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ESI

Patterned biocatalytic films via one-step self-assembly

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Materials

Horseradish peroxidase (HRP, Type II, EC 1.11.1.7), 4-aminoantipyrine, and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich and used as received. Acrylamide (AAm), N,N'-methylene bisacrylamide (bisAAm), hydrogen peroxide (H_2O_2, 30%), phenol, dimethyl sulfoxide (DMSO), ammonium persulfate ($(NH_4)_2S_2O_8$), N,N,N',N'-tetramethylethylenediamine (TMEDA), methanol, ethanol, and tetrahydrofuran (THF) were commercially obtained from Sinopharm Chemical Reagent Co. N-Acryloxysuccinimide, 4-dimethylaminoantipyrine, and sodium phosphotungstate were purchased from Aladdin and used without further purification. Polystyrene (PS, MW = 235000 g/mol, MWD = 2.89) was provided by Zhenjiang Chiemei Chemicals. Water used in all experiments was de-ionized and ultrafiltrated to 18.2 MΩ with an ELGA LabWater system. All other reagents were analytical grade and used without further purification.

Synthesis of vinyl HRP

100 mg of HRP and 10 mg of 4-dimethylaminoantipyrine as the stabilizer of HRP was firstly dissolved in 38 mL of phosphate buffer solution (PBS, pH 7.4, 50 mM). Then 40 mg of N-acryloxysuccinimide dissolved in 2 mL of DMSO was added in 1 h and the reaction was carried out for another 1 h at 25 ºC.[1] The acryloylated HRP (vinyl

HRP) was purified by centrifugal ultrafiltration with a molecular weight cutoff of 10 kD (Millipore). The centrifugal ultrafiltration was performed at 3500 rpm for 20 min and repeated three to four times. The concentrate was washed with PBS and ultrafiltrated at least three times.

Synthesis of HRP nanoparticles (HRP NPs)

A 1.0 mL of vinyl HRP solution was mixed with 38 mL of PBS (pH 7.4, 50 mM) and purged with nitrogen. The radical polymerization from the surface of vinyl HRP was started by adding 3 mg of $(NH_4)_2S_2O_8$ and 3 μL of TMEDA. Then AAm (7.11 mg) and bisAAm (1.54 mg) dissolved in 15 mL of deoxygenated and de-ionized PBS was evenly added to the flask in 1 h under nitrogen protection. The reaction proceeded for another 1 h and then the solution was purified by centrifugal ultrafiltration with a molecular weight cutoff of 10 kD. The centrifugal ultrafiltration was performed at 3500 rpm for 20 min and repeated three to four times. The concentrate was washed with PBS and ultrafiltrated at least three times.

HRP400 NP and HRP800 NP were prepared at a monomer/HRP molar ratio of 400 and 800, respectively. HRP400×2 NP was synthesized by using HRP400 NP as seed by repeating the protocol above at a monomer/HRP molar ratio of 400. In this work, HRP NP means HRP400 NP unless otherwise specified.

Synthesis of FITC-conjugated HRP

FITC-conjugated HRP (FITC-HRP) was prepared according to a standard protocol.[2] Firstly, HRP was dissolved in 0.1 M sodium carbonate (pH=9.5) at a concentration of about 10 mg/mL, and FITC was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mg/mL in the dark. Then, 0.1 mL of the FITC solution was added to each mL of the HRP solution. The reaction was carried out for at least 8 h at 4 °C in the dark. The reaction was quenched by the addition of ammonium chloride to

a final concentration of about 50 mM. The resultant FITC-HRP was purified by centrifugal ultrafiltration with a molecular weight cutoff of 10 kD.

FT-IR spectroscopy

FT-IR spectra were collected on a Nicolet FTIR/Nexus 470 spectrometer. Thirty-two scans were taken for each spectrum at a nominal resolution of 1 cm^{-1}. HRP preparations were dried by freeze drying.

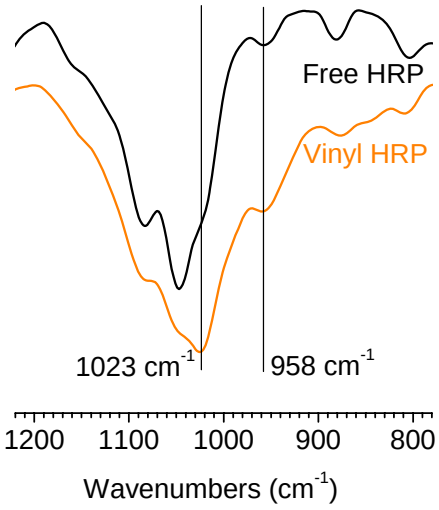

Figure S1. FT-IR spectra of free HRP and vinyl HRP. Bands at 958 cm^{-1} and 1023 cm^{-1} are induced by the out-of-plane bending vibration of =C–H.

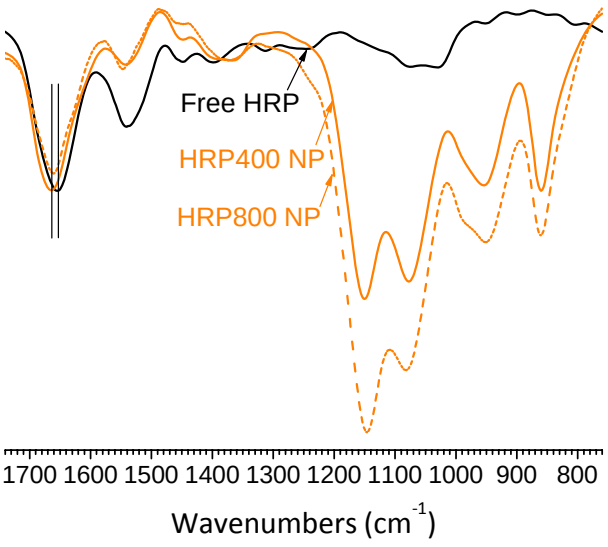

Figure S2. FT-IR spectra of free HRP, HRP400 NPs and HRP800 NPs. The absorption intensity of bands between 800~1000 cm^{-1} and 1000~1200 cm^{-1} increased obviously

because of the large amount of N–H and C–N bonds in poly(AAm), respectively. The peak attributed to carboxyl groups of HRP shows 9 cm^{-1} of blue shift changing from 1654 cm^{-1} to 1663 cm^{-1} after the introduction of $–CONH_2$.

Transmission electron microscopy (TEM)

TEM images of free HRP and HRP NPs were collected on Hitachi JEM-1200EX at 250,000x. Standard grids were photographed to determine the magnification. HRPs were diluted in water to give a concentration of ~0.1 mg/mL and a drop of the solution was added to carbon-coated TEM grids. After removing excess solution with a piece of filter paper, a drop of 1% sodium phosphotungstate was added. The sample was observed after dried in air.

Dynamic light scattering (DLS) measurements

The sizes of free HRP and HRP NPs were determined using a Nano-ZS nanosizer (Malvern Instruments, Worcestershire, UK). Before measurement the HRP solutions were filtered using PVDF membrane with 250 nm pores.

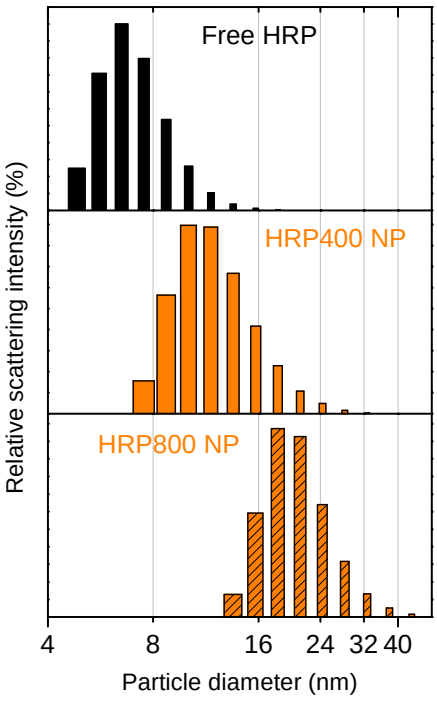

Figure S3. DLS results of free HRP, HRP400 NPs and HRP800 NPs.

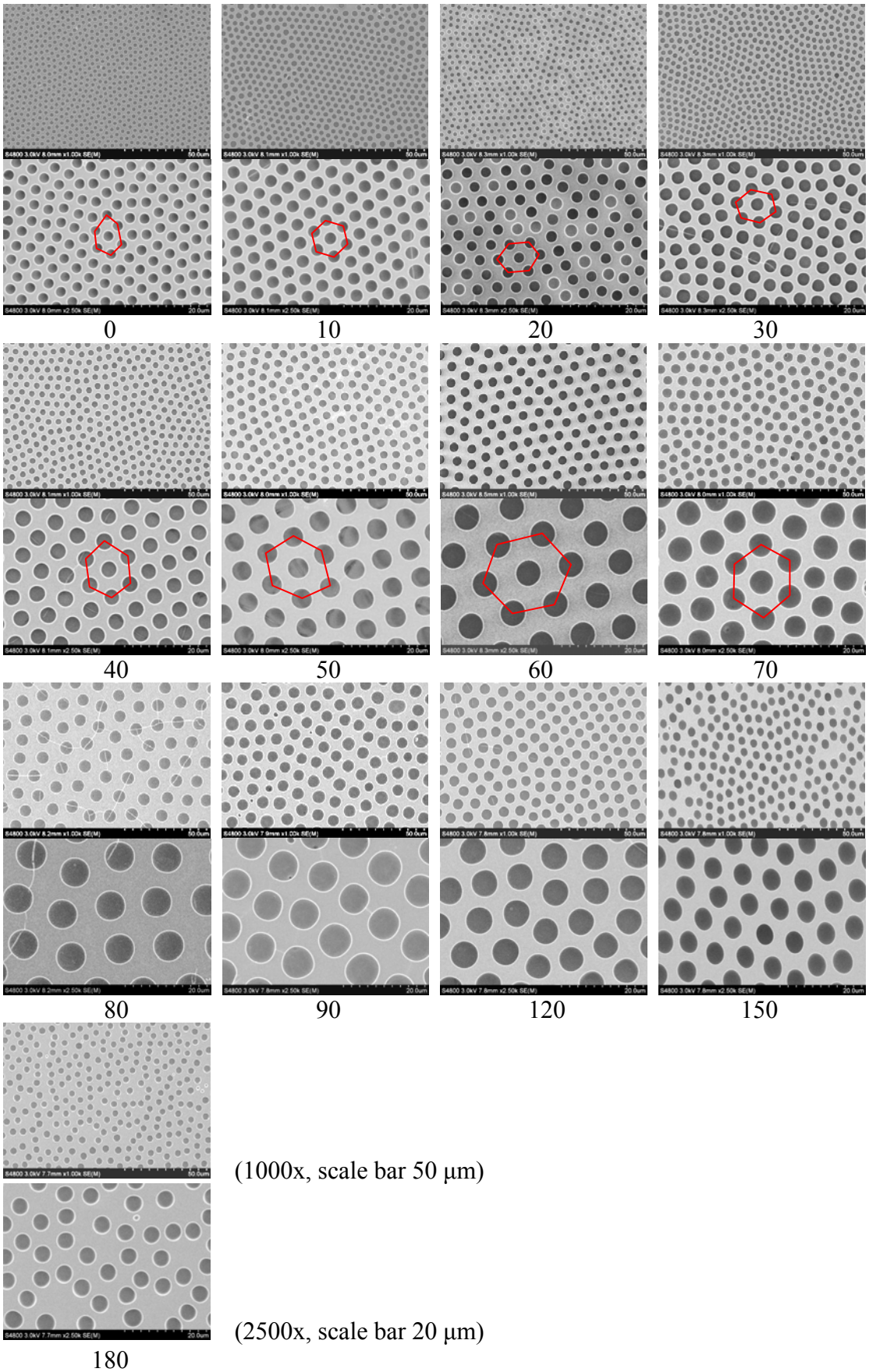

Figure S4. SEM images of patterned porous films prepared from mixtures of 1 mL PS solution and 0~180 μL of HRP NPs dispersed in ethanol (0~18%, v/v).

Preparation of HRP patterns via the breath figure method

PS was dissolved in chloroform to prepare a homogeneous solution with a concentration of 30 mg/mL. An aliquot of 100 μL for each polymer solution was cast onto a solid substrate (e.g., glass slide) placed in a humid environment (25 °C, ~70% RH). The relative humidity was measured by a hygro-thermograph (DT-321S, CEM Corporation). Owing to the condensation of water vapor on the solution surface during the evaporation of chloroform, the transparent solution soon turned turbid. The traces of condensed water droplets remained in the polymer film and formed honeycomb patterns. The film was then dried at room temperature.

HRP patterns were prepared according to the above-mentioned procedure. Differing from the preparation of PS film, a certain amount of HRP NPs dispersed in ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 120, 150, 180 μL) (1-18%, v/v) was added to 1 mL of PS solution to form a mixed casting solution.

Characterization of the HRP patterns

Field emission scanning electron microscope (FESEM, Hitachi S4800) was used to observe the surface morphology of films after being sputtered with gold using ion sputter JFC-1100. Pore diameter was analyzed using ImageJ (v1.42q, by Wayne Rasband). Height images were recorded by atomic force microscopy (AFM, SEIKO SPI3800N) under tapping mode. Films containing FITC-HRPs were analyzed by confocal laser scanning microscopy (CLSM), which was performed on a Leica TCS SP5 confocal setup mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Wetzlar) and was operated under the Leica Application Suite Advanced Fluorescence (LAS AF) program. Confocal Raman spectroscopy imaging of the HRP patterns was performed under a Renishaw Raman confocal microscope (inVia Reflex) using a 532 nm laser (50 mW) as the excitation light source. A 50× objective was used

to do imaging with ~1 μm laser spot size. Each Raman spectroscopy map contains at least 760 spectra with 10 s integration time for each spectrum. 1 μm step sizes were used in the high resolution images (Fig. 4). Mono-colored images were then created with red representing signal of PS at the Raman shift of 1003 cm^{-1}.

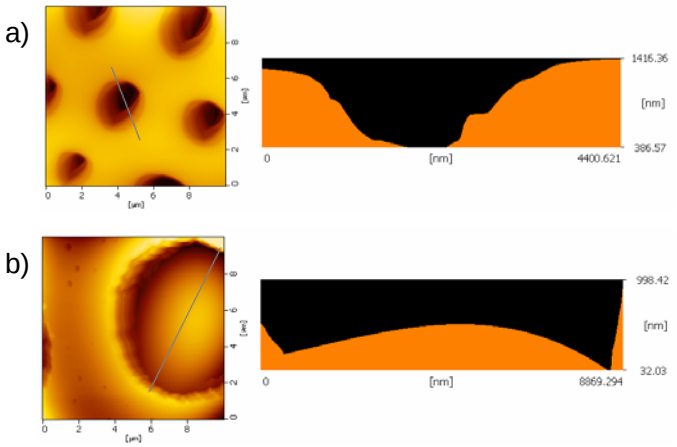

Figure S5. AFM height images and depth profiles of patterned porous films prepared from a) PS and b) the mixtures of 1 mL of PS solution and 60 μL of HRP NPs dispersed in ethanol (6%, v/v).

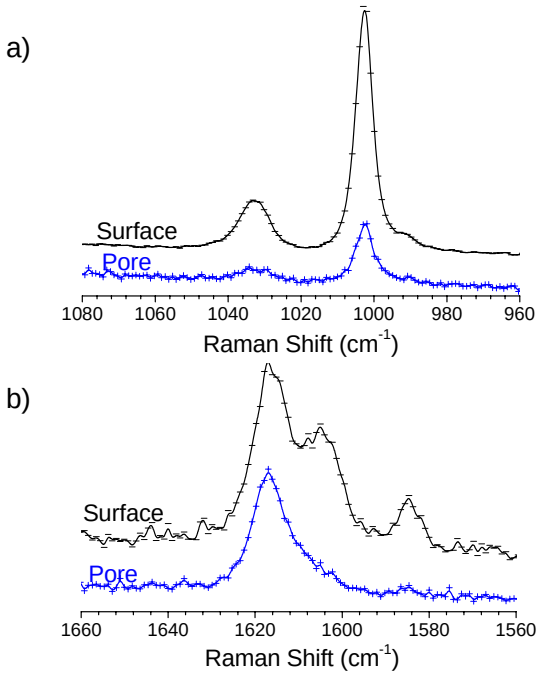

Figure S6. Raman spectra of patterned porous film containing HRP NPs with laser spot focused on the surface or pore area, showing signals of PS and HRP at Raman shift of a) 1003 cm^{-1} and b) 1618 cm^{-1}, respectively.

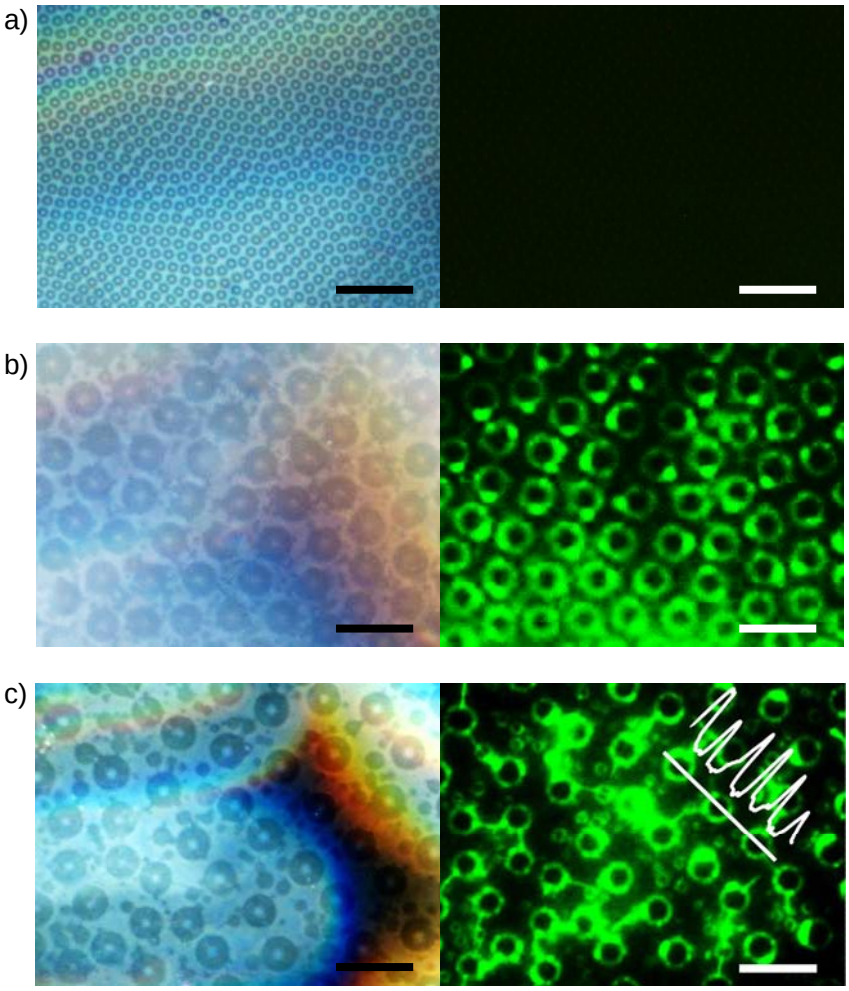

Figure S7. Optical (left column) and fluorescence (right column) images of a) PS film, b) PS film containing FITC-labeled HRP NP, and c) PS film containing FITC-labeled free HRP. Scale bar: 20 μm.

Activity assays of free HRP and HRP NPs

A colorimetric assay was used to measure the activity of HRP, as described by Nicell and Rao.[3,4] Phenol, 4-aminoantipyrine, and H_2O_2 were used as color-generating substrates. The product is in red-color solution, with a maximum absorbance at 510 nm, and the ε_{510} is 6.58 $cm^2 \cdot (\mu mol\ dye)^{-1}$. The mixture of 0.3 mL H_2O_2 solution (9.7 mM in pH 7.4, 50 mM PBS) and 2.7 mL phenol solution (containing 54 mM phenol and 12.9 mM 4-aminoantipyrine in pH 7.0, 50 mM PBS) was pre-incubated at 25 ºC for 10 min in the absence of light. Then 20 μL 0.035 mg/mL free HRP phosphate buffer solution (pH 7.4, 50 mM) was added into the mixture and the absorbance at 510 nm was

monitored every second for 1 min in the open cuvette following reaction initiation. One unit of specific activity (v) was defined as consumption of 1 μmol H_2O_2 per milligram enzyme in 1 min at 25 ºC and pH 7.4, which was calculated according to the following equation:

$$v = \frac{k \times V \times 60}{K \times Me}$$

where k is the slope of plot of absorbance at 510 nm vs. reaction time, s^{-1}; V is the volume of reaction solution, mL; K is the specific absorption coefficient of the products, ε_{510}, $cm^2 \cdot \mu mol^{-1}$; Me is the amount of HRP used in the reaction.

The same procedure of activity assay was also used for HRP NPs. The absorption intensity of HRP NPs at 403 nm was the same with that of free HRP by changing the concentration of HRP NPs. The HRP concentration was determined at 403 nm using an extinction coefficient of 102 $mM^{-1} \cdot cm^{-1}$ for the free HRP and HRP NPs.[1] The protein content was also determined by the method of Bradford[5] using BSA as protein standard.

Stabilities of HRPs

Effect of reaction temperature. Free HRP and HRP NP solutions were incubated for 10 min at different temperatures. Then the activity was measured according to the above-mentioned procedure.

Thermal inactivation kinetics. Free HRP and HRP NP solutions were incubated for different periods at 60 ºC or 65 ºC. Then the activity was measured according to the above-mentioned procedure.

Resistance to organic solvents. Free HRP and HRP NP solutions were mixed with organic solvents and kept for 30 min at 50 ºC. Then the activity was measured according to the above-mentioned procedure.

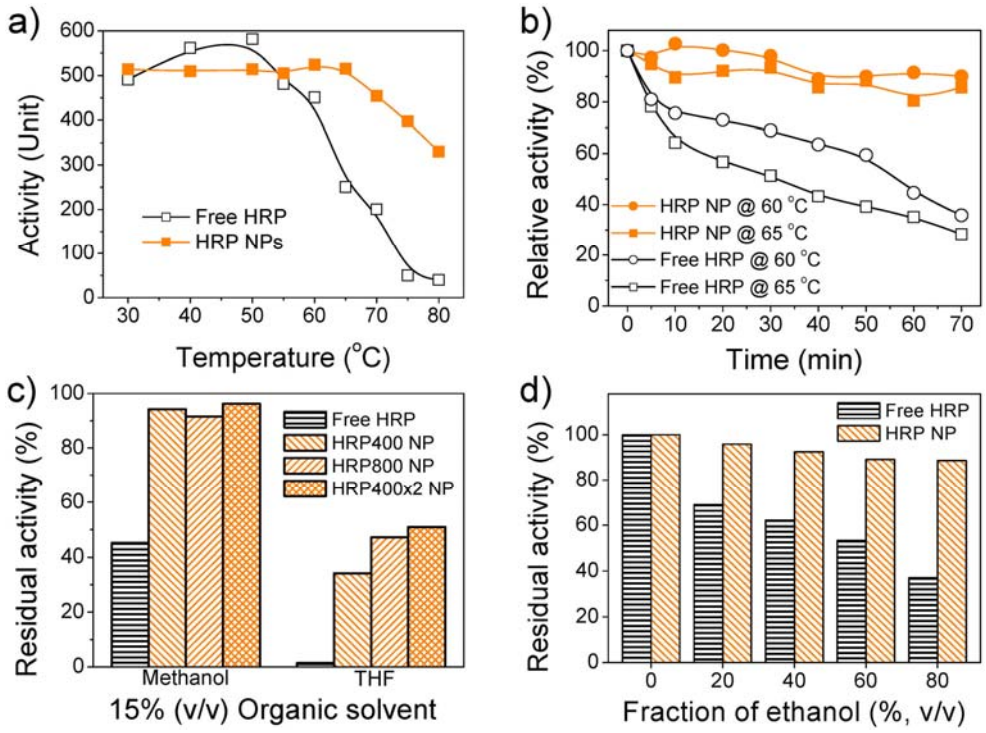

Figure S8. Comparison of activity and stability of free HRP and HRP NPs. a) Activity of HRPs incubated at different temperatures. b) Thermal deactivation kinetics at 60 °C and 65 °C. c) Residual activity of HRPs incubated in 15 vol% methanol and THF for 30 minutes at 50 °C. d) Residual activity of HRPs incubated in ethanol/water mixtures for 30 minutes at 50 °C.

Activity assays of HRP patterns

Residual activity of HRP patterns was measured according to the procedure for free HRP. Films containing HRP NPs were immersed in the substrate solution to start the reaction. The reaction was terminated by removing the film and then the absorbance was immediately recorded. Film containing free HRP was used as the control. The amount of HRP participating in the reaction was presumed to be that added to the casting solution.

References

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