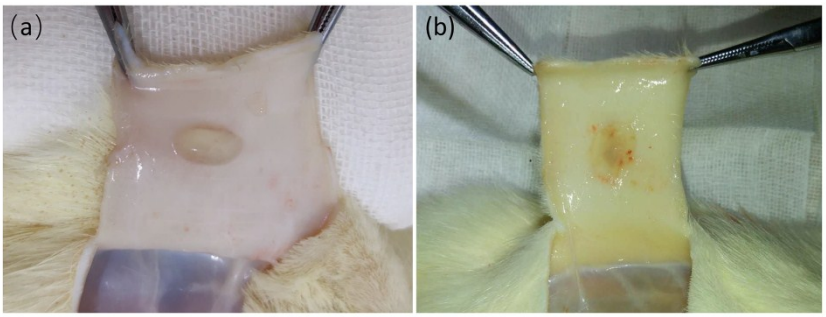

Fig. S6 The biodegradability of GH hydrogel of $GOX_{0.1U}HRP_{0.5U}$ on day3 (a) and day7 (b) after subcutaneous injection.

1.5 The criteria of scoring mNSS

Table S1 The criteria of scoring mNSS

Motor tests		Points
Raising rat by the tail		**3**
Flexion of forelimb	1	
Flexion of hindlimb	1	
Head moved >10° to vertical axis within 30 s。	1	
Placing rat on the floor (normal=0; maximum=3)		**3**
Normal walk	0	
Inability to walk straight	1	
Circling toward the paretic side	2	
Fall down to the paretic side	3	
Sensory tests		**2**
Placing test (visual and tactile test)	1	
Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	2	
Beam balance tests (normal=0; maximum=6)		**6**
Balances with steady posture	0	
Grasps side of beam	1	
Hugs the beam and one limb falls down from the beam	2	
Hugs the beam and two limbs fall down from the beam, or spins on beam (>60 s)	3	
Attempts to balance on the beam but falls off (>40 s)	4	
Attempts to balance on the beam but falls off (>20 s)	5	
Falls off: No attempt to balance or hang on to the beam (<20 s)	6	
Reflexes absent and abnormal movements		**4**
Pinna reflex (head shake when touching the auditory meatus)	1	
Corneal reflex (eye blink when lightly touching the cornea with cotton)	1	
Startle reflex (motor response to a brief noise from snapping a clipboard paper	1	
Seizures, myoclonus, myodystony	1	
Maximum points		18

1.6 NSE immunofluorescence staining

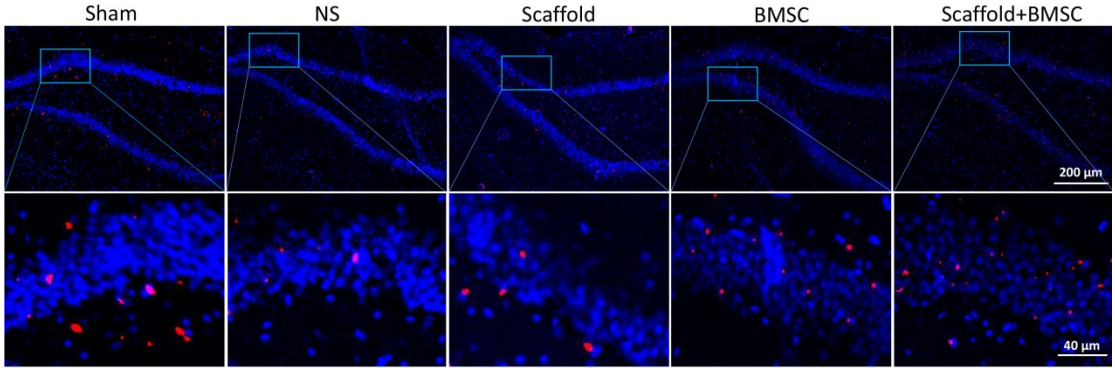

Fig. S7 NSE staining of the hippocampus of SD rats with cerebral injury after treatment for 28 days. The red color indicates positive expression of NSE.

1.7 Ki67 immunofluorescence staining

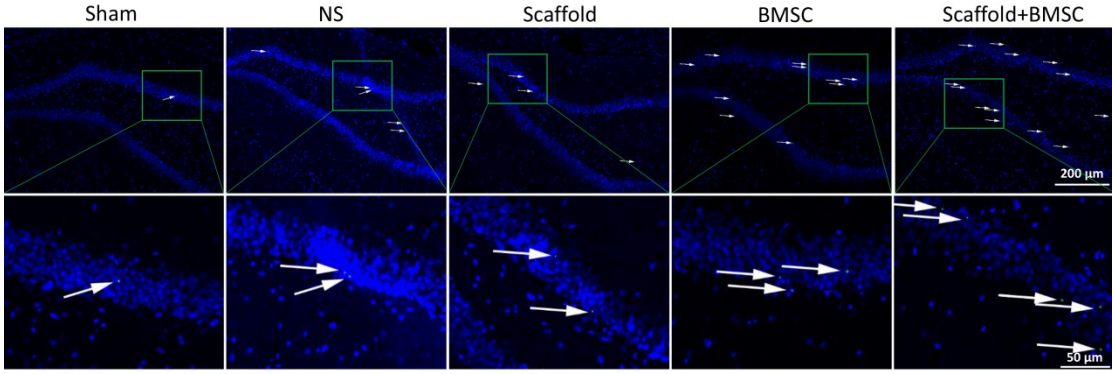

Fig. S8 Ki67 staining of the hippocampus of SD rats with cerebral injury after treatment for 28 days. The green color indicates positive expression of Ki67.