

Supplementary Information

Magnetically responsive horseradish peroxidase@ZIF-8 for biocatalysis

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Experimental section

Starting materials and characterization methods

Zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$), 2-methylimidazole (HmIm, $\text{C}_4\text{H}_6\text{N}_2$), Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), trisodium citrate dihydrate ($\text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \cdot 2\text{H}_2\text{O}$), aqueous ammonia solution (25%), horseradish peroxidase (HRP, 173 U/mg, lot number: BCBV2718), bovine serum albumin (BSA), sodium dodecylsulfate (SDS), and Bradford Reagent for 0.1-1.4 mg/ml protein were purchased from Sigma Aldrich / Merck with the highest degree of purity available, and used without further purification. Deionized water (18 M Ω) was produced from a Millipore Synergy purification system.

Scanning Electron Microscopy (SEM). Sample morphology was assessed using a TESCAN VEGA scanning electron microscope at 20 kV. Prior to SEM investigation, samples were sputter coated with gold.

Powder diffraction (XRD) analysis. PXRD patterns were collected on a Rigaku SmartLab at 9 KeV in Bragg-Brentano geometry, with samples deposited on flat silicon wafers and rotated. Scan speed was 3 deg min⁻¹, step 0.01°, and 2 θ was in the range between 5° and 50°.

Small- and Wide- Angle X-Ray Scattering (SAXS/WAXS) analysis. Small- and wide- angle X-ray scattering (SAXS/WAXS) measurements were performed at the Austrian SAXS beamline of the electron storage ring Elettra (Trieste, Italy)¹ using 8 keV branch corresponding to a wave-length of 1.54 Å; 1 s exposure time was used to collect the diffraction image. Data were analysed using Igor Pro software package (WaveMetrics Inc.).

Fourier transform infrared (FTIR) analysis. The measurements were performed using a Bruker ALPHA FT-IR spectrometer (Bruker Optik GmbH) in transmission mode. 128 scans were performed with 2 cm⁻¹ intervals.

Confocal Light Scanning Microscopy (CSLM). The images were acquired using a Leica DM5500 B microscope equipped with a Leica TCS SPE high resolution confocal system, using an excitation laser ($\lambda_{\text{exc.}} = 488 \text{ nm}$, 10 mW) and collecting the emission at 580 nm.

Atomic Force Microscopy (AFM). Topography measurements were performed using an Oxford Instruments Asylum Cypher and an Anton Paar Tosca 400 AFM, in tapping mode, using Budgetsensors Tap150 silicon probes and MikroMasch HQ:NSC15/AL BS silicon probes, respectively. Samples were diluted in ethanol (1:1000 for MNP, 1:100 for ZIF-8-based samples) and drop cast on clean Si (100) substrates.

Synthesis of Iron(II, III) oxide magnetic nanoparticles (MNPs)

The method has been reproduced from literature.² In a 250 mL, single-neck, round bottom flask equipped with a magnetic stir bar, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (3.24 g, 0.012 mol) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.80 g, 0.010 mol) were dissolved in 80 mL of water. Aqueous ammonia solution (10 mL, 25 % w/w) was added in 1 mL steps under vigorous stirring. The mixture was then heated

at 90 °C, then solid trisodium citrate (8.80 g, 0.030 mol) was added in one step and the stirring continued for additional 30 minutes at the same temperature. After removal of the stir bar and natural cooling at room temperature, the dark brown mixture was transferred in a cylindrical container, and a strong ND45 magnet (fitted into a glass tube) was immersed at the approximate centre of the solution for 5 minutes to collect the MNPs. The liquid part was removed and replaced by deionized (DI) water, then the magnet extracted. The suspension was briefly sonicated in a bath for 10 minutes, and the magnetic collection repeated. The process was repeated with water (3 x 100 mL), ethanol (3 x 100 mL) and water again (3 x 100 mL), then the suspension diluted to 250 mL and stored at room temperature in a polythene bottle.

Before use, few millilitres were collected, the water removed and the MNPs dried under vacuum at 60 °C overnight, then re-suspended to the desired concentration.

Synthesis of ZIF-8 and MNP@ZIF-8

The method has been adapted from literature.³ The procedure involves the preliminary preparation of three stock solutions, all in DI water: MNP suspension is prepared at a concentration of 6 mg/mL, and sonicated in a bath for 15 minutes to ensure homogeneous dispersion; zinc acetate stock solution is prepared with a concentration of 240 mM, whereas Hmlm stock solution is prepared with a concentration of 1.92 M.

To ensure the constant reproducibility of the sodalite (*sod*) phase, the metal-to-ligand ratio is kept as 1:16,⁴ their final concentrations being 40 mM and 640 mM, respectively. The amount of MNP is varied within a final concentration range of 0 ÷ 1 mg/mL, precisely 0.33, 0.67, and 1.00 mg/mL. For pure ZIF-8, the MNP is omitted and substituted with an equivalent amount of DI water.

In a typical procedure, Hmlm stock (600 µL), MNP stock (0, 100, 200, or 300 µL) and water (filling up to the 1.5 mL volume mark) were mixed in a 2 mL plastic vial with cap and briefly vortexed for few seconds. Zn acetate stock (300 µL) is then added: the solution containing MNPs becomes milky almost instantaneously, whereas for pure ZIF-8 this will occur in approximately 20 minutes. The mixture is briefly vortexed one more time for few seconds, and the vial put in a tube rotator (20 rpm, 16 hours).

General workup procedure

The reaction mixture is centrifuged (Eppendorf Minispin with rotor F-45-12-11, 12000 x g, 60 sec) and the supernatant separated for further analysis. An aqueous solution of SDS (1 % w/v, 1 mL) is added and the pellet vortexed until dispersion, with the aid of a brief step of sonication in bath (3-5 sec) if necessary. The suspension is left in the tube rotator for 2 hours, then centrifuged again (12000 x g, 60 sec). After removing the supernatant, the pellet is washed with water (3 x 1 mL), vortexed, and centrifuged as previously described. The pellet is then re-dispersed in water (or a suitable buffer), and stored at room

temperature (or 4 °C if enzyme is present). The samples were stored in water because ZIF-8 is highly hydrophobic in its dried form.^{5,6}

A portion (300 µL) of suspension is placed in pre-weighed vials and centrifuged (12000 x g, 5 min), the supernatant discarded and the pellet left to dry at room temperature overnight. The solid is weighed again to determine the amount of material. The final amount of composite is eventually adjusted to 10 mg/mL.

Synthesis of HRP@ZIF-8

The same procedure for the synthesis of MNP@ZIF-8 is followed, but using a stock solution of HRP in DI water (6 mg/mL) in place of the MNP suspension, with a final concentration in the range 0 ÷ 1 mg/mL, precisely 0.33, 0.67, and 1.00 mg/mL (0, 100, 200, 300 µL). The general workup protocol is then applied.

Synthesis of HRP/MNP@ZIF-8

The same procedure for the synthesis of MNP@ZIF-8 is followed, adding a stock solution of HRP in DI water (6 mg/mL) along with the MNP suspension, with a final concentration in the range 0 ÷ 1 mg/mL, precisely 0.33, 0.67, and 1.00 mg/mL (0, 100, 200 300 µL). Before adding Hmlm, the suspension is left standing for few minutes. The general workup protocol is then applied.

Scale-up synthesis

In a 100 mL glass bottle with cap and magnetic stirring, 30 mL of BSA or HRP solution (3 mg/mL), 15 mL of MNP suspension (6 mg/mL) and 30 mL of 2 mlm solution (1.92 M) were stirred for one minute, then 15 mL of Zn acetate solution (240 mM) were added in one step. If protein and/or MNP were omitted, water was added as replacement. The reaction mixture become turbid within one minute. After 18 hours, the mixture was centrifuged (3750 rcf, 10 minutes) and washed three times with water (ca. 30 mL each time), and dried at room temperature overnight, then in a vacuum oven (ca. 200 mbar) at 60 °C to determine the weight and the yield.

Bradford assay⁷

A calibration curve with HRP in water was determined in the 0 ÷ 1 mg/mL range. The resulting equation is:

$$A = 0.52536 \times [p.a.] - 0.00268$$

where *A* is the absorbance and [*p.a.*] the protein amount in mg/mL (see **Figure S10**).

To perform the assay, the supernatant of the reaction mixture after centrifugation (20 µL) is mixed with the Bradford Reagent (600 µL) in a plastic cuvette and the absorbance at 595 nm measured with a Nanodrop One^c (Thermofisher Scientific Inc.). The quantity is then calculated by difference with the initial amount of protein present in the reaction medium

(measured with the Bradford assay as well) and then applying the above equation. Protein loading in mg/g was determined from the amount of composite used in the assay.

Enzymatic activity

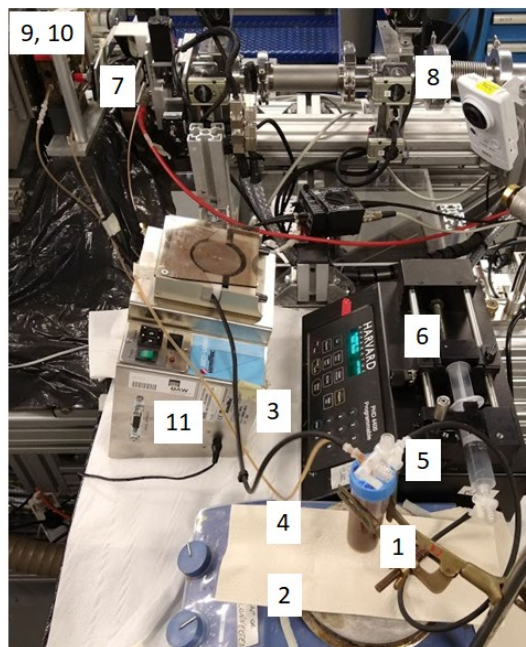
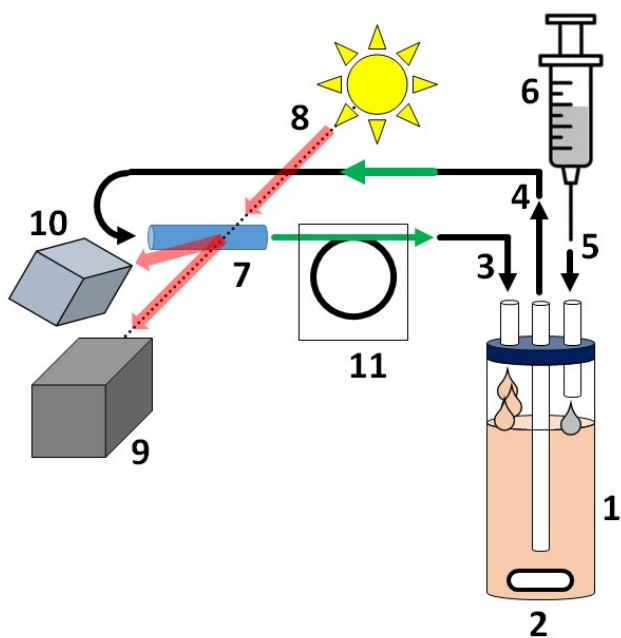
The assay is performed using pyrogallol and hydrogen peroxide as substrates.⁸ Activity is determined by monitoring the purpurogallin formation via UV-Vis spectrophotometry ($\lambda = 420$ nm). The assay procedure was modified by replacing PBS buffer with DI water (see **Figures S11** and **S12**).⁹ In a typical procedure, HRP@ZIF-8 (or ZIF-8), is suspended in DI water (10 mg/mL) and left to mix on a tube rotator (20 rpm, 2 hours) to ensure homogeneity. 100 μ L of the bio-composite suspension (1 mg), is added to 2420 μ L of DI water, 160 μ L of hydrogen peroxide (0.5 % v/v), and 320 μ L of pyrogallol (50 mg/mL, in DI water) in a 4 mL cuvette with stirring bar, monitoring the increase of the absorbance at 420 nm within 10 minutes. The enzymatic activity obtained by the assay is expressed as $U \text{ mL}^{-1}$, where U is defined as $\mu\text{mol min}^{-1}$ substrate converted (pyrogallol to purpurogallin) at the defined reaction conditions. From this, it is possible to calculate the specific activity of the enzyme bound, by applying the formula: $U \text{ mg}_{\text{bound HRP}}^{-1} = U \text{ mL}^{-1} / \text{mg mL}^{-1} \text{ HRP}$ (referred to the HRP in the composites, knowing the mg of enzyme per g of composite as previously determined).

Recyclability test

The assay is performed in DI water. The final volume is 1 mL. In a typical procedure, 100 μ L of HRP@ZIF-8 or HRP/MNP@ZIF-8 suspension (20 mg/mL) are added to a mixture containing 420 μ L of DI water and 160 μ L of hydrogen peroxide (0.5 % w/v). After adding 320 μ L of pyrogallol (50 mg/mL), the reaction is left for 15 minutes, then the suspension is centrifuged (12000 x g, 60 sec) or collected with a magnet for 2 minutes, and the absorbance of the supernatant measured at 420 nm. The material is washed and centrifuged three times with water before every cycle.

Recirculator setup

The system is illustrated in the image below, along with a photo of the real setup taken at the Elettra synchrotron:



A 50 mL plastic reactor (1) with magnetic stirring (2) is charged with an aqueous solution containing Hmlm, the enzyme and/or the magnetic nanoparticles. The reactor has an inlet (3), an outlet drawing out from the bottom of the reactor (4), and an additional inlet (5) for the injection (6) of the zinc acetate solution at the given time. The reactor is then connected with plastic tubing to a quartz capillary (7) put on the path of the synchrotron beam (8), with SAXS (9) and WAXS (10) detectors downstream the beam. The recirculation of the liquid is ensured by a peristaltic pump (11), withdrawing the liquid from the capillary. The green arrows illustrate the direction of flow in the system; the peristaltic pump is set so the mixture takes approx. 10 seconds to reach the capillary. The tubing volume is ca. 5 mL, the overall reaction volume is 48 mL (40 mL of ligand solution and 8 mL of metal ion solution). Starting concentrations used are [Hmlm]: 1.92 M, [Zn(OAc)₂]: 240 mM, HRP: 1 mg/mL, MNP: 1 mg/mL

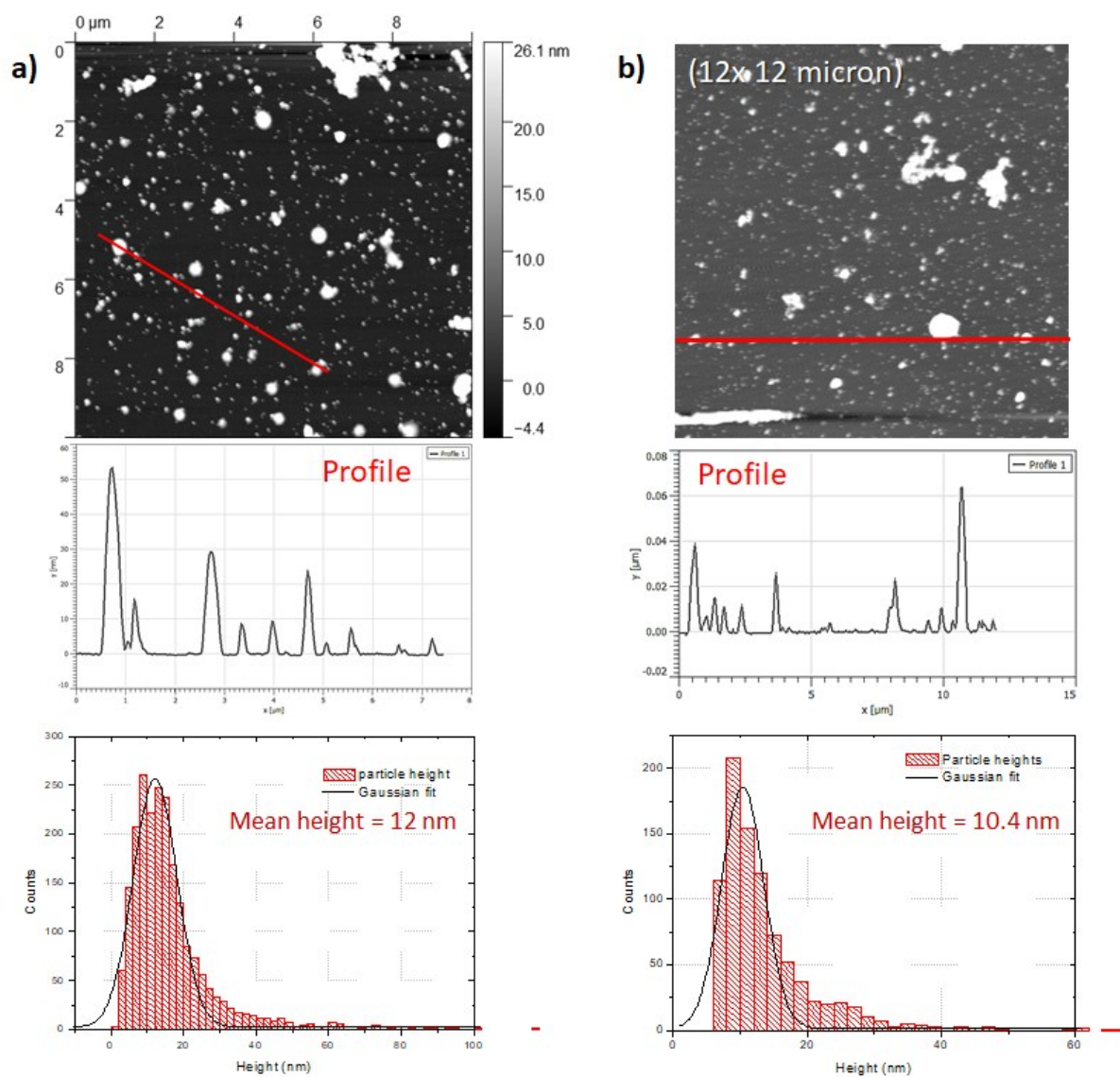


Figure S1. AFM maps, line profiles, and histograms including statistics over all measured regions of Fe₃O₄ magnetic nanoparticles before (a) and after (b) washing with ethanol to remove the citrate stabilizer. The average particle size is about 12 nm, with an inorganic core having a diameter of 10.4 nm.

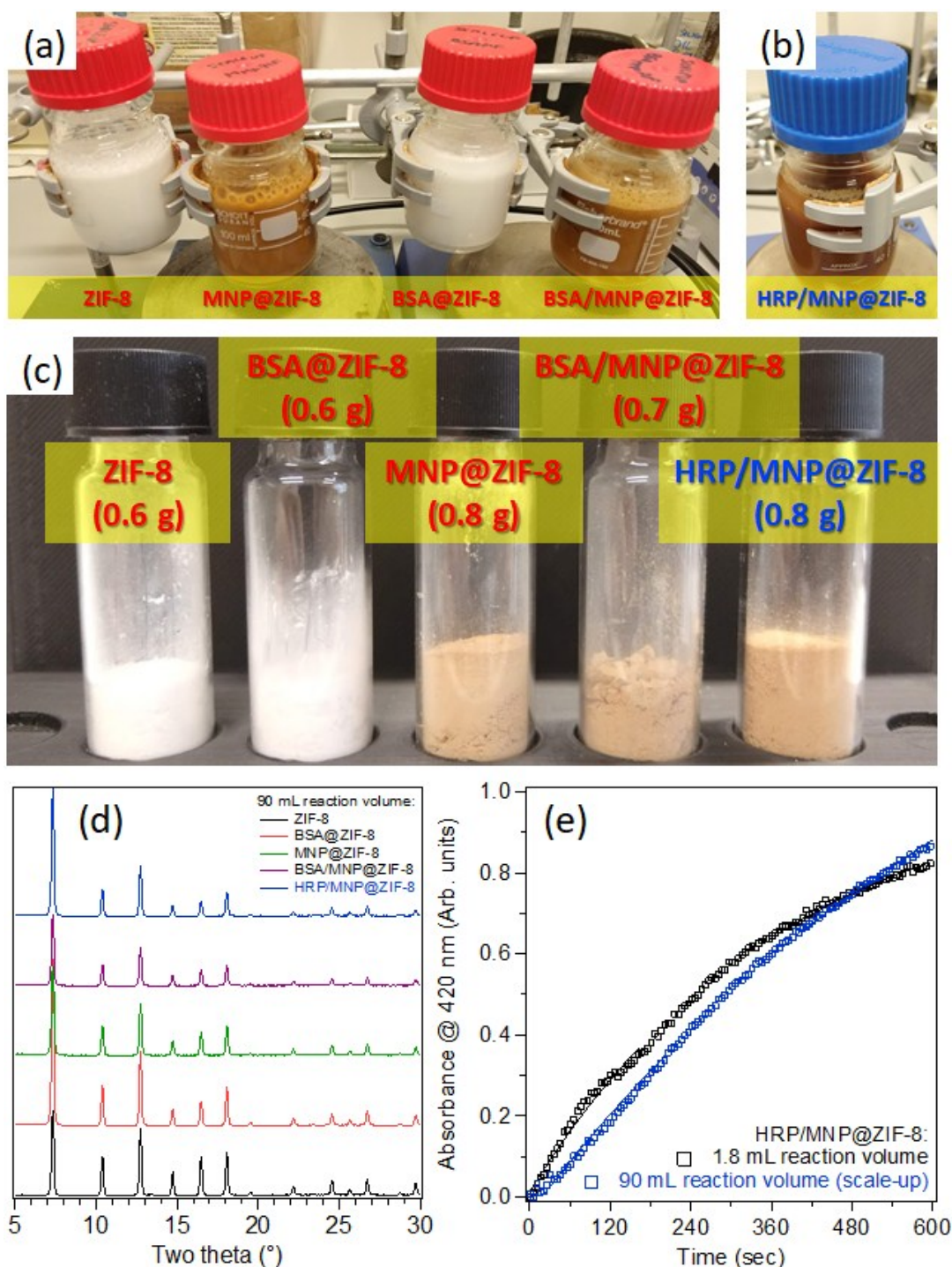


Figure S2. 90 mL reaction batches for ZIF-8, MNP@ZIF-8, BSA@ZIF-8, BSA/MNP@ZIF-8 (a) and HRP/MNP@ZIF-8 (b); resulting powders with weights (c) and XRD diffractograms (d). (e) Comparison of the catalytic evolution of the pyrogallol oxidation to purpurogallin, mediated by H_2O_2 , between the HRP/MNP@ZIF-8 synthesized in the 1.8 mL volume Eppendorf vial (black) and in the 90 mL reaction batch (blue).

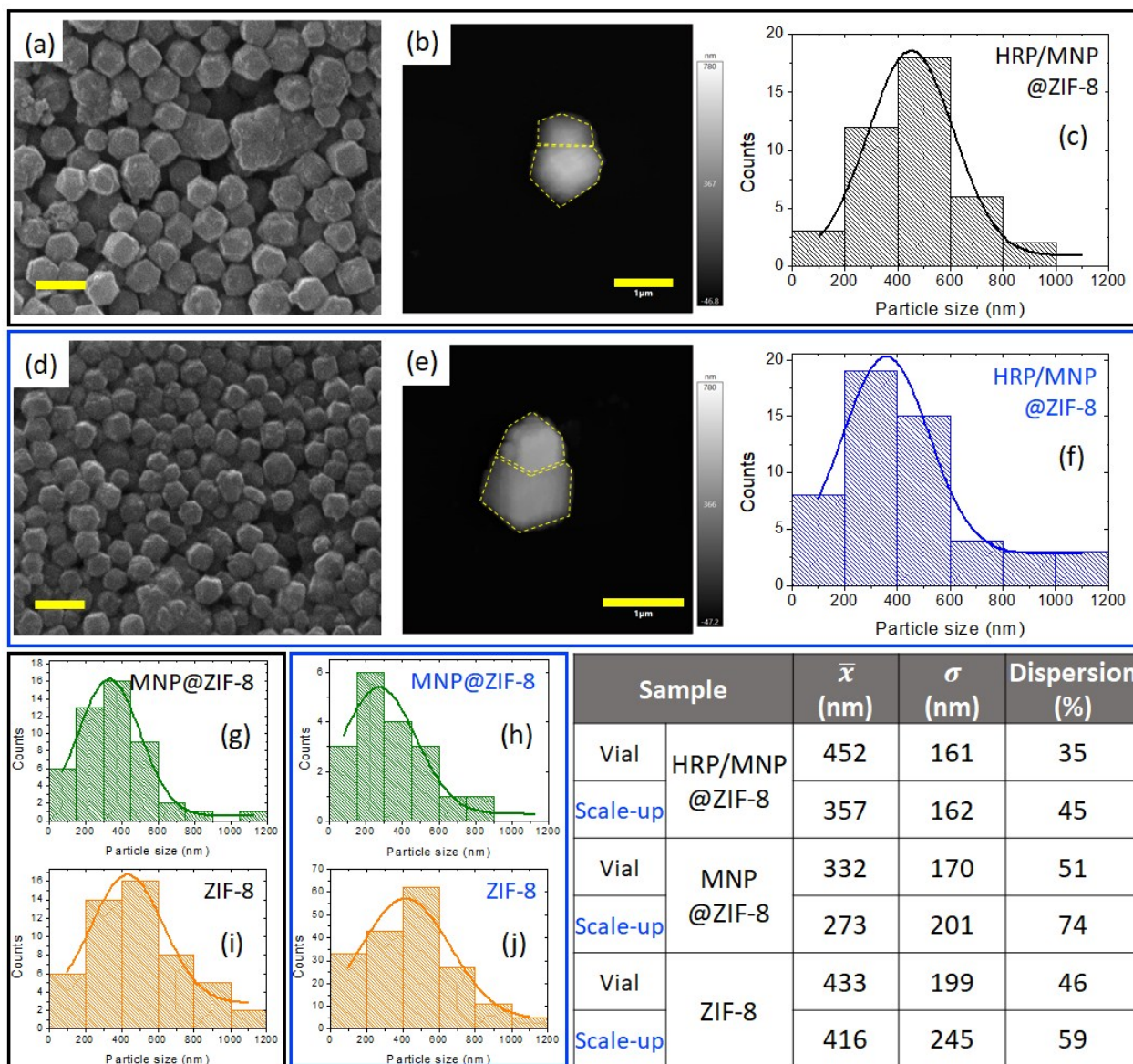


Figure S3. Size comparison of the HRP/MNP@ZIF-8 synthesized in 1.8 mL vial (black border) and with the 90-mL scale-up reaction (blue border), as reported in the previous Figure S2: (a, d) SEM, (b, e) AFM of representative particles, and (c, f) size distribution as calculated by AFM; scale bars: 1 μ m. (g-j) Size distribution for the MNP@ZIF-8 (g-h) and ZIF-8 (i-j) prepared in vial (g, i, black border) and in the 90-mL reaction vessel (h, j, blue border), as controls. The table reports the mean value (\bar{x}), standard deviation (σ), and percentage of dispersion of the obtained samples, calculated by AFM.

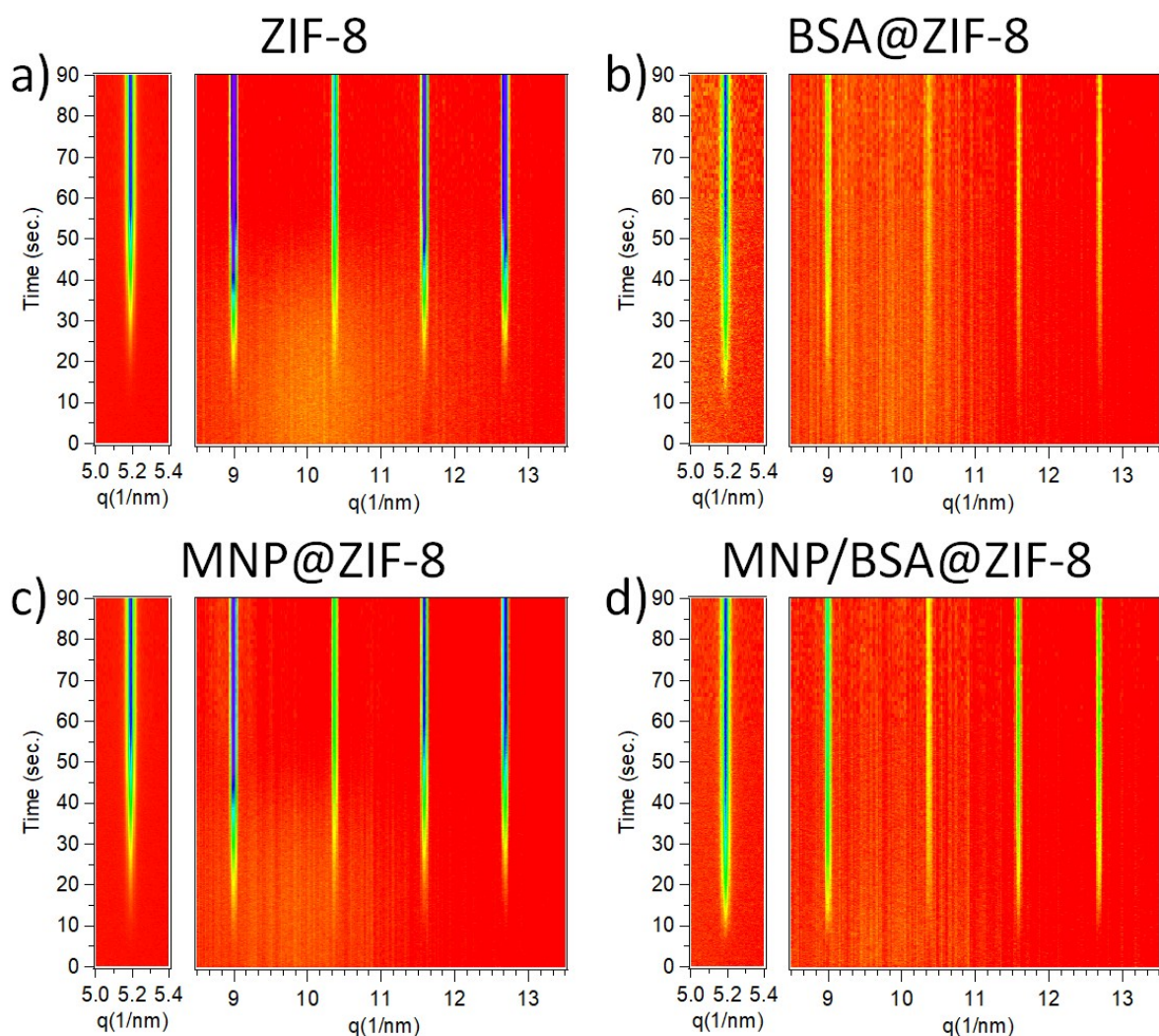


Figure S4. Evolution of the SAXS/WAXS signals for the (011), (112), (022), (013), and (222) crystal planes (from left to right) during the synthesis of (a) ZIF-8, (b) BSA@ZIF-8, (c) MNP@ZIF-8, and (d) MNP/BSA@ZIF-8 performed at the Elettra synchrotron with the recirculation setup described in the Experimental section. The (002) plane cannot be shown due to a gap between the SAXS and WAXS detectors. The data acquisition was started 10 seconds after the injection of the solution containing the ligand Hmlm.

The times when the signals started to appear are summarized in the following table:

Sample	Time (sec.)
ZIF-8	20''
BSA@ZIF-8	13''
MNP@ZIF-8	13''
BSA/MNP@ZIF-8	7''

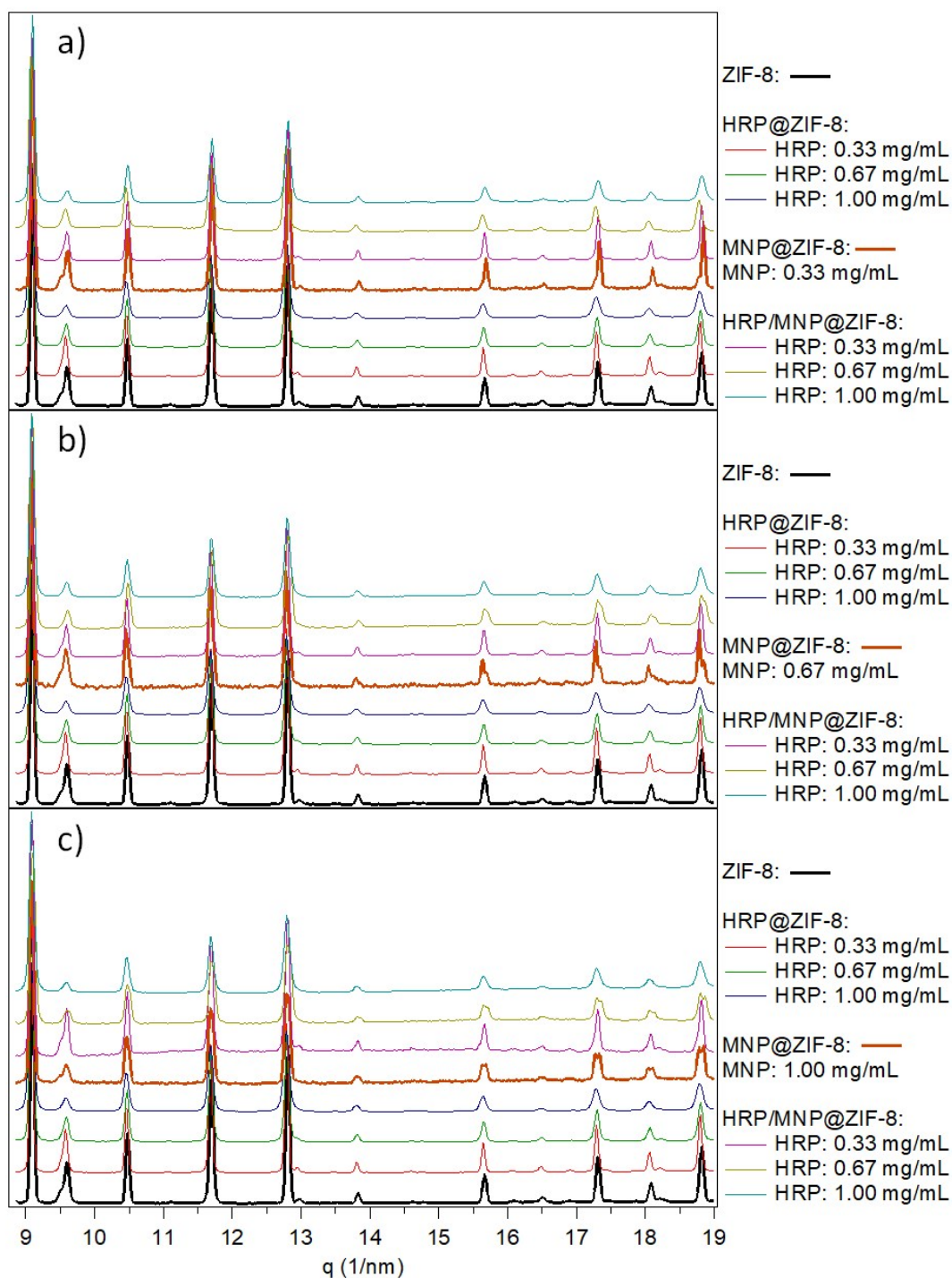


Figure S5. Normalized WAXS profiles of HRP@ZIF-8 and HRP/MNP@ZIF-8 acquired at the SAXS beamline of Elettra Synchrotron (Trieste, Italy), at different concentrations of enzyme. The dataset is divided for clarity according to the amounts of magnetic nanoparticles (MNP) in the reaction mixture: (a) 0.33 mg/mL; (b) 0.67 mg/mL; (c) 1.00 mg/mL.

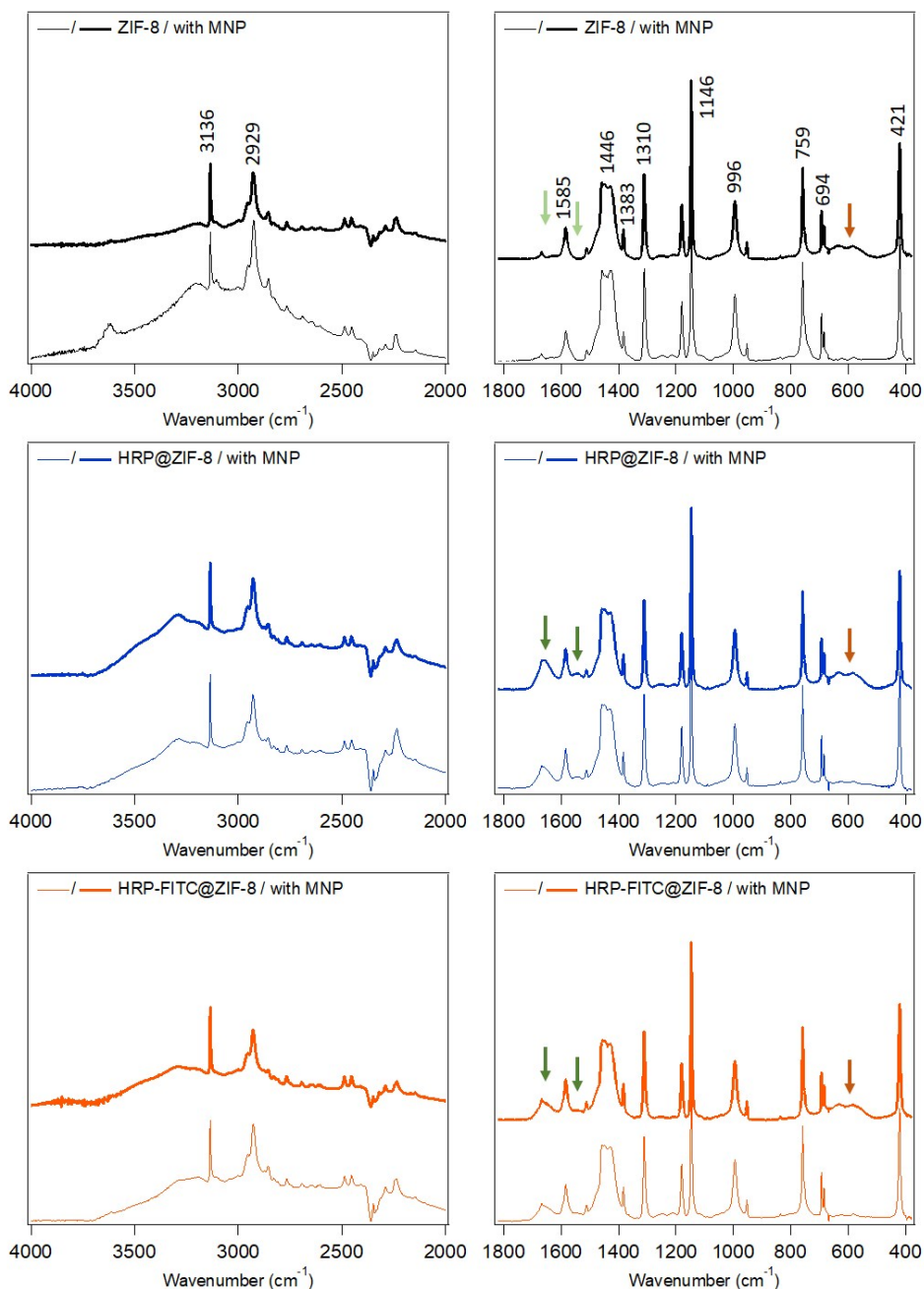


Figure S6. FTIR spectra related to Figure 2c of the main text. The bold spectra refer to the materials with MNP, the green arrows refer to the location of the Amide bands from HRP at 1650 and 1545 cm^{-1} , the brown arrow to the location of the Fe-O stretching from magnetic nanoparticles centred at 600 cm^{-1} . Assignments for ZIF-8: 3136 cm^{-1} (imidazole C-H stretching), 2929 cm^{-1} (methyl C-H), 1585 cm^{-1} (C=N stretching), 1446 cm^{-1} (ring stretching), 1383 cm^{-1} (ring bending), 1310 cm^{-1} (N-H wagging), 1146 cm^{-1} (C-N stretching), 996 cm^{-1} (in-plane ring bending), 759 cm^{-1} (C=N out-of-plane bending), 694 cm^{-1} (H-C=C-H out-of-plane wagging), 421 cm^{-1} (Zn-N stretching). The broad band between 3500 and 2500 cm^{-1} was attributed to the residual water due to the mild step of drying at room temperature.

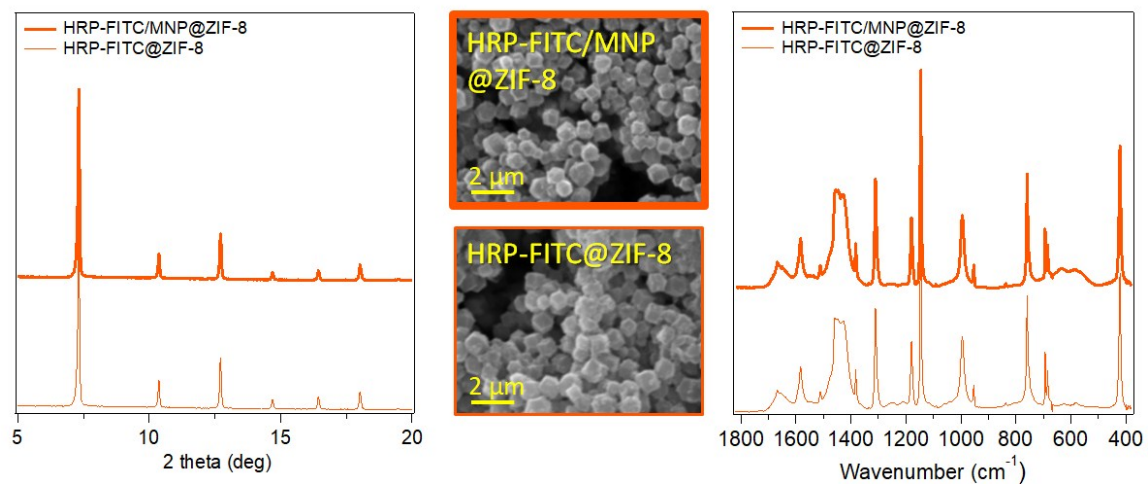


Figure S7. XRD (left), SEM (center), and FTIR (right) of HRP-FITC/MNP@ZIF-8 (thick lines and borders) and HRP-FITC/MNP@ZIF-8 as control (thin lines and borders)

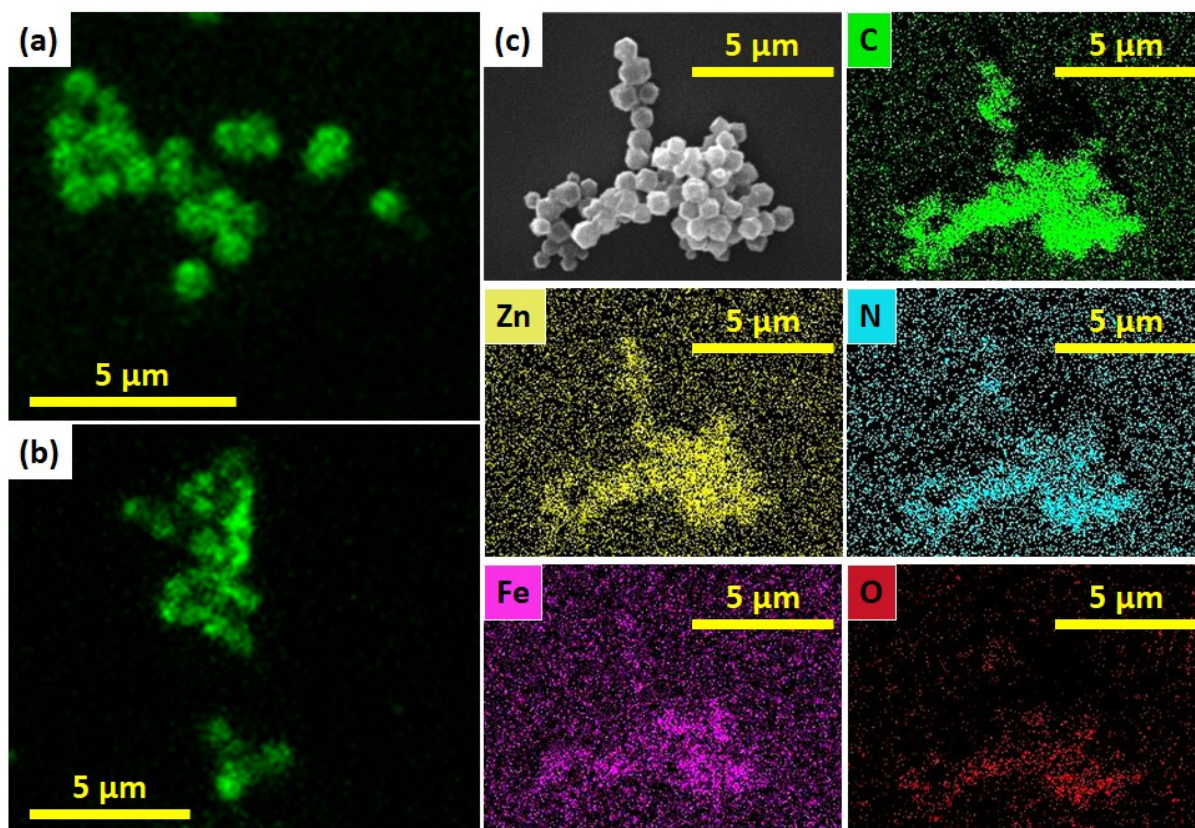


Figure S8. CSLM images of (a) HRP-FITC/MNP@ZIF-8, and (b) HRP-FITC@ZIF-8 (control); (c) EDX elemental maps of HRP-FITC/MNP@ZIF-8 synthesized with 1 mg/mL of HRP-FITC and 1 mg/mL of magnetic nanoparticles.

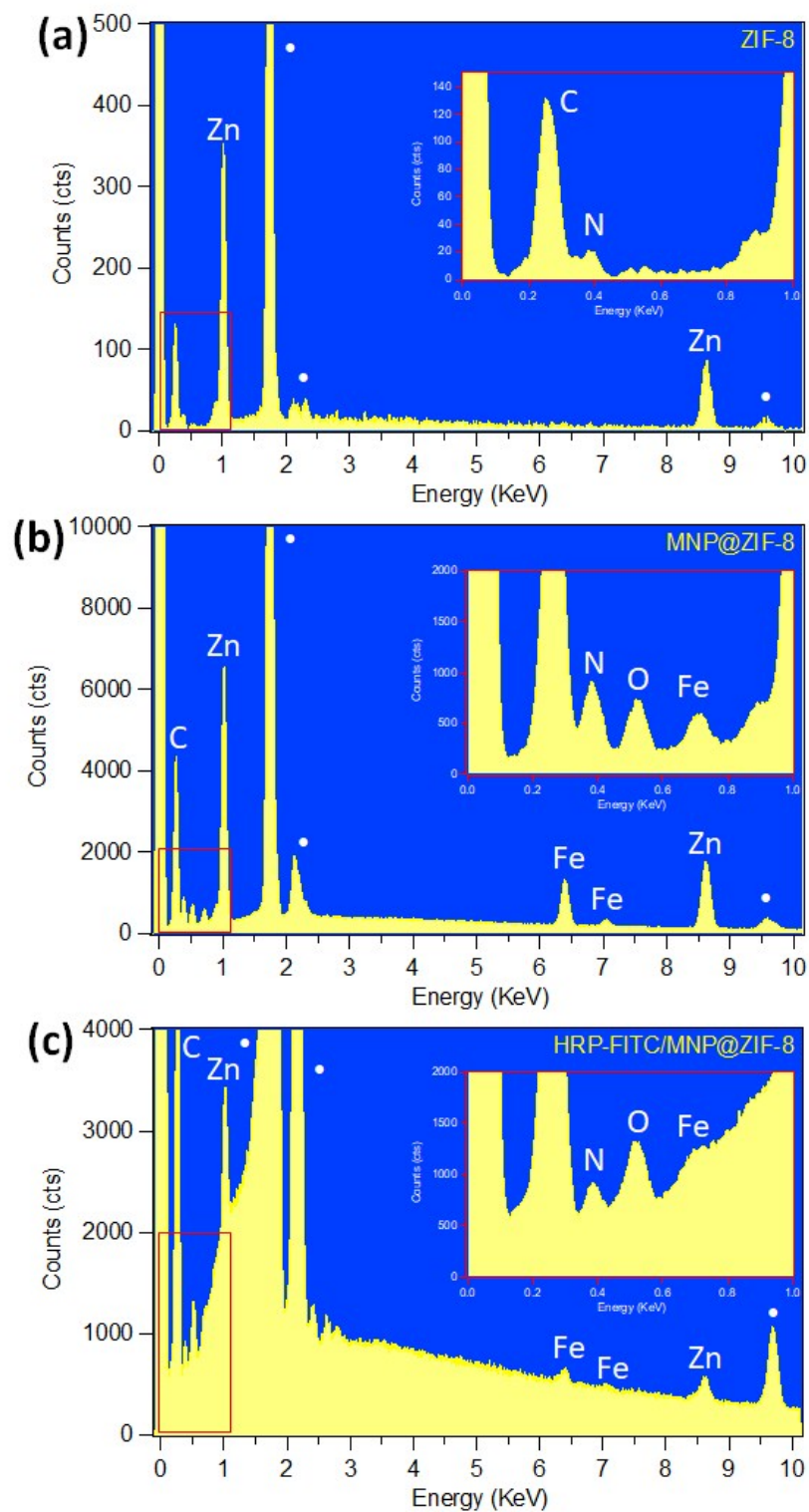
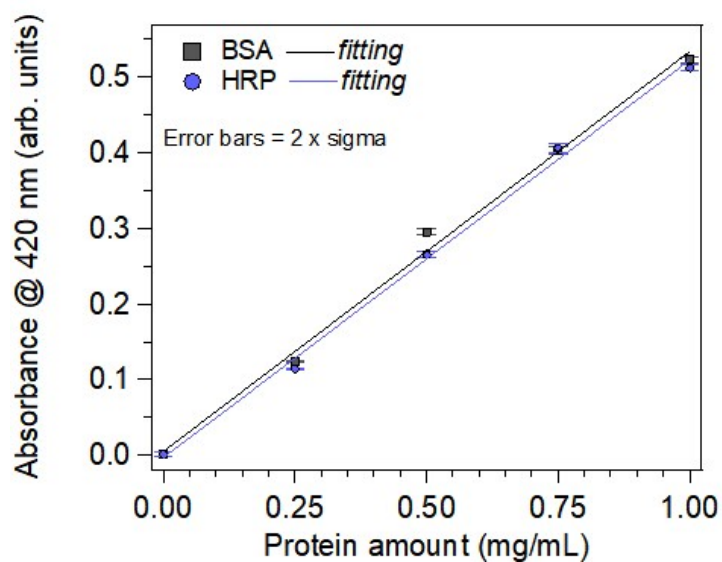


Figure S9. EDX spectra of (a) ZIF-8, (b) MNP@ZIF-8, and (c) HRP-FITC/MNP@ZIF-8. The peaks marked with ● belong to Si from the substrate (at 1.74 KeV) and to Au from the sputter coating (at 2.12 and 9.71 KeV). The red rectangles refer to the insets zooming on the 0-1 KeV region.



Type	Value		σ		R ²
	intercept	slope	intercept	slope	
BSA (water)	0.00532	0.52848	0.0140	0.0228	0.994445
HRP (water)	-0.00268	0.52536	0.0108	0.0176	0.996640

Figure S10. Calibration curves with the Bradford assay for BSA (grey squares) and HRP (blue circles) in DI water, in the 0 - 1 mg/mL range of protein concentration. The bold data is used in this manuscript. The table reports the values and relative errors for the intercept and slope of the four curves.

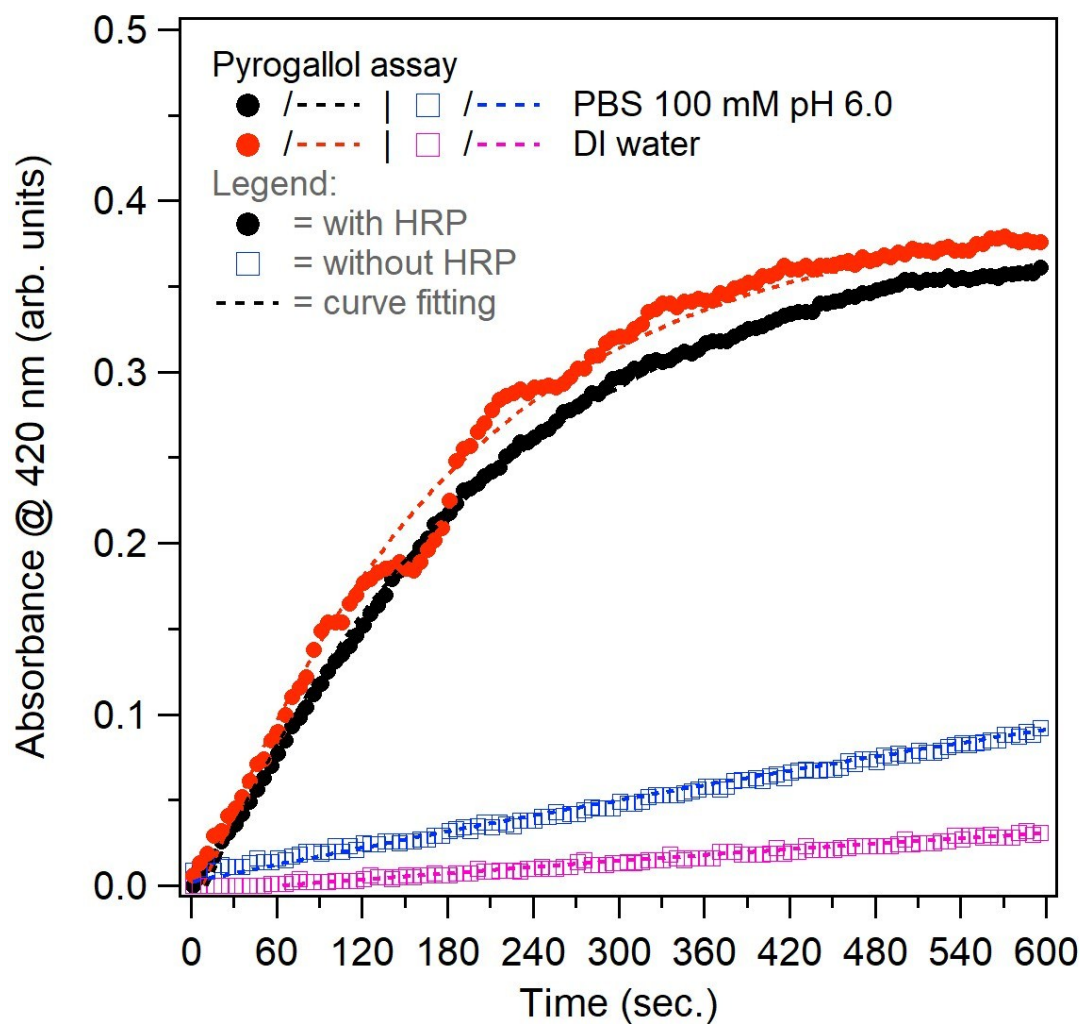


Figure S11. Kinetics of the enzymatic assay on free HRP performed in PBS buffer and in DI water, corresponding to the intensities at 420 nm referred in the following **Figure S8**. The outcome in water (red) is comparable to the one in PBS (black), with a lower background (purple vs blue line for water and buffer, respectively).

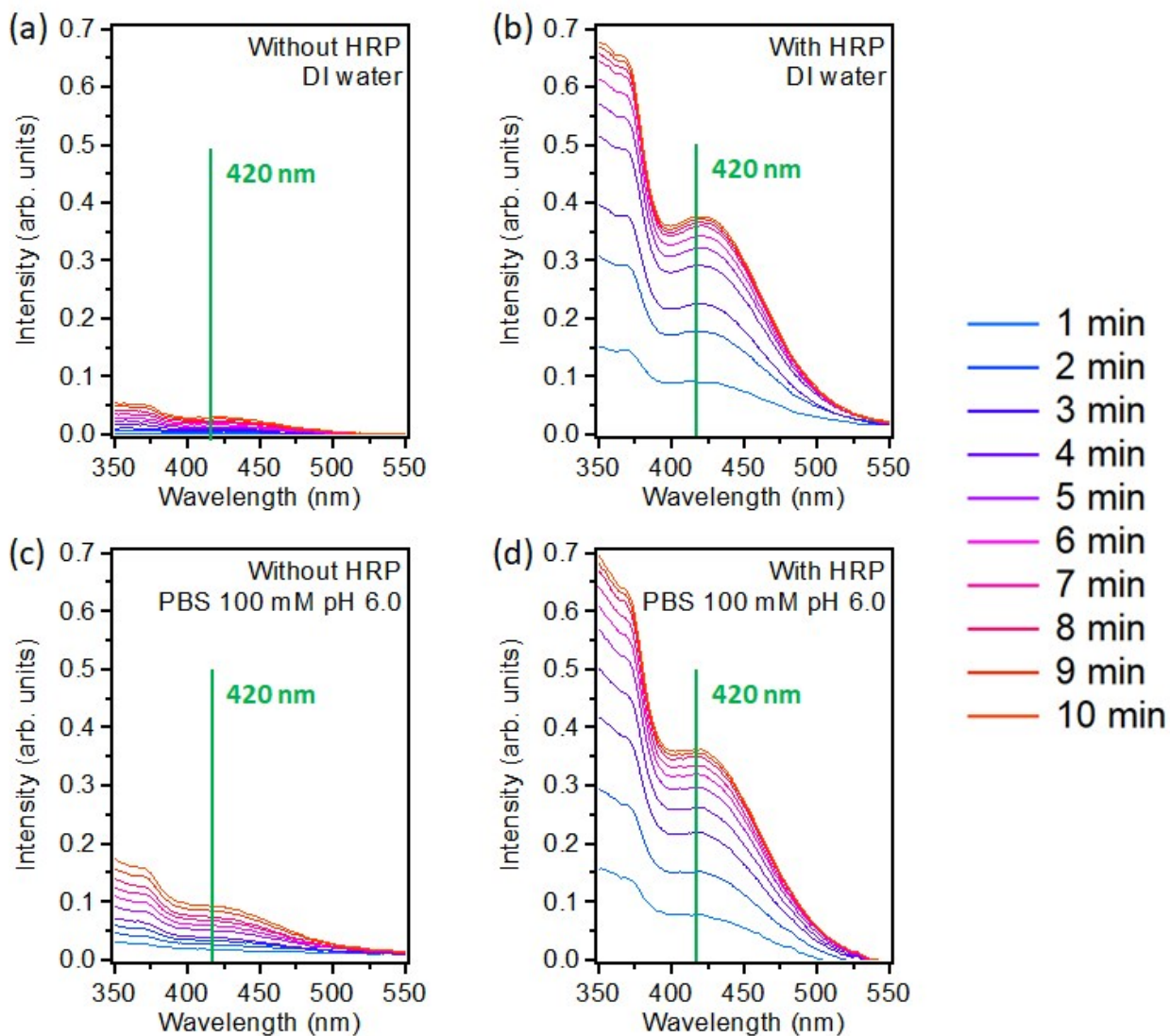


Figure S12. Comparison between the enzymatic assay on free HRP over time performed (a, b) using PBS 100 mM, pH 6.0, and (c, d) using only DI water. The results obtained with HRP (b and d) are comparable, however the background using PBS (a) increases more over the time than the one obtained in water only (c), indicating a superior stability of free pyrogallol in water rather than in buffer medium, without appreciable variation of absorption maximum. The green lines refer to the intensity at 420 nm.

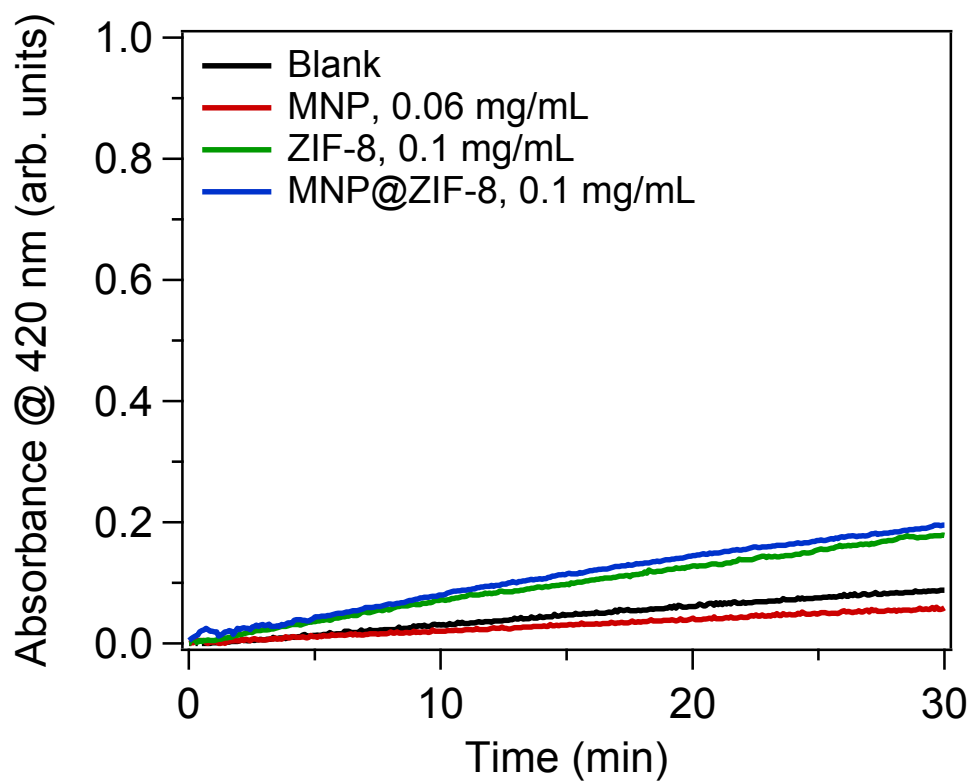


Figure S13. Influence of MNPs, ZIF-8, and MNP@ZIF-8 on the enzymatic assay using pyogallol as revealing dye.

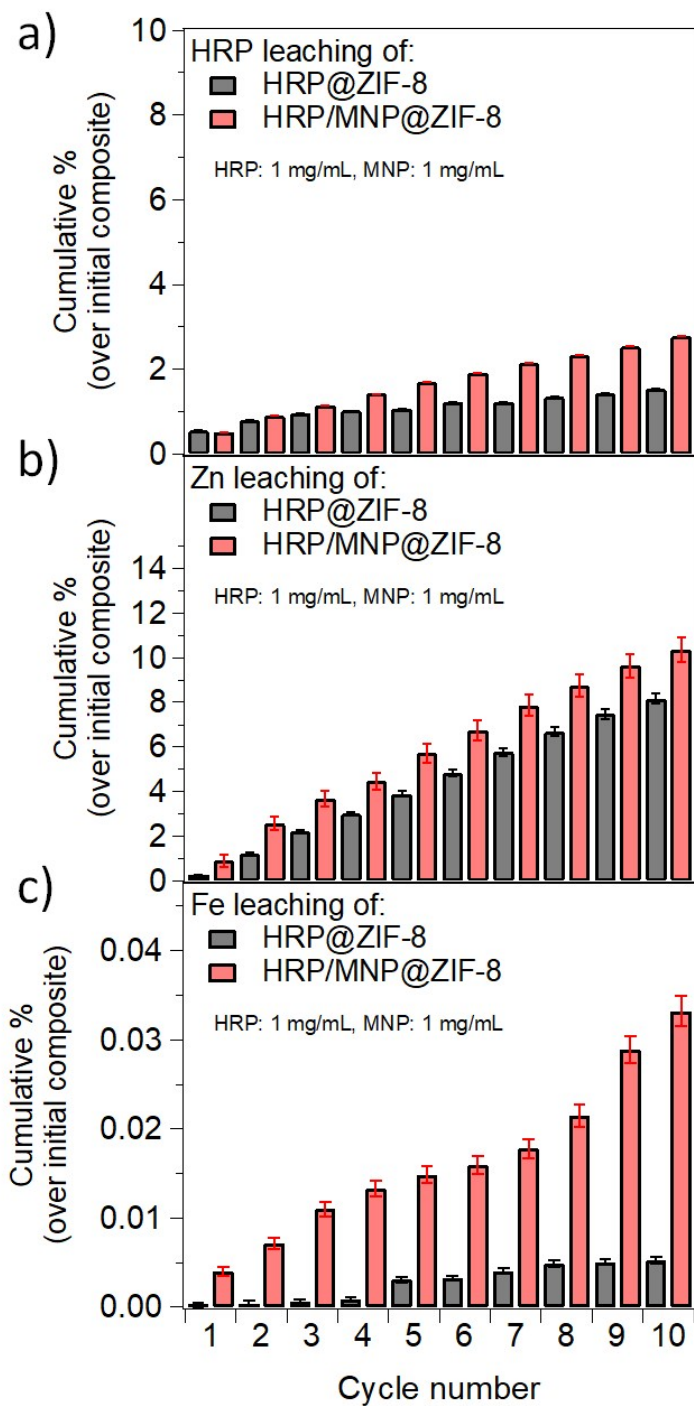


Figure S14. Cumulative percentage leaching of (a) HRP protein, (b) Zn, and (c), for the HRP@ZIF-8 (black bars) and HRP/MNP@ZIF-8 (red bars) after 10 repeated cycles of reaction. The HRP content was estimated by Bradford assay, the metal ions were assessed by ICP-OES (Zn) or ICP-MS (Fe). The percentages were calculated over the total composite amount (2 mg).

Table S1. Time-dependant yields for the scale-up reaction regarding HRP/MNP@ZIF-8, and the controls BSA/MNP@ZIF-8, BSA@ZIF-8, MNP@ZIF-8, and ZIF-8, prepared on a 90-mL scale.

Sample set	<i>Yield after 5 minutes</i>	<i>Yield after 18 hours</i>
HRP/MNP@ZIF-8	<u>48 %</u>	<u>88 %</u>
- Controls:		
BSA/MNP@ZIF-8	70 %	90 %
BSA@ZIF-8	46 %	98 %
MNP@ZIF-8	85 %	95 %
ZIF-8	77 %	91 %

Table S2. Data values of the HRP@ZIF-8 and the HRP/MNP@ZIF-8 sets depending on the starting quantity of the encapsulated species in the reaction mixture. The error is calculated from standard deviation.

Sample set	HRP (mg mL ⁻¹)	MNP (mg mL ⁻¹)	EE (%)	Loading (mg g ⁻¹)	SA (U mg ⁻¹)	SAC (U g ⁻¹)	η (%)
HRP @ ZIF-8	0.33	0.00	79 ± 5.	6.9 ± 0.4	1.6 ± 0.1	11.0 ± 0.9	10.1
	0.67	0.00	82 ± 5.	12.0 ± 0.7	1.5 ± 0.1	18.0 ± 1.6	9.5
	1.00	0.00	86 ± 2.	13.8 ± 0.4	0.8 ± 0.1	11.0 ± 1.4	5
HRP / MNP @ ZIF-8	0.33	0.33	84 ± 4.	5.1 ± 0.2	0.3 ± 0.1	1.5 ± 0.5	1.9
	0.67	0.33	86 ± 6.	4.7 ± 0.3	1.1 ± 0.2	5.2 ± 1.0	6.9
	1.00	0.33	88 ± 7.	4.1 ± 0.3	2.2 ± 0.1	9.0 ± 0.8	13.9
	0.33	0.67	87 ± 3.	8.8 ± 0.3	0.5 ± 0.3	4.4 ± 2.6	3.2
	0.67	0.67	92 ± 5.	5.4 ± 0.3	1.5 ± 0.3	8.1 ± 1.7	9.5
	1.00	0.67	93 ± 6.	4.4 ± 0.3	3.3 ± 0.1	14.5 ± 1.1	20.8
	0.33	1.00	91 ± 4.	9.1 ± 0.4	1.1 ± 0.4	10.0 ± 3.7	6.9
	0.67	1.00	94 ± 5.	5.5 ± 0.3	3.0 ± 0.3	16.5 ± 1.9	18.9
	1.00	1.00	94 ± 6.	5.6 ± 0.3	4.1 ± 0.1	23.0 ± 1.4	25.9

Legend:

EE = encapsulation efficiency

SA = Specific activity of the encapsulated enzyme (U mg_{immobilized enzyme}⁻¹)

SAC = Specific activity of the whole composite (U g_{material}⁻¹)

η = effectiveness factor: 100 x (U mg_{immobilized enzyme}⁻¹) / (U mg_{free enzyme}⁻¹).¹⁰

Table S3. Comparison of the catalytic activities of HRP immobilized in MOF systems and in this work. Other similar studies concerning HRP did not report the specific activity.¹¹⁻¹⁵

System (enzyme@MOF)	Specific activity			Assay conditions	Application	Reference
	U/mg _f	U/mg _i	U/g _m			
HRP@PCN-333	n.d.	(34)	(34090)	ABTS, water, 3.3 mM H ₂ O ₂ , 25 °C, cont.	Single molecule trap	16*
HRP/DNA@ZIF-8	300	293	73301	oPDA, PBS, pH 6.5, 0.3% H ₂ O ₂ , disc.	Simultaneous detection of Hg ²⁺ and phenol	17
HRP/GOx/MNP @HKUST-1	n.d.	79	9251	oPDA, PBS, pH 7, 0.3 mM Glucose, 25 °C, disc.	Co-immobilization of GOx & HRP	18**
HRP/MNP@ZIF-8	16	40	23	Pyrogallol, water, 0.5 % H ₂ O ₂ , 23 °C, cont.	Catalysis	This work

Legend:

U/mg_f = Units per mg of free enzyme

U/mg_i = Units per mg of immobilized enzyme

U/g_m = Units per g of material

ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

oPDA = o-phenylenediamine

PBS = phosphate buffer saline

cont. = continuous process

disc. = discontinuous process

Notes:

*: The Specific activity (in bracket) was calculated from k_{cat} , considering HRP mol. wt of 44.000;

** : Apparent Specific activity of GOx & HRP.

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