

## **Electronic Supplementary Information**

### **In Situ Forming Enzyme-free Hydrogels via Ferromagnetic Microbeads-assisted Enzymatic Cross-linking**

Jin Woo Bae, Bae Young Kim, Eugene Lih, Jong Hoon Choi, Yunki Lee,  
and Ki Dong Park\*

Department of Molecular Science and Technology, Ajou University  
5 Woncheon, Yeongtong, Suwon 443-749, Republic of Korea

## Experimental

### Materials

Horseradish peroxidase (HRP, type VI, salt-free, 250–330 units/mg solid), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), glycidyl methacrylate (GMA), methyl methacrylate (MMA), ethyleneglycol dimethacrylate (EGDMA),  $\alpha$ - $\alpha'$ -azoisobisbutyronitrile (AIBN), ferric chloride ( $\text{FeCl}_3$ ), sodium chloride (NaCl), bovine serum albumin (BSA) standard, and diammonium 2,2'-azino-bis(3-ethyl-benzothiasoline-6-sulfonate) (ABTS) were obtained from Sigma Aldrich (St. Louis, MO, USA). Gelatin-poly(ethylene glycol)-tyramine (GPT) was synthesized and characterized as previously reported.<sup>1</sup> All other chemicals were used as received without any further purification.

### Preparation of ferromagnetic microbeads

Ferromagnetic microbeads with epoxy groups were prepared using an aqueous dispersion polymerization of GMA and MMA with modification.<sup>2</sup>  $\text{FeCl}_3$  (4.87 g, 30 mmol) was dispersed in deionized (DI) water (100 mL) containing NaCl (2.34 g, 40 mmol) at room temperature. AIBN (0.1 g, 0.6 mmol) was added to toluene (10 mL) at 4 °C and sonicated for 30 min, followed by successive addition of GMA (4.01 g, 30 mmol), MMA (3.53 g, 40 mmol), and EGDMA (3.94 g, 20 mmol). To a solution of  $\text{FeCl}_3$  heated to 65 °C, the monomer solution was added dropwise and stirred at 75 °C for 2 h. The solution was then reacted at 85 °C for 2 h. The resulting beads were filtered, washed thoroughly with DI water and ethanol, and dried in vacuo. The yield of epoxy-functionalized beads obtained through 300 and 500  $\mu\text{m}$  sieves was approximately 50%.

### Preparation of HRP immobilized ferromagnetic microbeads (HRP-beads)

HRP was chemically immobilized onto ferromagnetic microbeads with amine-reactive epoxy groups. Beads (1 g) was immersed in 8 mL of PBS (0.01 M, pH 7.4). 2 mL of HRP solution (2.5 mg/mL) was then added and stirred at room temperature for 2 h. The beads were thoroughly washed with PBS (200 mL) and DI water (1 L) to completely remove physically adsorbed HRP. Finally, the HRP immobilized beads (HRP-beads) were dried and stored at 4 °C before use.

### **Characterization of HRP-beads**

The surface morphology and particle size of beads before and after HRP immobilization were observed by field emission scanning electron microscope (JSM-6700F, JEOL, Japan). The iron content in beads was measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES, OTIMA 5300DV; PerkinElmer, USA). Briefly, beads (40 mg) was placed in a microtube and PBS (2 mL) was added. After 3 h incubation at 50 rpm, the supernatant (1 mL) was withdrawn and subjected to analysis. The amount of immobilized HRP was quantitatively measured using a micro BCA protein assay kit (Pierce, Rockford, IL). BSA was used as a standard.

### **Catalytic activity**

Catalytic activity of HRP-beads in solution was measured using the ABTS assay.<sup>3</sup> To determine HRP-dependent activity, different amounts of HRP-beads were added to a 1 mL cuvette containing 900  $\mu$ L of PBS (0.01 M, pH 7.4) and equilibrated for 5 min. After adding a mixture (100  $\mu$ L) of 1 mM ABTS and 0.01 wt%  $H_2O_2$ , absorbance changes at 414 nm were recorded as a function of time using a UV-Vis spectrophotometer (V-570, Jasco, Japan). Soluble HRP was used as a control. For  $H_2O_2$ -dependent activity, 20 mg of HRP-beads (or beads without HRP) was added to a 1 mL cuvette containing 900  $\mu$ L PBS (0.01 M, pH 7.4) and equilibrated for 5 min. A series of 1 mM ABTS solutions (100  $\mu$ L) containing various concentrations of  $H_2O_2$  ranging from 0.0001 to 0.1 wt% were prepared and added to beads-containing cuvettes. Absorbance at 414 nm was measured after 10 min.

### **Gelation time, rheological measurement and surface morphology**

Gelation time was measured by a vial tilting method. 5 wt% GPT and 0.01 wt%  $H_2O_2$  were mixed to a total volume of 300  $\mu$ L. The solution was then added to different amounts of HRP-beads placed in 2 mL microtubes and stirred at 100 rpm. Gelation time was determined when the tilted solution exhibited no flow.

The elastic modulus ( $G'$ ) of hydrogels was measured using a rheometer (Advanced Rheometer GEM-150-050; Bohlin Instruments, USA) as previously described.<sup>4</sup> HRP-beads (30 mg) were packed into a 1 mL syringe equipped with a needle (26G), followed by addition of PBS (300  $\mu$ L) to equilibrate the beads for 1 min. After removal of PBS, a GPT solution (300  $\mu$ L) containing 0.01 wt%  $H_2O_2$  was loaded into

a HRP-beads packed syringe. The GPT solutions injected after 5, 30, and 60 seconds were applied to the bottom plate, and the modulus was recorded as a function of time at 37 °C.

Internal structures of hydrogels were analyzed using a scanning electron microscopy (SEM; JSM-6380, JEOL, Japan). As described above, four kinds of hydrogels were prepared in a circular Teflon mold (diameter, 0.50 cm; height, 0.15 cm). The formed hydrogels were quenched into liquid nitrogen, cross-sectioned and freeze-dried for 3 days. The morphology of hydrogels was observed using the SEM after gold sputter coating.

### **Fluorescent detection of HRP in hydrogels**

Fluorescein isothiocyanate-labeled HRP (FITC-HRP) was used to visualize HRP incorporated into hydrogels. FITC-HRP was immobilized onto the beads using the same protocol for the HRP-beads. Two kinds of GPT hydrogels were prepared using FITC-HRP and FITC-HRP-beads, respectively. First, each 5 wt% GPT solution (50  $\mu$ L) containing either 0.02 wt% H<sub>2</sub>O<sub>2</sub> or FITC-HRP (10  $\mu$ g) was prepared separately, and rhodamine phalloidin (Rho, 1 U/mL, Molecular Probes, Eugene, OR) was added to each solution. After loading into a dual syringe, the solutions were injected into a circular Teflon mold to form a cylindrical-shaped hydrogel. For the preparation of GPT hydrogel using FITC-HRP-beads, the beads (10 mg) were packed into a 1 mL syringe and hydrated with PBS for 1 min. GPT solution (100  $\mu$ L) containing 0.01 wt% H<sub>2</sub>O<sub>2</sub> and Rho was loaded and then injected into a mold after 30 s. The formed hydrogels were examined under an inverted fluorescence microscope (Motic AE31, Motic, UK), and images were acquired using a digital camera (Moticam Pro 205A, Motic, UK).

### ***In vitro* cell studies**

Cytotoxicity of HRP-beads was evaluated using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Tokyo, Japan). Different amounts of HRP-beads (1, 10, and 100 mg) were placed in a 24-well plate. Human dermal fibroblasts (hDFBs) ( $2 \times 10^4$  cells/well) were seeded and cultured with DMEM supplemented with 10% FBS and 1% PS at 37 °C under a humidified atmosphere of 5% CO<sub>2</sub>. After 1 day of culture, 20  $\mu$ L of CCK reagent was added to each well, and the absorbance was measured at 450 nm.

Cell viability of GPT hydrogels was evaluated by *in vitro* 3D culture of hDFBs. hDFBs ( $2 \times 10^5$  cells) were suspended in 3 wt% GPT solution (200  $\mu$ L) containing 0.01 wt% H<sub>2</sub>O<sub>2</sub>. The resulting cell-polymer solution was loaded into a syringe packed with 20 mg of HRP-beads and then placed in a 48-well plate by injecting 30 s after bead-solution contact. As a control, cells were suspended in GPT solution (50  $\mu$ L) containing 0.02 wt% H<sub>2</sub>O<sub>2</sub> and mixed with GPT solution (50  $\mu$ L) containing HRP (20  $\mu$ g). After 1, 4, and 7 days of culture, viable cells were assessed by the CCK-8 assay. For confocal microscopy, the hDFBs cultured in hydrogels were fixed with 4% paraformaldehyde for 90 min at room temperature. Immunofluorescent staining with Rho and Hoechst 33258 was performed as previously described.<sup>1</sup> Confocal images were taken at 200 $\times$  magnification using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss AG, Germany).

### Statistical analysis

Statistical analysis between the experimental data of two groups was performed using a Student's *t*-test. Statistical significance was set to a *p*-value < 0.001. All results were presented as mean  $\pm$  standard deviation (*n* = 4).

### References

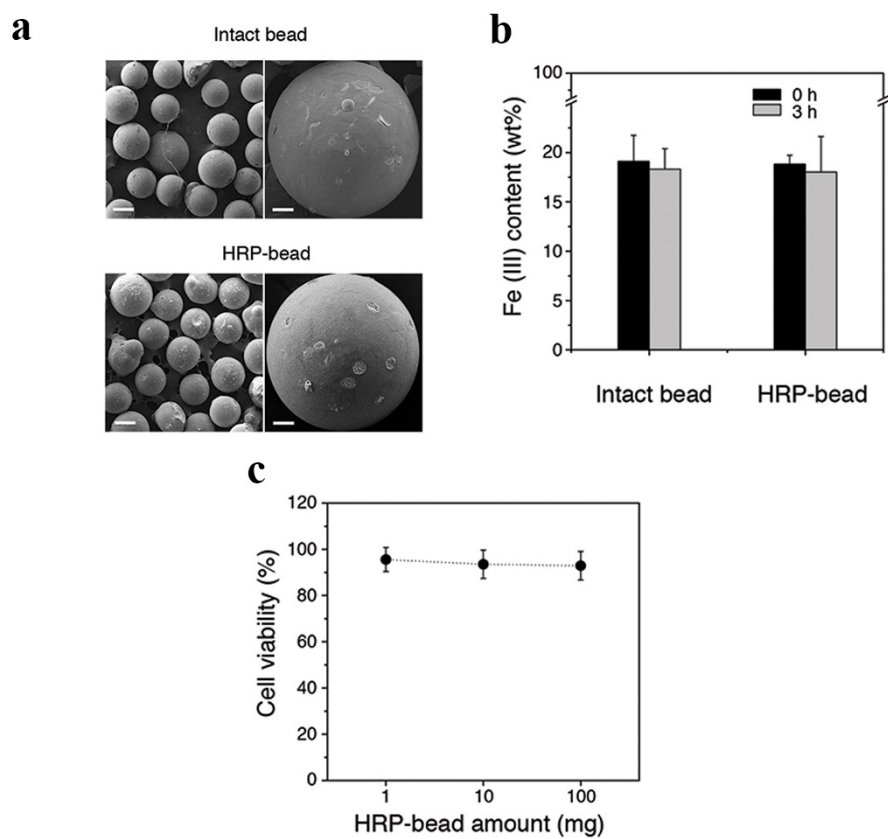
1. K. M. Park, K. S. Ko, Y. K. Joung, H. Shin and K. D. Park, *J. Mater. Chem.*, 2011, **21**, 13180-13187.
2. G. Bayramoglu and M. Y. Arica, *J. Hazard. Mater.*, 2008, **156**, 148-155.
3. A. F. Naves, A. M. Carmona-Ribeiro and D. F. Petri, *Langmuir*, 2007, **23**, 1981-1987.
4. K. M. Park, Y. Lee, J. Y. Son, J. W. Bae and K. D. Park, *Bioconjug. Chem.*, 2012, **23**, 2042-2050.

**Table S1.** HRP-beads with different iron contents.

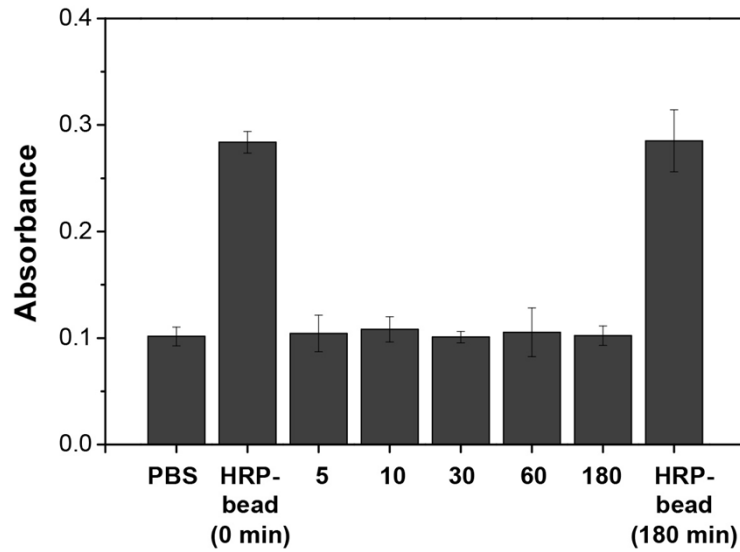
Samples	Feed amount of FeCl <sub>3</sub> (wt% of bead)	Fe content (wt% of bead)	Feed amount of HRP (mg/g of bead)	Immobilized amount of HRP (mg/g of HRP- bead)
HRP-bead/F0	0	0	5	1.18 ± 0.09
HRP-bead/F25	25	19.1 ± 1.0	5	1.03 ± 0.18
HRP-bead/F50	50	39.7 ± 1.5	5	0.75 ± 0.09

**Table S2.** HRP-beads with different HRP amounts.

Sample	Feed amount of HRP (mg/g of bead)	Immobilized amount of HRP (mg/g of HRP-bead)	Immobilization efficiency (%)
HRP-bead/F25	2	0.32 ± 0.02	16.1 ± 1.1
	5	1.03 ± 0.18	20.7 ± 3.7
	10	2.26 ± 0.35	22.6 ± 3.5
	20	4.67 ± 0.97	23.4 ± 4.9



**Fig. S1.** (a) SEM images of intact beads and HRP-beads/F25. Scale bars indicate 200  $\mu\text{m}$  (left) and 50  $\mu\text{m}$  (right). (b) Changes in iron content of HRP-beads/F25 after HRP immobilization. (c) Dose-dependent cytotoxicity of HRP-beads/F25 against hDFBs after 1 day of culture.



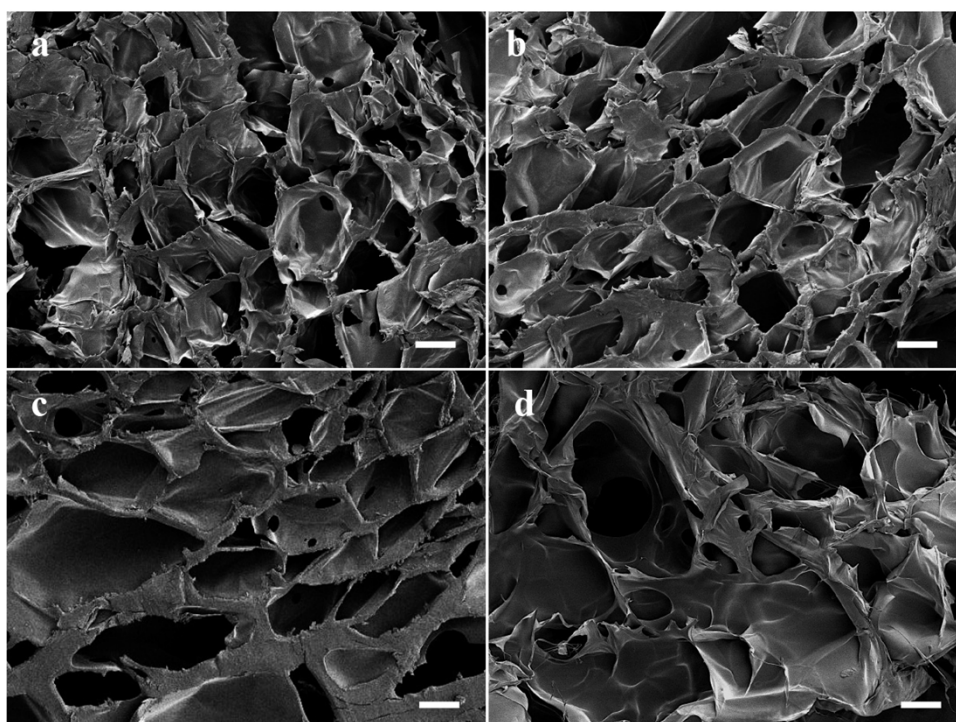
**Fig. S2.** Stability of HRP immobilized on beads.

#### **Further characterization of HRP-beads/F25**

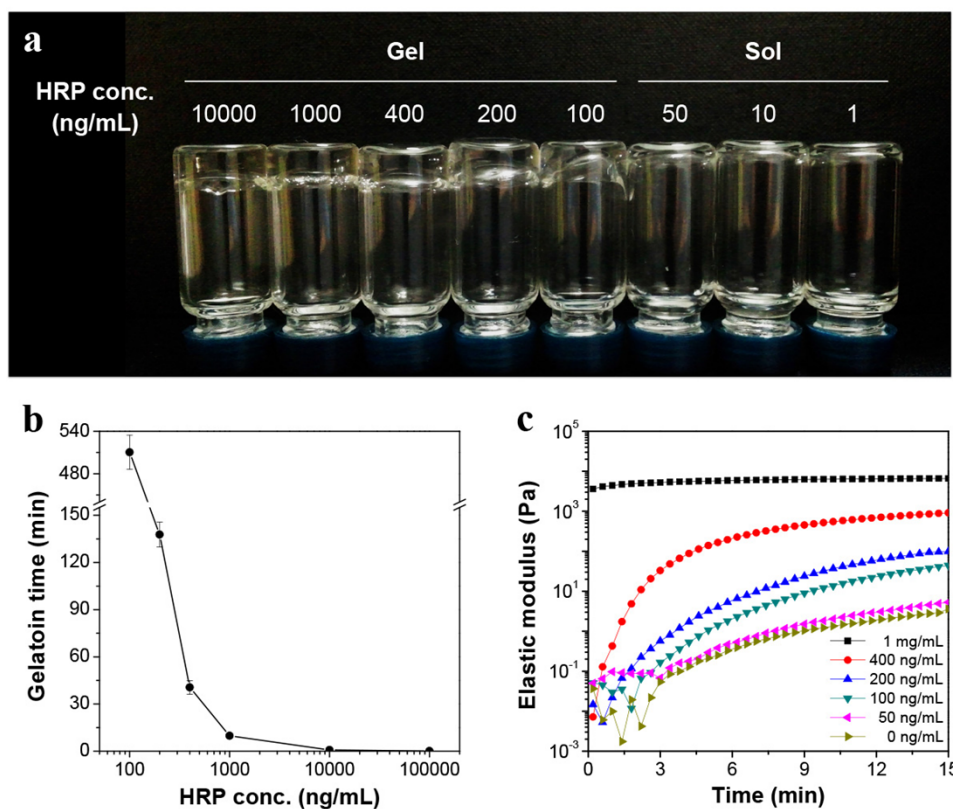
It was observed that the surface morphology of HRP-beads/F25 was almost the same compared to intact beads (Fig. S1a). Both beads were spherical in shape with 300–500  $\mu\text{m}$  in diameter. To investigate whether iron particles were released during the immobilization step, we measured the iron contents in intact beads and in HRP-beads/F25, and found that there was a negligible change in the iron content (Fig. S1b). It also appeared that the iron particles were hardly released after additional 3 h incubation. These results support that the iron content of HRP-beads/F25 was retained after HRP immobilization. We also confirmed that the initial amount of immobilized HRP was consistent with the amount of HRP in HRP-beads/F25 measured after 3 h of further incubation. For cell delivery, cells of interest need to mix with a phenol-rich polymer solution containing  $\text{H}_2\text{O}_2$  and then be injected using a syringe packed with HRP-beads. From the viewpoint of cell-bead contact, the dose-dependent cytotoxicity of HRP-beads/F25 was investigated. We found that hDFBs were almost 100% viable for 1 day even at the highest concentration of HRP-beads/F25 (Fig. S1c).

To confirm whether immobilized or adsorbed HRP was released from HRP-bead/F25, 10 mg of HRP-bead/F25 was incubated in 2 mL of PBS for 3 h. At predetermined time intervals, the media were withdrawn from each sample and the withdrawn samples were analysed using a micro BCA assay kit. As shown in Figure S2, there was no HRP release from HRP-beads. In addition, the initial immobilized amount of HRP was retained after 3 h incubation.





**Fig. S3.** SEM images of cross-sectioned GPT hydrogel constructs. (a) GPT hydrogel formed after mixing with soluble HRP, (b-d) GPT hydrogels formed using a HRP-beads/F25 packed syringe. Injection time interval: (b) 5 s, (c) 30 s, and (d) 60 s. Scale bars indicate 100  $\mu\text{m}$ .



**Fig. S4.** (a) Digital images of GPT hydrogels formed after mixing with different HRP concentrations. (b) Gelation time of GPT with varying HRP concentrations. (c) Elastic modulus of GPT hydrogels formed by soluble HRP.