Supplementary Information (SI) for Nanoscale.

Enhanced Peroxidase-like Activity of Palladium-Iron Bimetallic Nanoparticles Supported on *N*- doped Mesoporous Carbon for Colorimetric Detection of Ascorbic Acid and Dopamine

Zahra Bahreini,[a] Siyavash Kazemi Movahed,[a]* Keun Hwa Chae[b]*, and Cheol-Hwee Shim[b]

[a]Department of Chemistry, Isfahan University of Technology, Isfahan 8415683111, Islamic Republic of Iran, Email: skazemi@iut.ac.ir, Phone: +98 31 3391 3282

[b] Advanced Analysis Center, Korea Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea, Email: khchae@kist.re.kr

1.1. Characterization

Diffraction data were collected on a Asenware AW-DX300 and Cu Kα radiation (λ = 1.5406 Å). XPS analysis was performed using a Nexsa (Thermo Fisher Scientific) equipped with an Al Ka (1486.6 eV) X-ray source, with charging corrected using carbon (284.8 eV). Field emission scanning electron microscopy (FESEM) images were characterized using an electron microscope FEI QUANTA FEG-450. Transmission electron microscopy (TEM) images were obtained using a transmission microscope Philips CM-30. High-resolution-transmission electron microscopy (HR-TEM) images were obtained using an FEI Tecnai F20 G2. The concentration of palladium was estimated using an inductively coupled plasma optical emission spectrometer (ICP-OES) AMETEK Materials Analysis Division (Acros). The pore volume of the samples was obtained from Nitrogen adsorption/ desorption isotherms, recorded on Belsorp Mini II at 77.3 K. The Brunauer–Emmett–Teller (BET) was used to calculate the specific surface area. UV-Vis spectra were recorded employing a Shimadzu UV-2600 and JENWAY Genova.

1.2. Material

All the chemicals used in this study were sourced from commercial suppliers and were employed without undergoing additional purification steps. Poloxamer 407 (Pluronic F127®), 3-aminophenol (99%), hexamethylenetetramine (HMT, 99.0%), ammonia solution (28-30%), hydrogen peroxide (35.0%), 3,3,5,5-tetramethylbenzidine (TMB), *ortho*-phenylenediamine (OPD), hydrochloric acid (HCl), sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate, citric acid, iron(III) nitrate 9-hydrate ($Fe(NO_3)_3.9H_2O$), palladium chloride (99%), and sodium borohydride (99%) were purchased from Merck and Sigma-Aldrich.

1.3. Preparation of *N*-MC

Nitrogen-doped mesoporous carbon structures were prepared by modifying the procedure reported by Huang [1]. First, a 10 mL aqueous solution containing 0.52 g of Pluronic F-127 was stirred for at least 3 h until a homogeneous state was achieved. Next, 3-aminophenol (0.654 g) and hexamethylenetetramine (HMT) (0.420 g) were dissolved in 70 mL of DI water and stirred for 1 h 30 min. Then, 10 mL of the F-127 solution was slowly added to the latter solution. After stirring at 80 °C for 24 h, the product (AFPS) was collected by centrifugation, washed three times with DI water, and finally dried in an oven at 50 °C. The *N*-MC was

obtained by heating the AFPS to 250 °C for 2 h at a rate of 2 °C·min^{-1}, followed by heating to 600 °C for 3 h at a rate of 2 °C·min^{-1} under an Ar atmosphere.

1.4. Preparation of FePd@*N*-MC

The powder obtained from the previous step (0.3 g) was dispersed in 130 mL of DI water for 30 minutes using an ultrasonic bath. Next, solutions of palladium (dissolving of palladium chloride (13.2 mg) in 20 µL of 12 M HCl and further diluting to 10 mL of DI water) and iron (10 mL, 0.0142 M solution of iron (III) nitrate) were prepared. The iron solution was then added drop by drop, followed by the palladium solution. The mixture was stirred for 23 h. Then, a 16% sodium hydroxide solution was gradually added to the mixture until the pH reached 10. After one hour, a fresh solution of sodium borohydride (0.56 g) in water (10 mL) was prepared and added drop by drop to the above mixture, and was stirred for 1 h. The resulting structure was collected using an external magnetic field and was washed three times with DI water, and finally dried in an oven at 50°C.

1.5. Preparation of Pd@*N*-MC

The synthesis procedure for the Pd@*N*-MC was identical to that of the FePd@*N*-MC nanozyme except for the addition of palladium chloride (26.3 mg in 40 µL of 12 M HCl and further diluting to 20 mL of DI water).

1.6. Preparation of Fe@*N*-MC

The synthesis procedure for the Pd@*N*-MC was identical to that of the FePd@N-MC nanozyme except for the addition of iron(III) nitrate nonahydrate (20 mL of 0.0142 M solution of iron (III) nitrate).

1.7. Preparation of $Pd_{2.5\%}$@*N*-MC

The synthesis procedure for the Pd@*N*-MC was identical to that of the FePd@*N*-MC nanozyme except for the addition of palladium chloride (13.1 mg in 20 µL of 12 M HCl and further diluting to 20 mL of DI water).

1.8. The peroxidation of 3,3,5,5-tetramethylbenzidine (TMB)

Before assessing the peroxidase-like activity of TMB peroxidation, an experiment at pH 5 (2.76 mL, citrate buffer), TMB (60 µL, 0.09 mM), H_2O_2 (60 µL, 1.10 mM), and nanozyme (120 µL, 100 µg/mL) was carried out. It was determined that the maximum wavelength of the diimine complex was 650 nm.

1.8.1. pH optimization for TMB peroxidation

A TMB (60 µL, 0.09 mM), H_2O_2 (60 µL, 1.10 mM), FePd@*N*-MC nanozyme (100 µL, 100 µg/mL suspension), and citrate buffer solution at different pH levels (2.76 mL, pH: 3, 4, 5, 6 and 7) were poured into a quartz cuvette (3 mL). The UV-Vis spectrum was obtained from this mixture after allowing it to react for ten minutes.

1.8.2. Steady-state kinetics assays of FePd@*N*-MC nanozyme toward TMB peroxidation

Steady-state kinetics of the FePd@*N*-MC nanozyme toward TMB peroxidation were studied using different concentrations of H_2O_2 and TMB. Kinetic assays of FePd@*N*-MC (120 µL, 100 mg/mL) with TMB were evaluated using H_2O_2 (60 µL, 1.10 mM), citrate buffer (pH: 5.0, 2.76 mL) and varying concentrations of TMB aqueous solution (0.030, 0.045, 0.060, 0.090 and 0.120 mM). The kinetic assays of FePd@*N*-MC (120 µL, 100 mg/mL) with H_2O_2 were studied with TMB (60 µL, 0.09 mM), citrate buffer (pH: 5, 2.76 mL) and different concentrations of H_2O_2 solution (0.26, 0.36, 0.46, 0.72, 1.10, and 1.80). The absorbance was measured every twenty seconds with a single wavelength UV instrument at 650 nm until it did not change.

1.9. The peroxidation of *o*-phenylenediamine (OPD)

Before assessing the peroxidase-like activity of OPD peroxidation, an experiment at pH 5 (2.76 mL, citrate buffer), OPD (60 µL, 0.122 mM), H_2O_2 (60 µL, 9 mM), and nanozyme (120 µL, 100 µg/mL) was carried out. It was determined that the maximum wavelength of the OPDox was 450 nm.

1.9.1. pH optimization for OPD peroxidation

An OPD (60 µL, 0.122 mM), H_2O_2 (60 µL, 9 mM), FePd@*N*-MC nanozyme (120 µL, 100 µg/mL suspension) and citrate buffer solutions at different pHs levels (2.76 mL, pH: 3, 4, 5, 6, and 7) were poured into a quartz cuvette (3 mL). The UV-Vis spectrum was obtained from this mixture after allowing it to react for ten minutes.

1.9.2 Steady-state kinetics assays of FePd@*N*-MC nanozyme toward OPD peroxidation

Steady-state kinetics of the FePd@*N*-MC nanozyme toward OPD peroxidation were studied using different concentrations of H_2O_2 and OPD. Kinetic assays of FePd@*N*-MC (120 µL, 100 mg/mL) with OPD were evaluated using H_2O_2 (60 µL, 9 mM), a citrate buffer solution (pH: 5, 2.76 mL) and various concentrations of an OPD aqueous solution (0.01, 0.038, 0.066, 0.094, and 0.122 mM). The kinetic assays of FePd@*N*-MC (120 µL, 100 mg/mL) with H_2O_2 were studied with OPD (60 µL, 0.122 mM), citrate buffer (pH: 4, 2.76 mL) and different concentrations of H_2O_2 solution (0.6, 3.0, 4.5, 6.0, and 9.0 mM). Absorbance was measured

every twenty seconds with a single wavelength UV instrument at 450 nm until it did not change.

1.10. Dopamine detection

In a typical procedure, the following reagents were added sequentially to a citrate buffer solution (pH 5.0, 2.76 mL): TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), FePd@*N*-MC (120 μL, 100 mg/mL), and 60 μL of DA at various concentrations (0-10 μM). After incubation for 15 min at room temperature, the absorbance spectrum was measured using a UV-Vis spectrometer. The calibration curve was generated by plotting the absorbance at 652 nm against DA concentration.

1.11. Ascorbic acid detection

For the colorimetric detection of ascorbic acid (AsA), the following reagents were sequentially adde the to citrate buffer (pH 5.0, 2.70 mL): TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), FePd@*N*-MC (120 μL, 100 mg/mL), and 60 μL of AsA at various concentrations (0-12 μM). After a 15 min incubation period at room temperature, the absorbance spectrum was measured using a UV-Vis spectrometer. A calibration curve was constructed by plotting the absorbance at 652 nm against the AsA concentration.

1.12. Competitive oxidation of TMB in the presence of AsA

The effect of ASA on TMB oxidation was investigated in 2.7 mL of citrate buffer solution (pH 5.0) containing TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), and FePd@*N*-MC (120 μL, 100 mg/mL), and 60 μL of AsA at final concentrations of 0, 4, 8, and 12 μM. The mixture was incubated at room temperature, and the absorbance at 652 nm was recorded over 20 min.

1.13. Oxidation of AsA under different conditions

The maximum absorption of AsA in citrate buffer (pH 5.0) was found to be at λ_{max}= 265 nm (Fig. S11). Using this wavelength, the absorbance of AsA (60 μL, 50 μM) was monitored under the following conditions for 20 min: alone; with H_2O_2 (60 μL, 1.10 mM); with FePd@*N*-MC (120 μL, 100 mg/mL); and with both H_2O_2 (60 μL, 1.10 mM) and FePd@*N*-MC (120 μL, 100 mg/mL). This was done to evaluate its oxidation behavior and electron transfer interactions.

Redox cycling of TMB with successive AsA additions

TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), and FePd@*N*-MC (120 μL, 100 mg/mL) were incubated in citrate buffer (pH 5.0, 2.7 ml), and the absorbance at 652 nm was recorded.

Then, 20 μL of 12 μM AsA was added and the absorbance was measured. After a few minutes a second 20 μL aliquot was added to monitor the reduction and regeneration of ox-TMB.

1.14. Detection of ascorbic acid in fresh fruits

The fresh juices from apple, orange, and lime were centrifuged at 10,000 rmp for 15 min and filtered through filter paper. For the assay, 0.5 ml of each juice was diluted to 5 ml. Then, 60 μL of the diluted juice was mixed with TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), and FePd@*N*-MC (120 μL, 100 mg/mL) in citrate buffer (pH 5.0 2.7 ml), and incubated at room temperature for 15 min. The absorbance at 652 nm was recorded.

1.15. Recovery experiment in orange juice

First, the clarified orange juice filtrate was analyzed under the optimized assay conditions without adding AsA to determine its natural AsA content. Then, 0.5 mL of the juice was diluted to a final volume of 5 mL. ،The reaction mixture containing citrate buffer (pH 5.0 2.74 ml), TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), and FePd@*N*-MC (120 μL, 100 mg/mL) was then prepared and incubated for 15 minutes. Finally, 10 μL of the juice and 10 μL of an AsA standard (0-4 μM) were added, and the absorbance at 652 nm was recorded. The AsA concentrations were calculated using the calibration curve.

1.11. Selectivity test

The selectivity of the FePd@*N*-MC-based colorimetric assay was rigorously evaluated against various potential interfering substances. Specifically, 60 μL of 100 μM solution containing several amino acids (histidine, lysine, valine, alanine, proline, glycine, aspartic acid, phenylalanine, methionine, and phenylglycine) and various metal ion solutions (Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Ni^{2+}, Al^{3+}, and Ba^{2+}) and dopamine (DA) were examined separately. Each solution was mixed with TMB (60 μL, 0.09 mM), H_2O_2 (60 μL, 1.10 mM), and FePd@*N*-MC (120 μL, 100 mg/mL) in a citrate buffer solution (pH 5.0, 2.76 mL). The reaction mixtures were incubated for 15 min at room temperature, after which the absorbance was measured at 652 nm using UV-Vis spectroscopy.

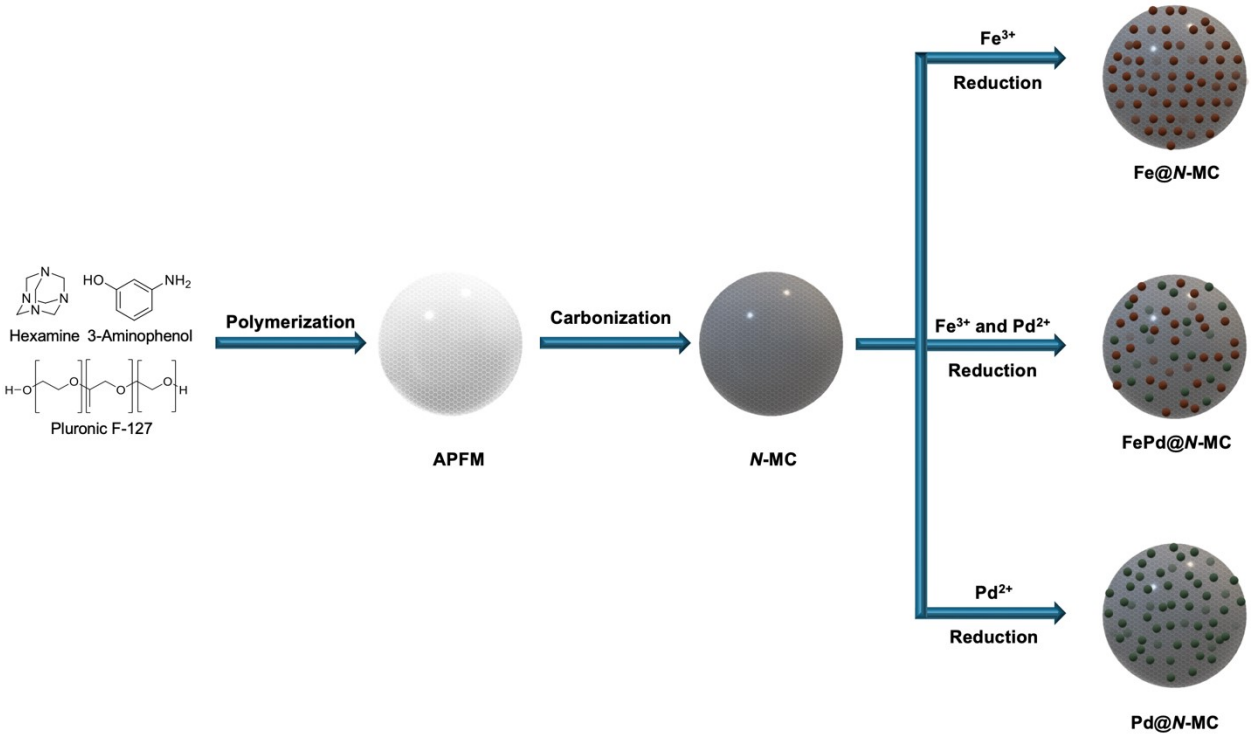
1.12. Electrochemical measurements

Electrochemical measurements were conducted using a conventional three-electrode system coupled with a μAUTOLAB III electrochemical workstation. A modified glassy carbon electrode (GCE) served as the working electrode, while an Ag/AgCl electrode and platinum wire functioned as the reference and counter electrodes, respectively. Cyclic voltammetry (CV)

was used to evaluate the electrochemical response toward H_2O_2 in phosphate-buffered saline (PBS, 0.1 M, pH 7.0), both in the absence and presence of 5 mM H_2O_2. The CV measurements were performed within a potential window of -0.8 to 0.4 V at a scan rate of 50 mV s^{-1}.

1.13. Time-resolved FT-IR measurements

FT-IR spectra of the catalyst were recorded using a Rayleigh WQF-510A spectrometer. To prepare the sample, the catalyst was mixed homogeneously with KBr and compressed into a pellet. An initial spectrum was acquired for the catalyst/KBr pellet. Then, 50 µL of a 10 mM H_2O_2 solution was added directly to the pellet surface. Time-resolved FT-IR spectra were then recorded at two-minute intervals to monitor changes in the catalyst-peroxide interaction over time.



Scheme S1. Schematic presentation for the preparation of *N*-MC, Fe@*N*-MC, Pd@*N*-MC, FePd@*N*-MC

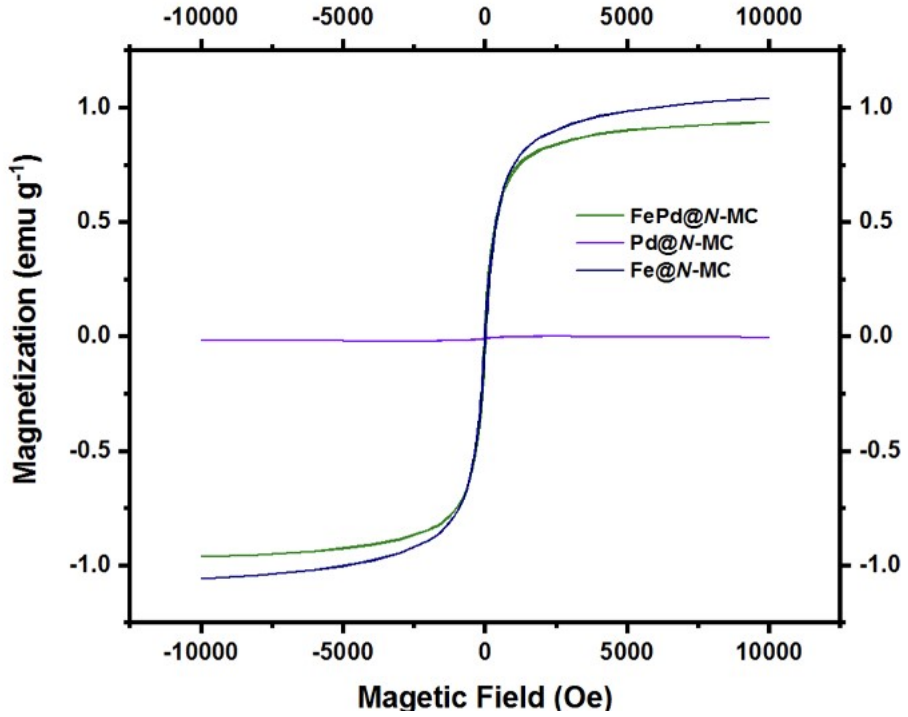


Fig. S1. VSM plots of synthesized structures.

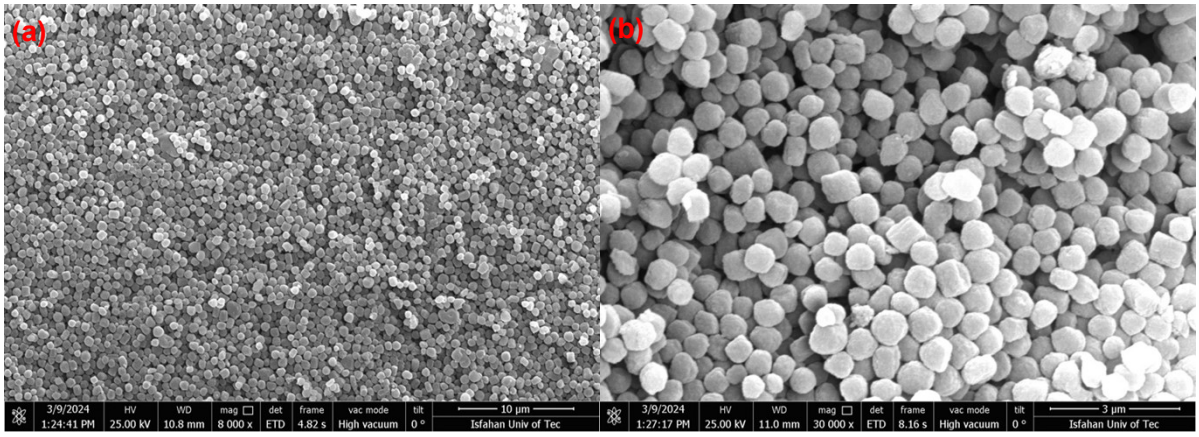


Fig. S2. FESEM images of 3-aminophenol-formaldehyde polymer (AFP) structures

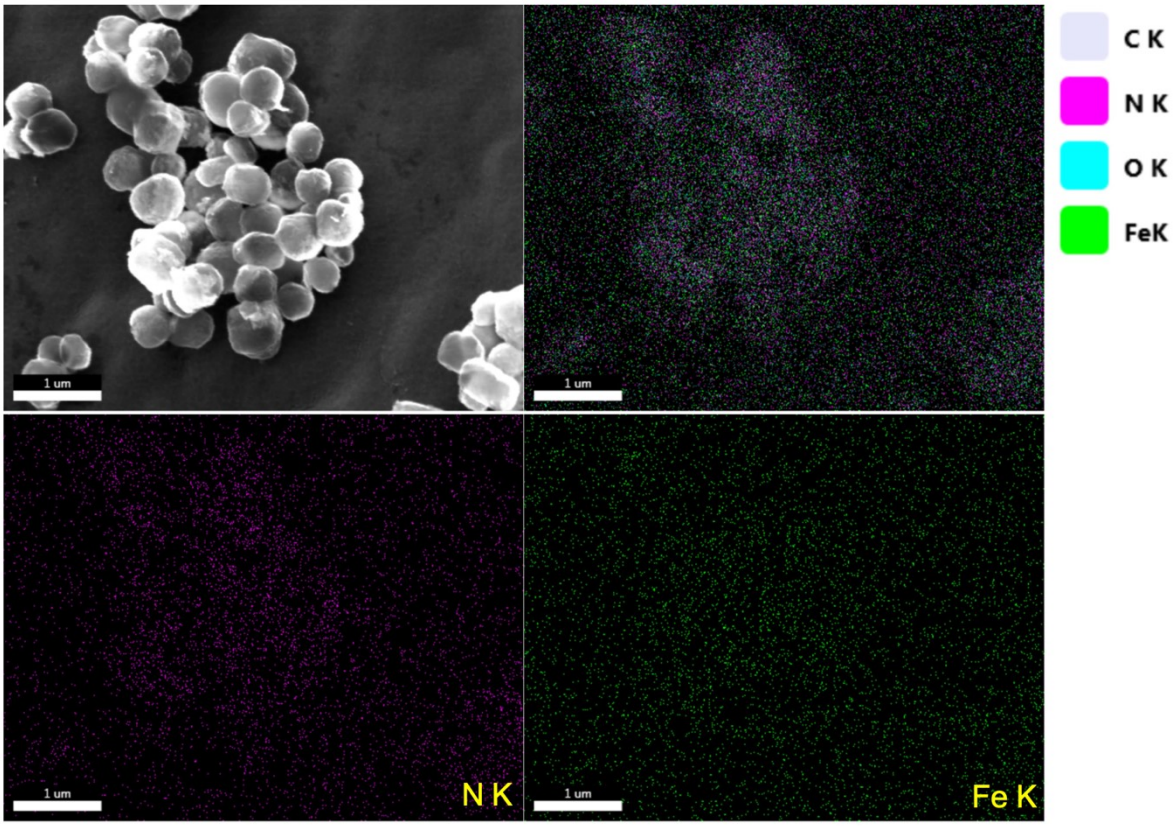


Fig. S3. EDS mapping of Fe@*N*-MC

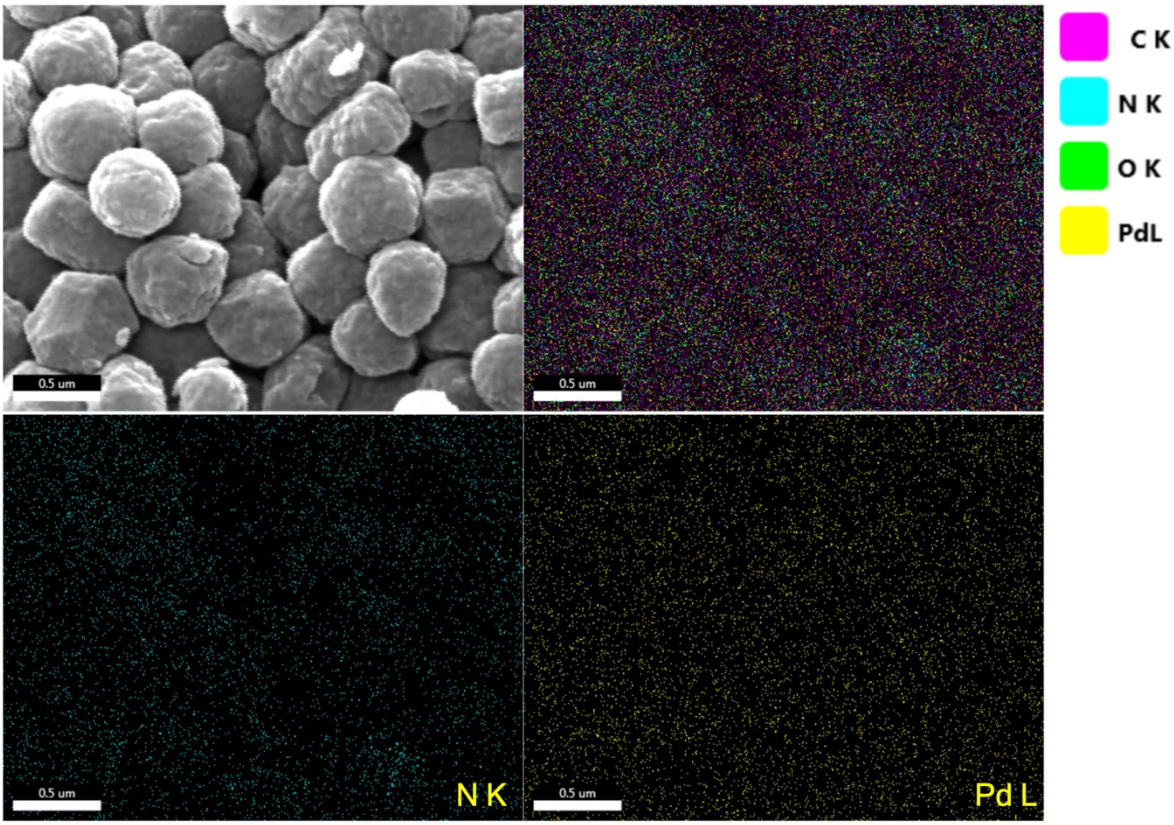


Fig. S4. EDS mapping of Pd@*N*-MC

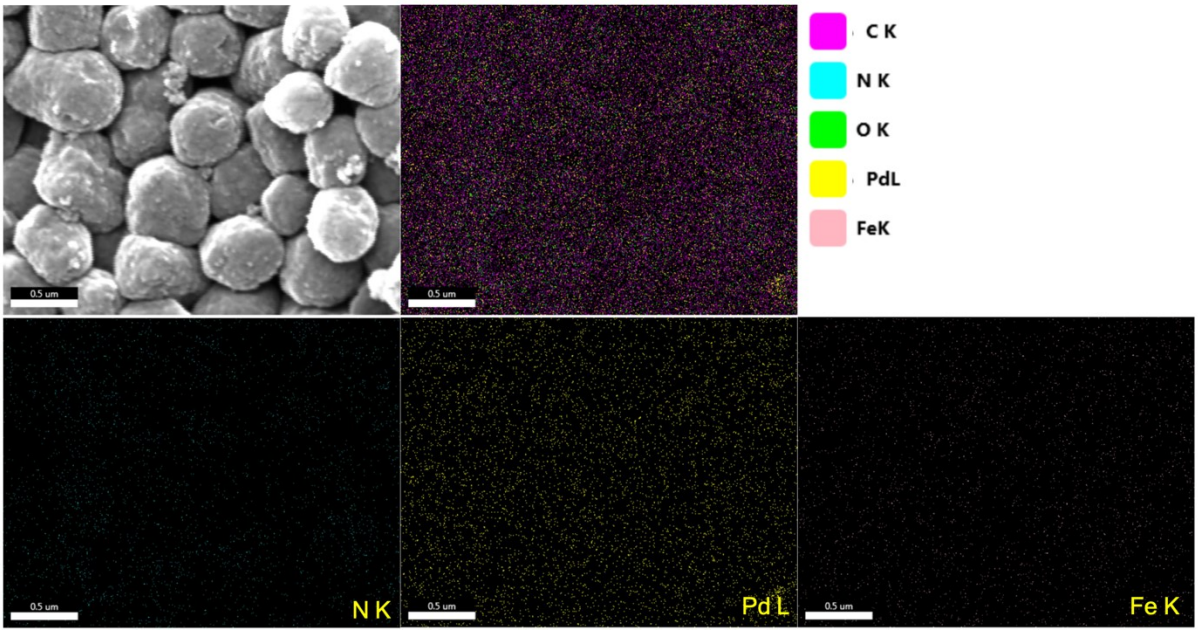


Fig. S5. EDS mapping of FePd@*N*-MC

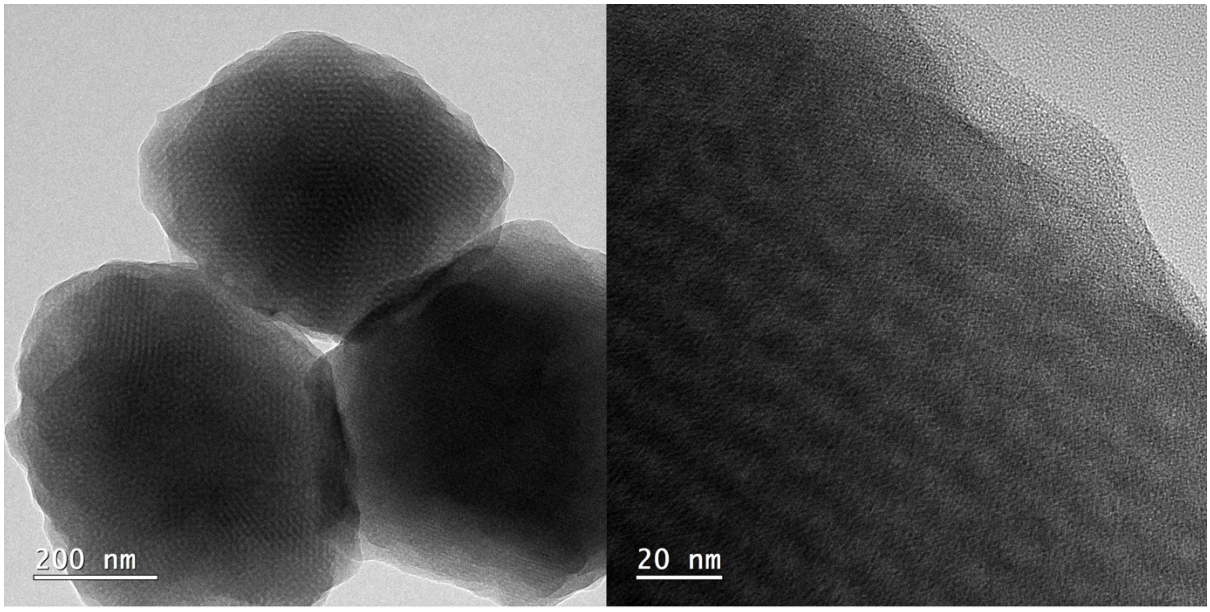


Fig. S6. TEM images of *N*-MC

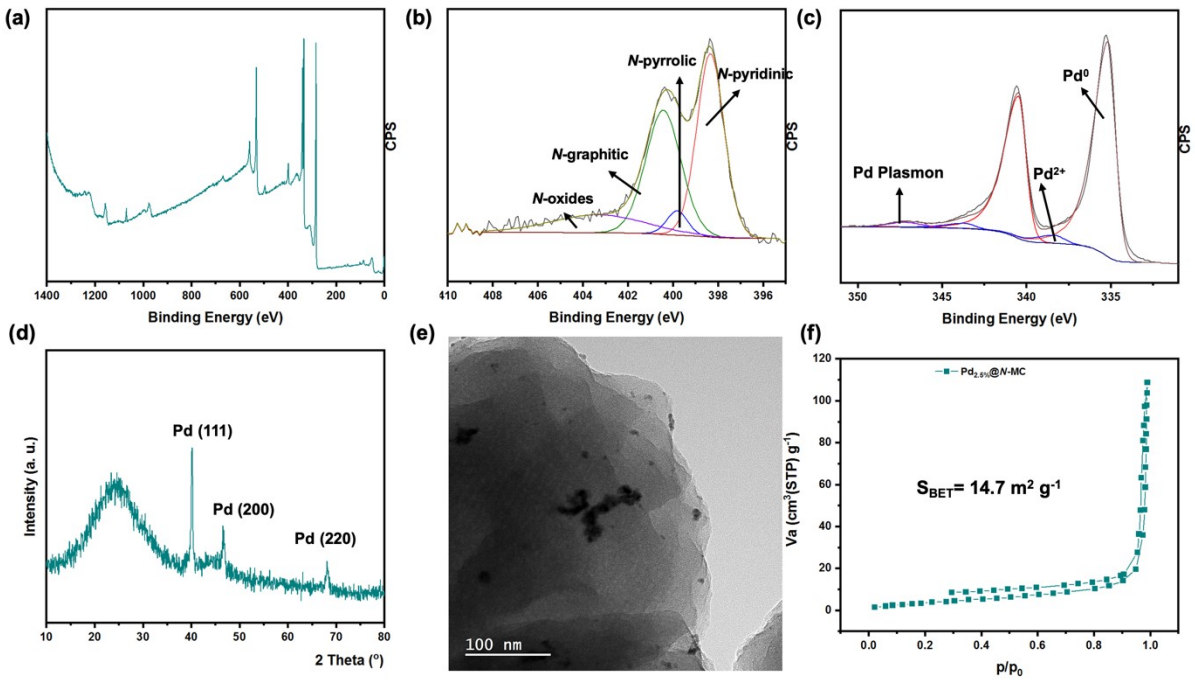


Fig. S7. a) XPS survey, b) N 1s core level, c) Pd 3d core level, d) XRD pattern, e) TEM image, and f) N_2 physisorption of $Pd_{2.5\%}$@*N*-MC

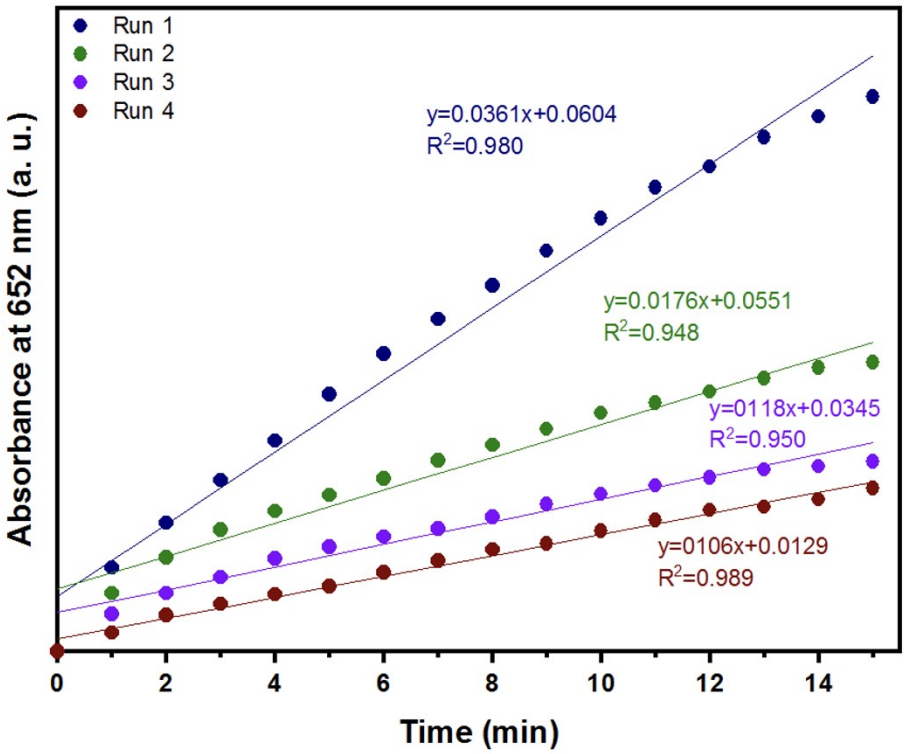


Fig. S8. Reusability of FePd@*N*-MC nanozyme in the peroxidation of TMB substrate

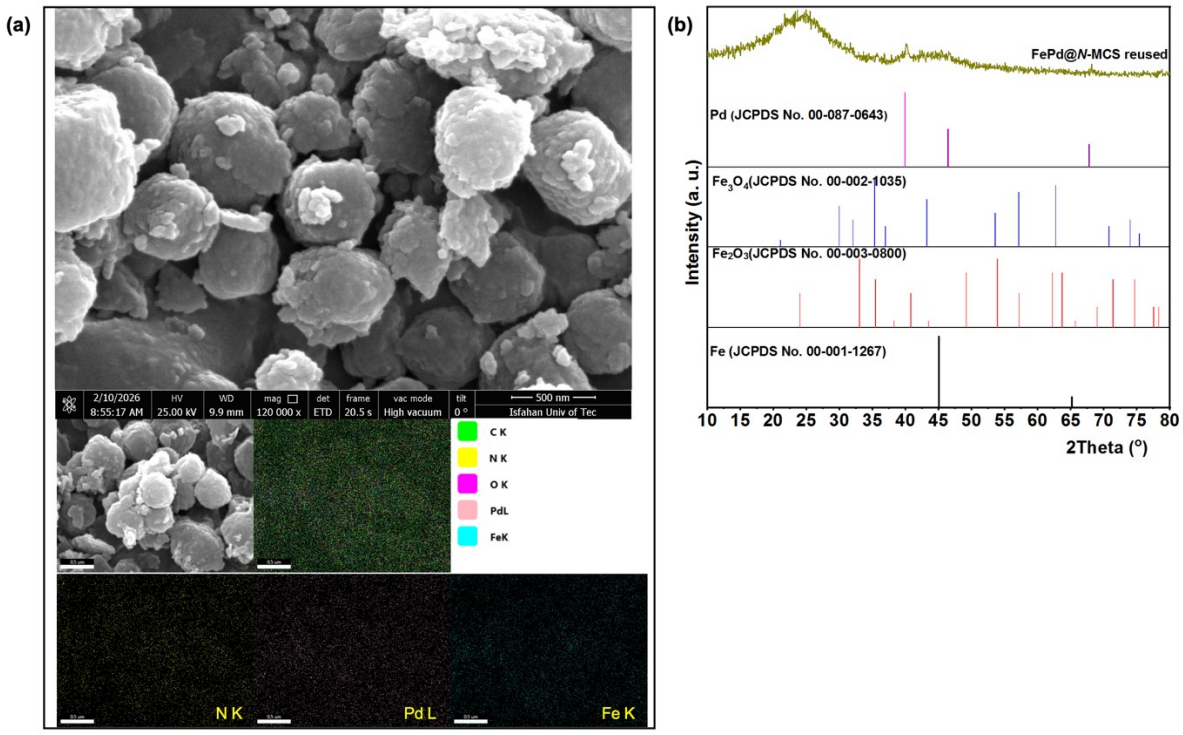


Fig. S9. a) FESEM and EDS mapping, b) XRD pattern of FePd@*N*-MC nanozyme after four runs.

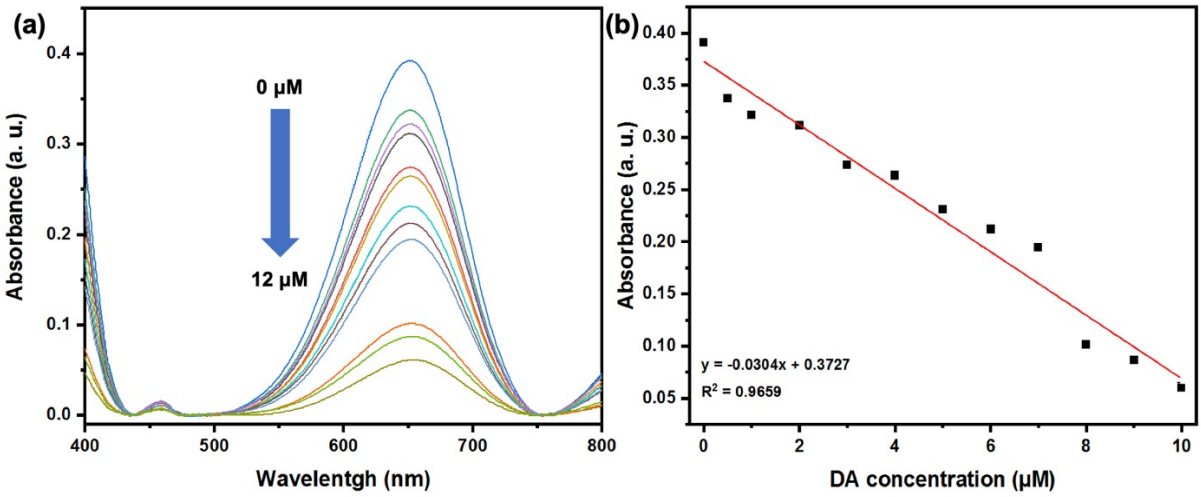


Fig. S10. a) Absorption spectra of ox-TMB in the presence of various concentrations of DA and b) Linear calibration curve of DA detection.

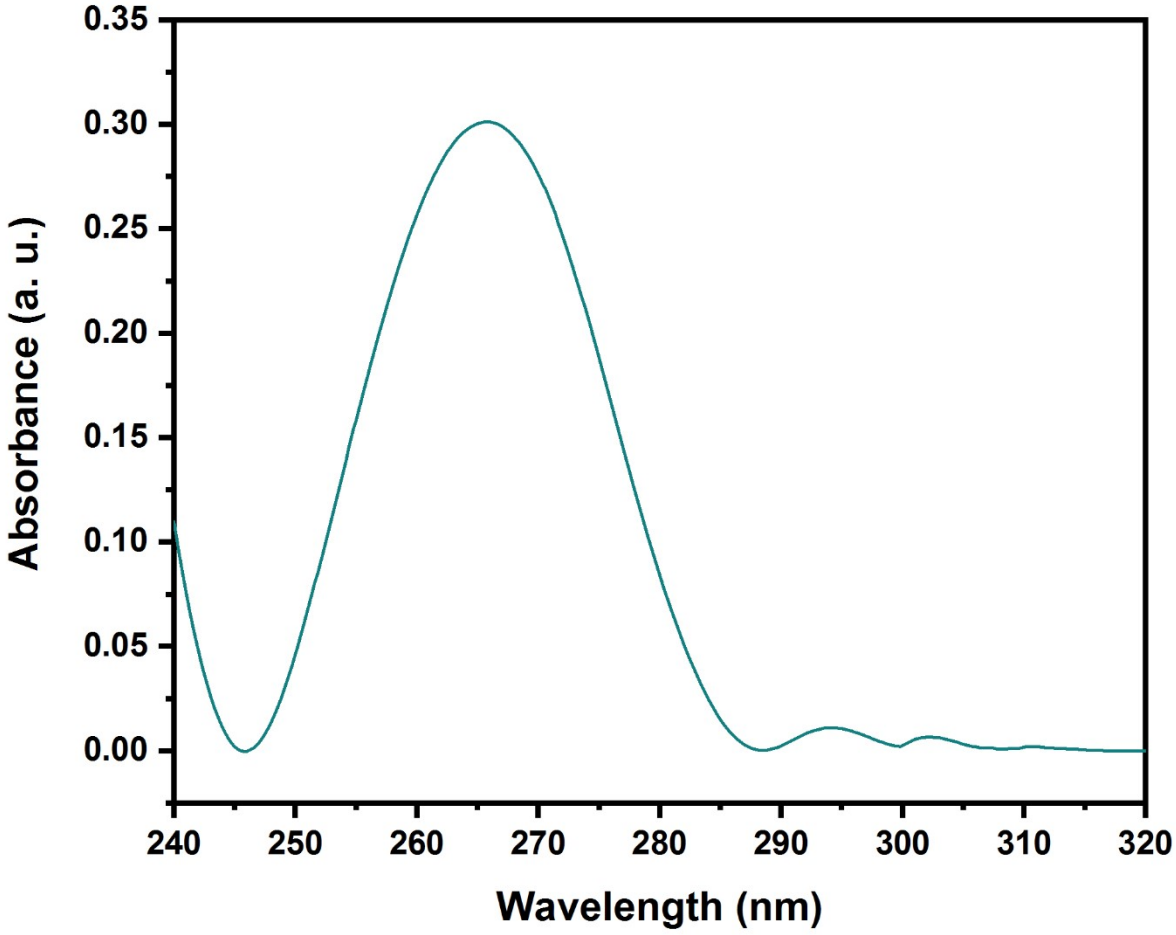


Fig. S11. UV-Vis spectrum of ascorbic acid (AsA) at pH 5

References:

[1] Z. Tian, H. Jiang, M. Huang, G.-H. Wang, Facile Synthesis of Size-Controlled Nitrogen-Doped Mesoporous Carbon Nanosphere Supported Ultrafine Ru Nanoparticles for Selective Hydrogenation of Quinolines, Chemistry – A European Journal 26 (2020) 17000–17004. https://doi.org/10.1002/chem.202003492.