Electronic Supplementary Information for

HRP-mediated polymerization forms tough nanocomposite hydrogels with high biocatalytic performance

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Contents

1. Materials

Horseradish peroxidase (HRP, EC 1.11.1.7, MW = 40 kDa, 300 U mg^{-1}) was purchased from Shanghai Baoman Biotechnology Co., Ltd. Human serum albumin (HSA, MW = 66.3 kDa, 96-99%, lyophilized powder) was obtained from Sigma-Aldrich. Silica nanoparticles (SNPs) in an aqueous dispersion (SNOWTEX-C, Silica content 21 wt %) were obtained from Nissan Chemical Industries, Ltd. Hydrogen peroxide (H_2O_2, 30 wt %) was obtained from Sinopharm Chemical Reagent Co., Ltd. All other chemicals and solvents were of the highest purity commercially available and were used as received.

2. Hydrogelation via HRP-mediated Polymerization

Acryloylation of HSA

HSA (600 mg) and NAS (54 mg) were dissolved in 30 mL of distilled water and the reaction was carried out with 2.5 h stirring at 25 °C. The acryloylated HSA was purified by dialyzing in a semi-permeable membrane against 1.5 L of deionized water for three days. The water was changed once a day. Then, the dialyzate was freeze-dried to give acryloylated HSA as a white solid product, which was stored at 4 °C.

According to Tada's method, [1] the average number of acryloyl groups introduced to one HSA molecule was estimated to be 4.2 from the measurement of the amount of reaction by-product, *N*-hydroxysuccinimide.

Preparation of hydrogels via HRP-mediated polymerization

In a typical example of hydrogelation, DMAA (100 mg), acryloylated HSA (60 mg), and deionized water (0.58 mL) were added to 1143 mg of an aqueous dispersion of SNOWTEX-C (240 mg SNPs) in a vial under vigorous vortexing to give a clear homogeneous solution. The pH value of the above solution was adjusted to about 6.0 by the addition of 0.1 M acetic acid. Then, 8 μL of acetylacetone (ACAC, 0.078 mmol), 100 μL of a HRP solution (42 mg mL^{-1}), and 6μL of a H_2O_2 solution (4.86 M) were added successively. The vial was sealed and allowed to stand at room temperature (ca. 25 ^{o}C) for about 6 min to give a yellowish, transparent tough hydrogel (Fig. S6, ESI). Without the addition of SNPs, the same procedure gives a control hydrogel. Without the addition of the SNPs, the same procedure gives the control hydrogel, which is easily fractured by a manual application of force. The obtained gels are referred to as SHH-x, where x is the content of SNPs.

3. Pre-treatments and characterizations

Pre-treatment of SEM samples

The hydrogel samples for SEM analysis were cut into cubes of about 2×2×2 mm^3 from the inner parts of hydrogels. The samples were rapidly quenched into liquid nitrogen, and then freeze-dried for 48 h. A thin layer of gold (about 5 nm) was sputter-coated before scanning with a field emission SEM (Hitachi S 4800) at an accelerating voltage of 3 kV.

Pre-treatment of TEM samples

The hydrogel samples for TEM analysis were cut into cubes of about 1×1×1 mm^3. The samples were dehydrated with ethanol / water mixture solution at increasing ethanol concentrations of 50%, 70%, 90% (vol. %) for 15 min in each solution. Then, the samples were immersed sequentially in a mixture of 90% ethanol and 90% acetone (1/1, vol. /vol.) for 30 min, a mixture of 90% acetone and embedding resin (1/1, vol. /vol.) for 12 h, and in the pure resin for 3 h at room temperature. The embedding procedure was conducted in an oven at 37 °C for 12 h, at 45 ^oC for 12 h and at 60 °C for 48 h. The ultrathin sample slices of about 70 nm in thickness were prepared using a Leica ultramicrotome. The slices were stained with 3% uranyl acetate and observed using a JEOL model JEM-1230 microscope at an accelerating voltage of 80 kV.

Compressive stress–strain measurement

The compressive measurements of the hydrogels were performed on a FR-108B testing machine (Farui Co., China) at a crosshead speed of 1 mm min^{-1}. The cylindrical gel samples were 15 mm in diameter and 7–8 mm in thickness. The compressive stress (σ) was approximately calculated as $\sigma = Load/\pi R^2$, where R is the original radius of the specimen. The strain (ε) under compression is defined as the change in the thickness relative to the original thickness of the freestanding specimen. The stress and strain between $\varepsilon = 0$ and 10% were used to calculate the initial compressive modulus. At least three specimens were tested for each hydrogel.

Rheological measurement

The rheological properties of hydrogels were measured using an ARES controlled-strain rheometer (TA Instruments) with a parallel plate geometry (25 mm diameter, 1.5 mm gap) at 25°C. Dynamic time sweep measurements were carried out to measure the storage modulus (G') and loss modulus (G'') as a function of time at a constant frequency of 1 rad s^{-1}. The

strain amplitude (γ) was fixed at 1% as it has been found to be well within the linear strain amplitude response regime. The recovering process of the hydrogel in response to applied shear forces was investigated using continuous step strain sweep test with alternate small oscillation force (γ = 1%) and large one (γ = 100%).

NMR measurement

All proton NMR spectra were recorded using a Bruker ARX-400 (400 MHz) spectrometer at 25 °C. In this work, when the concentrations of monomers and ternary initiation system were fixed, the content of SNPs has barely any influence on the gelation rate according to the free radical polymerization theory and the experimental measurements of gelation time. Therefore, we used a gelation system without SNPs to prepare the D_2O-substituted sample to investigate the polymerization kinetics during the hydrogelation process. Dioxane was used as an internal standard to calculate the conversion of vinyl double bonds, because its only peak at 3.65 ppm separates from the signals of other compounds in the system. In a typical measurement, DMAA (100 mg, 1.009 mmol), Dioxane (11.1 mg, 0.126 mmol), acryloylated HSA (60 mg), and D_2O (1715 mg) were mixed under vigorous vortexing to give a homogenous solution. After the successive addition of 8 μL of ACAC (0.078 mmol), 100 μL of HRP in D_2O solution (42 mg mL^{-1}), and 6μL of H_2O_2 in D_2O solution (4.86 M), the time counting began while 0.6 mL of the above solution was transferred as quickly as possible to a NMR tube and placed into the probe of the spectrometer. The time-dependent spectra of the sample were recorded *in situ* at pre-determined time intervals using a Bruker Topspin program. In all experiments, the first spectrum was acquired after 5 min reaction.

Electron Paramagnetic Resonance (EPR) measurement

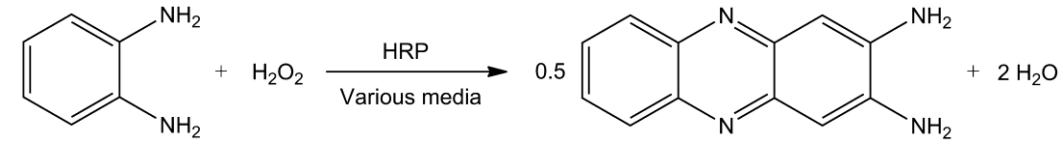
The EPR spectra were recorded on a Bruker EMX-8/2.7 Spectrometer operating at 9.873 GHz (microwave power: 20 mW; modulation frequency: 100 kHz; modulation amplitude: 0.5 G; receiver gain: 4×10^5; 25 °C). As for the signal of initiation radical, the mixture of ACAC/HRP/H_2O_2 ternary initiation system and the spin trap (POBN) in deionized water was rapidly transferred to a standard quartz capillary (1 mm in diameter) and placed into the EPR spectrometer. The spectrum recorded after 5 min reaction is shown in Figure S2A. When the monomer (DMAA, 7.5 wt %) was added to the reaction system, the spectrum recorded in the same procedure is shown in Figure S2B.

4. Test of the leach of enzyme in hydrogels

The leach of enzyme from the hydrogels (SHH-0, SHH-6, SHH-12, and SHH-18) to water could be measured by incubating 0.5 g of different hydrogel samples (containing 1.05 mg of HRP) in 5 mL of phosphate buffer solution (0.1 M, pH 6.0) or organic solvents. Then the released amounts of HRP from different hydrogels were measured by the Bradford method. The absorbance of the complex of protein and coomassie brilliant blue G-250 is measured at 595 nm. After 24h incubation, the 100 μL release solution was added to 2 mL of the G-250 solution and the absorbance at 595 nm was measured. At the same time, the 100μL of native HRP standard solution (with 0.21 mg mL^{-1} concentration) was treated with the same procedure. By comparing their absorbance at 595 nm, the leached amounts of HRP in SHH-0, SHH-6, SHH-12, and SHH-18 hydrogels were 12.5%, 8.8%, 6.5%, and 5.2%, respectively. Due to the insolubility of HRP in the selected organic solvents (*e.g.*, toluene and dioxane), which can be easily confirmed by the observation during enzymatic reaction, the absorbance of the leached HRP in these solvents is less than 0.0001 (the lowest testing limit), therefore the leached amount of HRP in these solvents is negligible.

5. Test of catalytic activity

We used the oxidation of *o*-phenylenediamine (OPD) by H_2O_2 as a model reaction to characterize the catalytic activity of the different catalysts (SHH-18 hydrogel, SHH-0 hydrogel and native HRP). Typically, 20 μg HRP (or a piece of hydrogel containing 20 μg HRP) was added into a solution containing the substrate (OPD, 10 mM) and H_2O_2 (20 mM) in 100 mL of different media for catalyzing the reaction. During the reaction course, the reaction mixture was slightly stirred at room temperature (approx. 25 °C). The increase in absorbance at 450 nm due to the oxidation product (phenazine-2, 3-diamine) was measured by using a UV-Vis spectrometer (UV-2700, Shimadzu) at 0.2-min intervals. The concentrations of the colorful product in different solvents were corrected according to the molar extinction coefficients in aqueous buffer, toluene, and dioxane, respectively.



S: *o*-phenyldiamine P: phenazine-2, 3-diamine

$\varepsilon_{450\ nm}$ is 16300 M^{-1} cm^{-1} in 50 mM pH 7.0 phosphate buffer, 10600 M^{-1} cm^{-1} in toluene, and 15000 M^{-1} cm^{-1} in dioxane, respectively.

The average slope in the first minute can be used to calculate the initial reaction rate due to the zero-order kinetics. By changing the substrate concentration from 10 mM, 7.5 mM, 5 mM, 3 mM, 2 mM, and 1 mM with a fixed H_2O_2 concentration at 20 mM, we obtained the corresponding initial reaction rates for constructing the Lineweaver-Burk plot to estimate the kinetic constant values, the maximum reaction rate (V_{max}), Michaelis-Menten constant (K_m), and turnover number (K_{cat}). The turnover number, $K_{cat} = V_{max}$ / [Enzyme], can represent the activity of the enzyme (or the enzyme-immobilizing hydrogel), which is independent of the concentrations of substrate and enzyme.

6. Test of reusability

To test the reusability of the enzyme-immobilizing hydrogel, we used the fresh and recovered SHH-18 hydrogel to catalyze the oxidation of OPD (5 mM) by H_2O_2 (20 mM) in 100 mL toluene or phosphate buffer solution. The randomly shaped hydrogel particles (about 0.5-1.5 mm in diameter) were used during reaction. The total amount of product (phenazine-2, 3-diamine) was measured by a UV-Vis spectrometer after 15 min reaction. The recovered hydrogel particles were separated from the reaction mixture easily by decantation, then washed with 50 mL fresh toluene (for reaction in toluene) or phosphate buffer (for reaction in buffer solution) for 2 times to remove the product. The recovered hydrogel particles were then subjected to the next catalytic run.

7. Figures

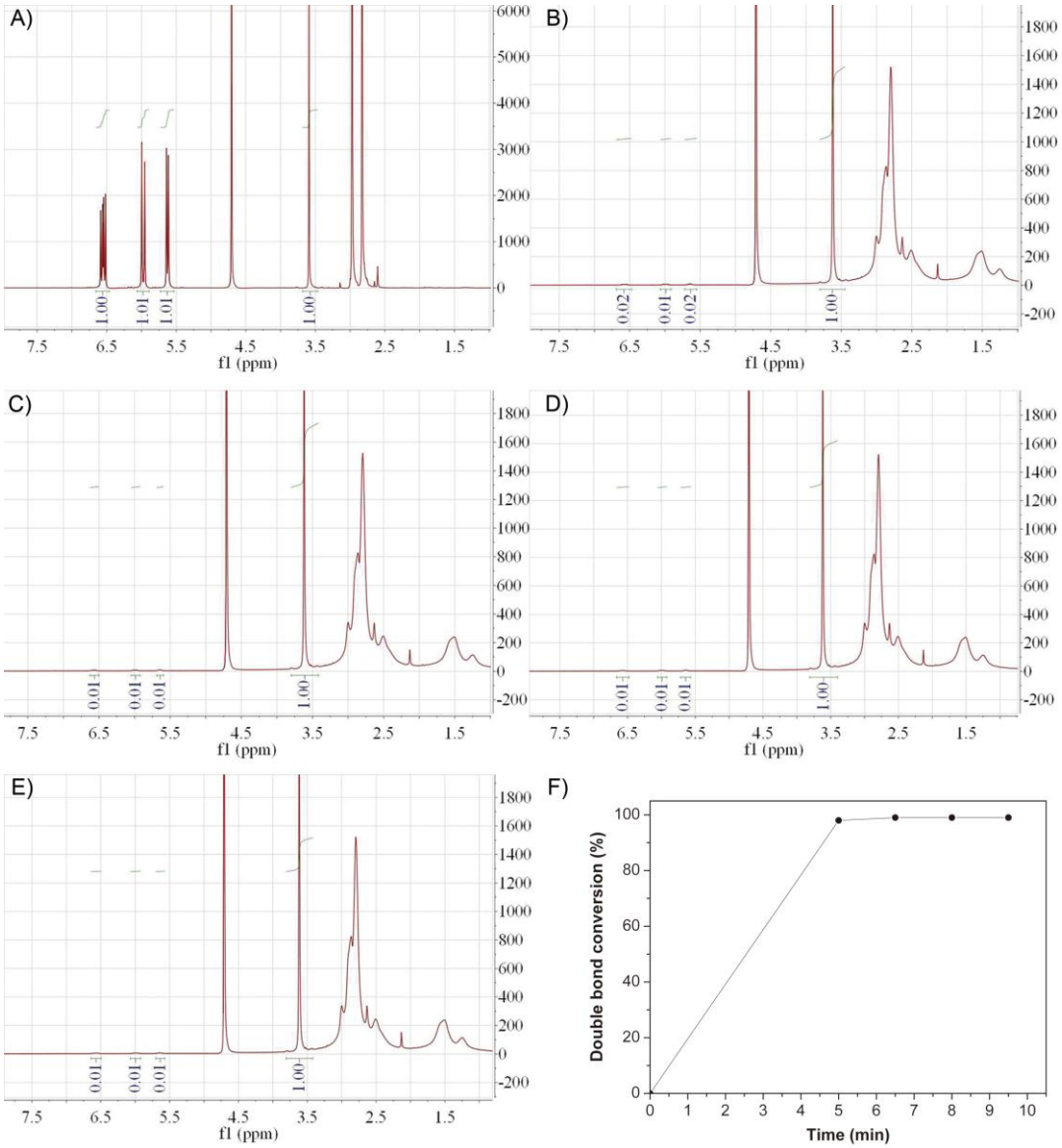


Figure S1. The NMR spectra of a D_2O-substituted precursor (5% DMAA, 3% acryloylated HSA) in the presence of the HRP-mediated initiation system ([ACAC]/[HRP]/[H_2O_2] = 42.0/0.056/15.7 mM) at different reaction times: 0 min (A); 5 min (B); 6.5 min (C); 8 min (D); 9.5 min (E). The conversion of vinyl double bonds in the gel is calculated using dioxane as an internal standard (F).

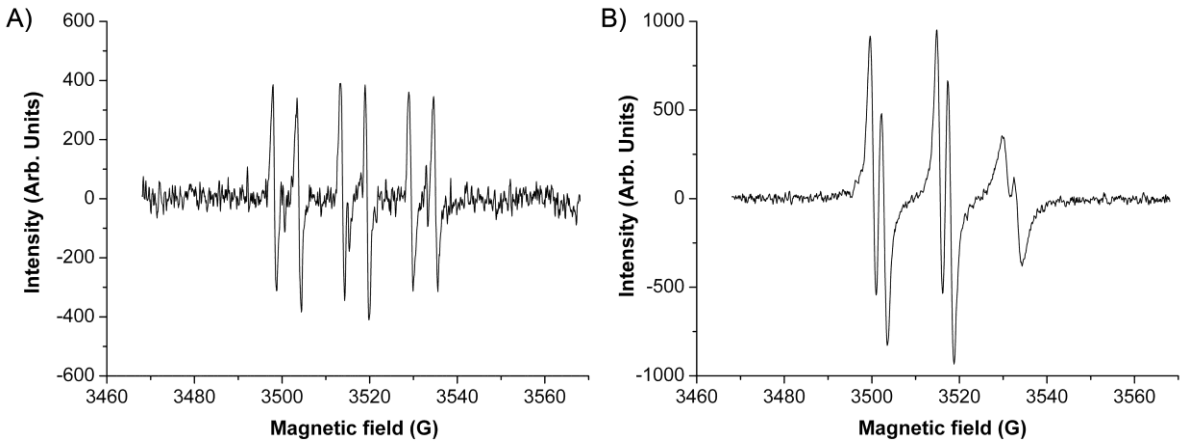


Figure S2. EPR spectra of the POBN radical adducts formed in the HRP-mediated ternary initiation system without (A) and with (B) the addition of DMAA (7.5 wt %).

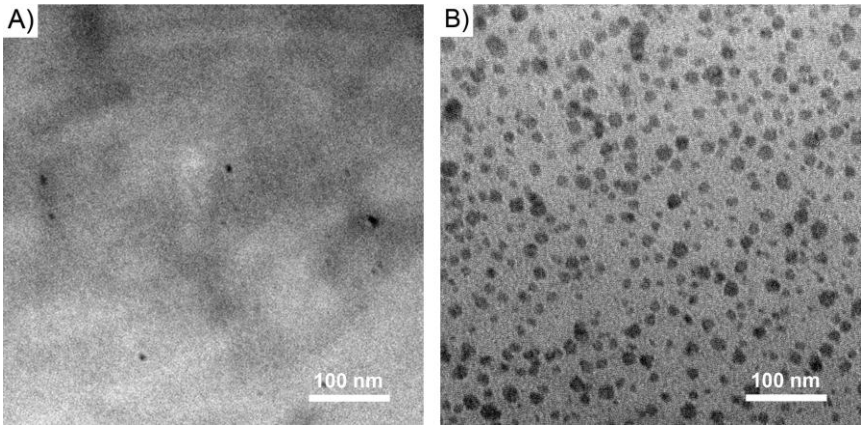


Figure S3. The TEM images of SHH-0 (A) and SHH-18 (B) hydrogels.

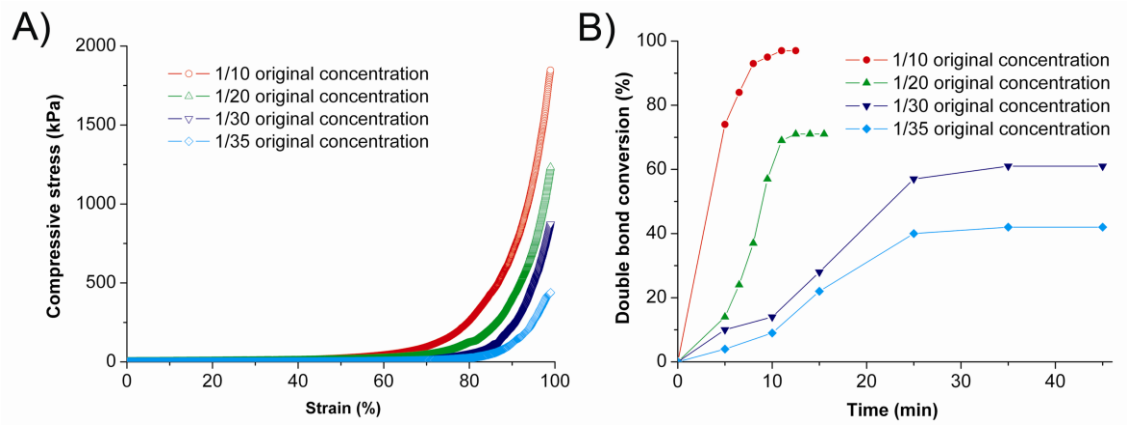


Figure S4. (A) The representative compressive stress-strain curves of the hydrogels (18.0% SNPs, 3.0% acryloylated HSA, 5.0% DMAA) prepared with decreasing the concentration of the HRP-mediated ternary initiation system to 1/10 (○), 1/20 (△), 1/30 (▽), 1/35 (◇) of the original concentration ([ACAC]/[HRP]/[H_2O_2]=42.0/0.056/15.7 mM), respectively. (B) Double bond conversion profile of the D_2O-substituted precursor (3% acryloylated HSA, 5% DMAA) in the presence of the diluted HRP-mediated initiation system in D_2O with varying concentrations. The conversion of vinyl double bonds was determined by time-dependent proton NMR using dioxane as an internal standard.

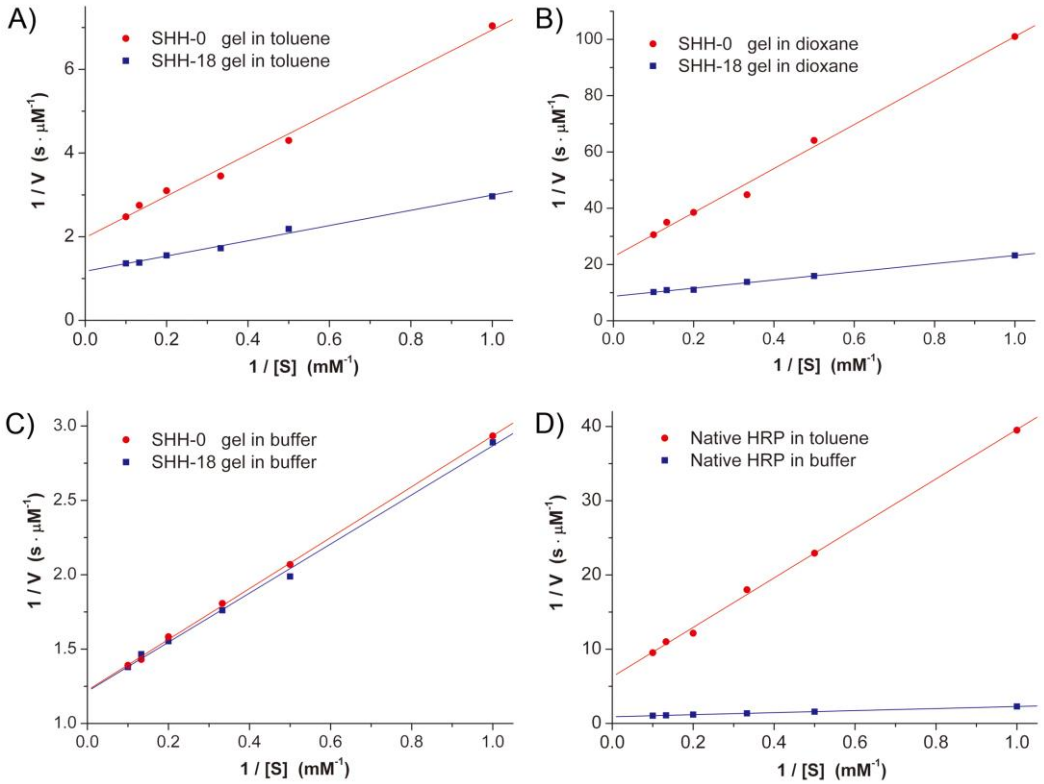


Figure S5. Lineweaver-Burk plots of the SHH-0 and SHH-18 hydrogels in toluene (A), in dioxane (B), and in aqueous buffer (C) and the native HRP in toluene and buffer (D).

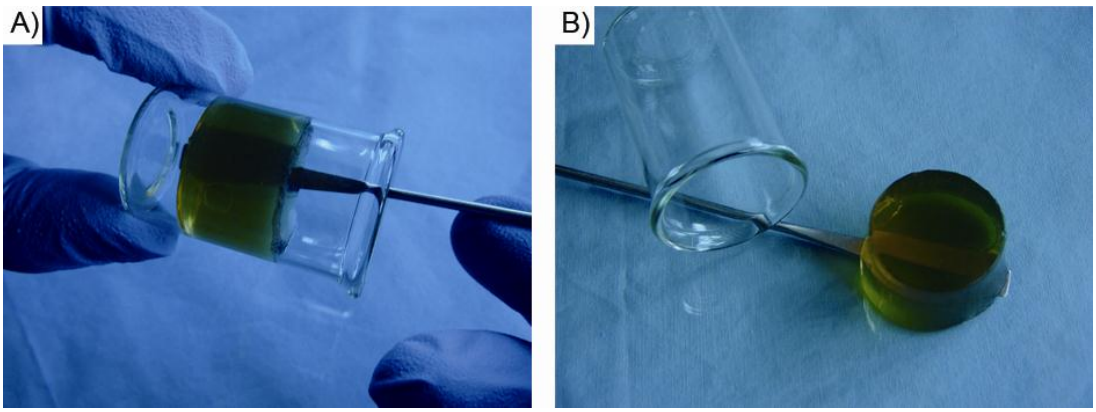


Figure S6. Optical image of a transparent cylindrical hydrogel formed via HRP-mediated polymerization (A) that can be easily separated from the glass vial (B).

8. Tables

Table S1. The compressive properties of the hydrogels.

Gel samples	Silica content (wt %)	Compressive strength (kPa)	Compressive modulus (kPa)	Strain (%)	Appearance after test
SHH-0	0	35.9 ± 2.9	4.15 ± 0.47	76.0	Fractured
SHH-3	3.0	436.5 ± 44.2	4.54 ± 0.09	95.0	Deformed
SHH-6	6.0	1021.2 ± 60.7	6.99 ± 0.62	99.0	Deformed
SHH-9	9.0	1199.4 ± 67.5	9.29 ± 0.53	99.0	Recovered
SHH-12	12.0	1349.3 ± 58.2	14.67 ± 1.04	99.0	Recovered
SHH-15	15.0	1676.9 ± 38.9	15.98 ± 1.07	99.0	Recovered
SHH-18	18.0	1884.5 ± 46.1	21.55 ± 2.08	99.0	Recovered

Table S2. The gelation ability of silica nanoparticles.

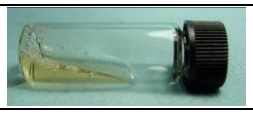
Composition (wt %)	Optical image	Compressive strength (kPa)	Compressive modulus (kPa)	Strain (%)
Acryloylated HSA/DMAA =1.5/5.0		-	-	-
Acryloylated HSA/DMAA/SNPs =1.5/5.0/6.0		551.0 ± 45.2	4.78 ± 0.09	95.0
DMAA/SNPs =5.0/6.0		-	-	-

Table S3. The kinetic parameters for the oxidation of OPD catalyzed by the hydrogels and the native HRP in different solvents.

	Solvent	V_{max} ($\mu M\ s^{-1}$)	K_m (mM)	K_{cat} (s^{-1})
SHH-18 gel	Toluene	0.76	1.55	152
	Dioxane	0.12	1.67	24
	Buffer	0.82	1.36	164
SHH-0 gel	Toluene	0.51	2.51	102
	Dioxane	0.044	3.44	8.8
	Buffer	0.81	1.40	162
Native HRP	Toluene	0.16	5.32	32
	Dioxane	<0.0001	[a]	<0.02
	Buffer	1.11	1.50	222

[a] Undetected data due to the large relative error for the initial rate measurement.

9. References

[S1] a) J. Kim, J. W. Grate, *Nano Lett.* **2003**, 3, 1219; b) D. Tada, T. Tanabe, A. Tachibana, K. Yamauchi, *J. Biosci. Bioeng.* **2005**, 100, 551.

[S2] Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations: A Collection of Processes*. 2nd ed. Wiley-VCH: Darmstadt, 2006.