

Electronic Supplementary Information

Quantitative Evaluation of O₂ Activation Half-Reaction for Fe-N-C in Oxidase-Like Activity Enhancement

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Chemical materials.

o-Phenylenediamine (o-PD), ammonium persulfate (APS), iron (III) chloride hexahydrate (FeCl₃·6H₂O), hydrochloric acid (HCl), acetic acid (HAc), sodium acetate trihydrate (NaAc·3H₂O), perchloric acid (HClO₄), dimethyl sulfoxide (DMSO), and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Cyanamide (CA) was obtained from Energy Chemical (China). Carbon black (CB, Ketjenblack EC 600 JD) was obtained from Akzo Nobel N.V. (Netherlands). Mannitol was purchased from Aladdin Chemistry Co., Ltd. (China). Dicyandiamide (DCDA), melamine (MELA), 3,3',5,5'-tetramethylbenzidine (TMB), and superoxide dismutase (SOD, from bovine erythrocytes, ≥3,000 units mg⁻¹ protein) were purchased from Sigma-Aldrich (USA). 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and 2,2,6,6-Tetramethyl-4-piperidone hydrochloride (TEMP) were purchased from DOJINDO (Japan). Pt/C (HiSPEC™ 3000, 20 wt.% Pt on carbon black) were purchased from Johnson Matthey (UK). Unless otherwise specified, all other chemicals were of analytical grade and used without further purification. Ultrapure water (18.2 MΩ cm) was obtained from a Direct-Q 3 UV pure water purification system (Millipore, USA).

Synthesis of Fe-N-C nanozymes.

The Fe-N-C nanozymes were synthesized via the high-temperature pyrolysis. Taking preparation of Fe-N_{Ctrl}-C as an example, briefly, o-phenylenediamine (2 g) and iron (III) chloride

hexahydrate (2 g) were first stirred together with carbon black (1 g) in 1 M HCl (50 mL). The polymerization of o-PD was triggered by using ammonium persulfate (APS, 5 g) in an ice bath. The final Fe-N-C was obtained by pyrolyzing the precursor (3 g) at 900 °C under N₂ for 2 h and acid-etching of metallic iron and iron oxide impurities with concentrated hydrochloric acid (30 mL) for 24 h. The other Fe-N-C with the secondary nitrogen precursors added (2 g for CA, DCDA, and MEA, respectively) and the varied CA content (i.e., 0.5 g, 1 g, 6 g and 10 g, respectively) were prepared similarly, and denoted as Fe-N_{xn}-C, where x referred to the type of the secondary N precursor and n was used to distinguish samples prepared with different secondary N precursor content in the precursor.

Characteristic of Fe-N-C nanozymes.

The scanning electron microscopy (SEM) images were taken from a FEI Inspect F50 (FEI, USA). The transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HR-TEM) images were obtained from a JEM-2100F (JEOL, Japan) at an accelerating voltage of 200 kV. The N₂ adsorption-desorption (77 K) isotherms were collected by an Autosorb-iQ (Quantachrome, USA). The Raman spectra were collected on LabRAM HR UV-Visible (Horiba, Japan). The X-Ray diffraction (XRD) patterns were measured by an Ultima IV (Rigaku, Japan). The aberration-corrected high-angle annular dark field scanning transmission electron microscopy (AC HAADF-STEM) images were carried out by Themis Z (FEI, USA) equipped with energy-dispersive X-ray spectroscopy (EDS) mapping at an accelerating voltage of 300 kV. The X-ray photoelectron spectroscopy (XPS) was investigated on an ESCALAB 250XI electron spectrometer (Thermo Fisher, USA) with monochromatic Al K α X-rays (h ν = 1486.6 eV) as the excitation source, and the peak positions were corrected by the C 1s peak at 284.6 eV. The UV-Vis absorption spectra were taken by a Cary 100 UV-is (Agilent, Singapore). The oxygen concentration was recorded by an oxygen electrode on JPSJ-605 (Leici, China). The electron paramagnetic resonance (EPR) spectra were measured on EMX-10/12 (Bruker, Germany). The electrochemical measurements were performed with a 4 mm diameter glassy carbon disk-Pt ring working electrode rotated at 1600 rpm on RRDE-3A (ALS, Japan).

Oxidase-like activity and kinetic assay of Fe-N-C nanozymes.

The catalytic activity of Fe-N-C for the oxidation of TMB was assessed by monitoring the time dependent absorbance (at 652 nm for the TMB_{ox}) change via the kinetic mode using a Cary 100 UV-vis spectrometer (Agilent, Singapore). Typically, the Fe-N-C nanozyme (2 mg mL⁻¹, 10 μL) was added firstly into 0.1 M HAc-NaAc buffer solution (pH 3.6, 990 μL) containing TMB (100 mM in DMSO, 10 μL), then the test was started quickly at room temperature. The initial reaction velocity was calculated as follow:

$$v = \frac{\Delta A / \Delta t}{\epsilon \times l} \quad (1)$$

Where v is initial reaction velocity, $\Delta A/\Delta t$ is the initial rate of change in absorbance at 652 nm s⁻¹, ϵ is the molar absorption coefficient of TMB ($\epsilon_{652 \text{ nm}} = 39,000 \text{ M}^{-1} \text{ cm}^{-1}$), and l is the path length of light traveling in the cuvette ($l = 0.2 \text{ cm}$).

The steady-state kinetic assay of Fe-N-C was performed under above condition with varied volume of TMB solution (0, 0.5 ,1, 2, 4, 6, 10, 15 and 20 μL), respectively. The Michaelis-Menten constant (e.g., K_m and v_{max}) was calculated as follow based on the Michaelis-Menten saturation curve:

$$v = \frac{v_{max} \times [S]}{K_m + [S]} \quad (2)$$

Where v is the initial reaction velocity, v_{max} is the maximal reaction rate, $[S]$ is the concentration of the TMB and K_m is the Michaelis constant.

The specific activity (SA) of Fe-N_{CA1}-C, defined as activity units per milligram of nanozyme, was carried out at the similar condition with a series of Fe-N_{CA1}-C dosage (5, 10, 15, 20 and 25 μL, respectively). One unit is defined as the amount of nanozyme that catalytically produces 1 μmol of product per minute at standard condition. The SA was obtained by the following formula:

$$SA = \frac{V \times v}{[m]} \quad (3)$$

Where SA is the specific activity expressed in units per milligram (U mg⁻¹), V is the total volume of reaction solution (μL), v is initial reaction velocity (μM min⁻¹), and $[m]$ is the nanozyme weight (mg) of each assay.

The Michaelis–Menten constant was obtained based on the Michaelis–Menten saturation curve using Origin 2018 (OriginLab).

Oxygen concentration monitoring.

The dissolved O₂ concentration during the catalytic oxidation of TMB by Fe-N_{CA1}-C was studied via the oxygen electrode on JPSJ-605 (Leici, China). Briefly, TMB (100 mM, 0.1 mL) was added into the 0.1 M HAC-NaAc buffer solution (pH 3.6, 9.98 mL) containing Fe-N_{CA1}-C (2 mg mL⁻¹, 0.1 mL, or no Fe-N_{CA1}-C for control) under stirring at room temperature and the dissolved oxygen concentration (mg L⁻¹) was recorded once every 10s.

Free radical identification.

The reactive oxygen species (ROS) participated in the oxidation of TMB were investigated by comparing the activity in the absence or presence of ROS scavengers (SOD and mannitol scavenges superoxide and hydroxyl radical, respectively). In the catalytic oxidation of TMB, the Fe-N_{CA1}-C nanozyme (2 mg mL⁻¹, 10 μL) was added firstly into 0.1 M HAC-NaAc buffer solution (pH 3.6, 990 μL) containing SOD (2 mg mL⁻¹, 10 μL; or 1 M for mannitol) and TMB (0.1 M in DMSO, 5 μL) then the test was started quickly at room temperature.

The EPR spectra were taken to further explore the ROS generated during the activation of H₂O₂ by the Fe-N-C. 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and 2,2,6,6-Tetramethyl-4-piperidone hydrochloride (TEMP) were adopted to trap the short-lived hydroxyl radical (superoxide radical was trapped in methanol solution) and singlet oxygen in 0.1 M HAC-NaAc aqueous solution, respectively. Typically, the Fe-N_{CA1}-C (0.4 mg mL⁻¹, 10 μL) was added into 0.1 M HAC-NaAc buffer solution (pH 3.6, 190 μL) containing DMPO (2 M, 10 μL; 0.4 M for TEMP) first and then the test was started at room temperature. The EPR spectra of blank references were also obtained by the same method except that no Fe-N_{CA1}-C was added.

Electrochemical measurement.

Electrochemical measurement was performed in a three-electrode electrochemical cell using a CHI 700E potentiostat (CHI, USA) at room temperature. Rotating disk (ring disk) glassy carbon electrode (RRDE-3A, ALS, Japan) supported electrocatalyst (Fe-N_{Ctrl}-C and different Fe-N_{CA}-C) was used as the working electrode, the Ag/AgCl (saturated KCl) electrode and platinum wire were used as reference and counter electrodes, respectively. Electrode potentials measured in acid media were converted to the RHE scale using the following relationship: $E(\text{RHE}) = E(\text{Ag}/\text{AgCl}) + (0.197 + 0.0592 \times \text{pH}) \text{ V}$. The freshly prepared aqueous solution of HClO₄ (0.1 M) was used as

electrolyte. The electrocatalyst ink (2 mg mL^{-1}) was prepared by ultrasonic treatment of the mixture of the catalyst powder (2 mg) and H_2O (1 mL) for 15 min. Then, 15 μL electrocatalyst ink was dropped onto the polished RRDE (dia. 4 mm), resulting in a catalyst loading of 0.239 mg cm^{-2} . After dried at $60 \text{ }^\circ\text{C}$ for 15 min, 5 μL Nafion (0.05 wt%) was further dropped onto the surface of the electrode and dried in the same condition. As a control experiment, commercial Pt/C working electrode was also prepared in the same way.

The linear sweep voltammetry (LSV) measurement of modified RRDE was performed in O_2 - and N_2 -saturated 0.1 M HClO_4 , respectively, with the scan rate of 10 mV s^{-1} and the ring potential of 1.21 V (vs. RHE). The four-electron selectivity of catalysts was evaluated based on the H_2O_2 yield, calculated from the following equation.

$$H_2O_2(\%) = \frac{200 \times I_R / N}{I_D + I_R / N} \quad (4)$$

$$n = \frac{4 \times I_D}{I_D + I_R / N} \quad (5)$$

where I_D and I_R are the disk and ring current, respectively, and N is the ring collection efficiency. The N was determined to be 0.42 in a solution of 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ + 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$.

The potentiostatic electrochemical impedance spectroscopy (EIS) was collected in 0.1 M KCl containing 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ + 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$.

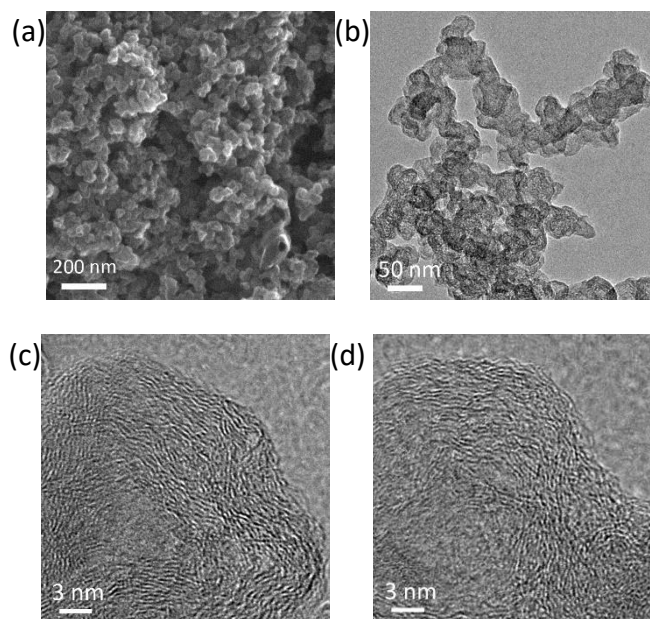


Figure S1. SEM (a), TEM (b) and HR-TEM image (c) of the Fe-N_{ctrl}-C. (d) HR-TEM image of the Fe-N_{CA1}-C.

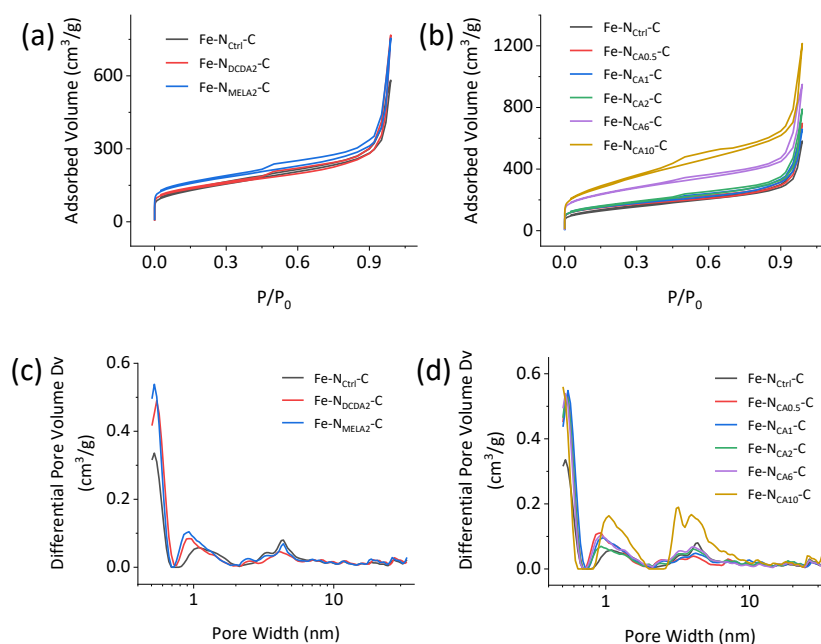


Figure S2. N₂ adsorption and desorption isotherms (a, b) and pore size distribution (imitated using QSDFT; c and d) of all the Fe-N-C.

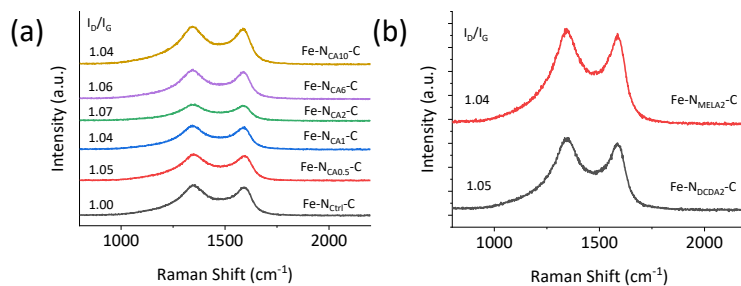


Figure S3. Raman spectra of the Fe-N_{Ctrl}-C (a), all the Fe-N_{CA}-C (a), the Fe-N_{DCDA2}-C (b), and the Fe-N_{MELA2}-C (b).

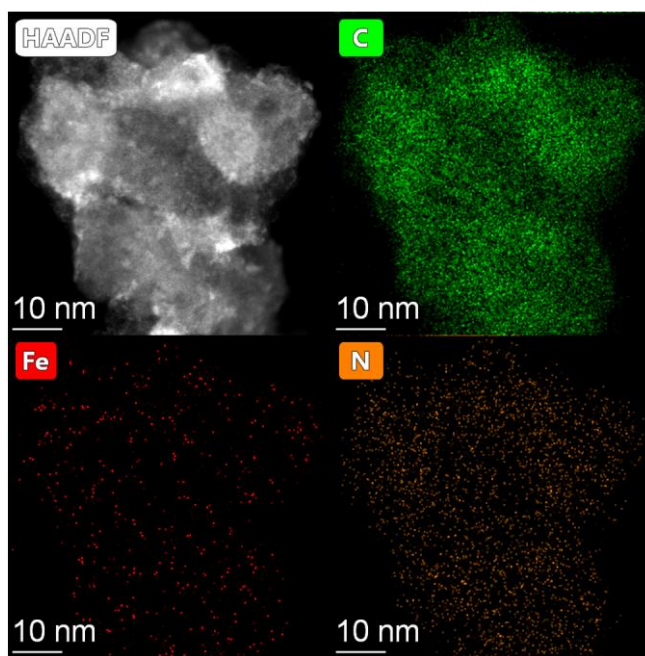


Figure S4. EDS mapping images of the Fe-N_{CA1}-C.

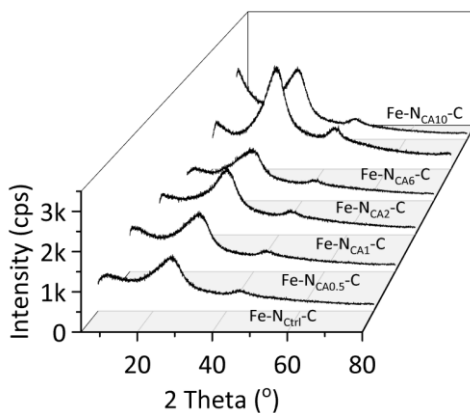


Figure S5. XRD spectra of all Fe-N-C samples.

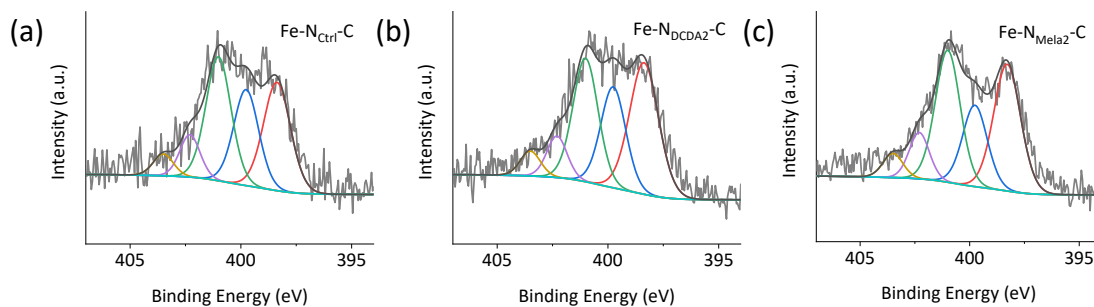


Figure S6. Deconvoluted high-resolution N 1s spectra of the Fe-N_{Ctrl}-C, Fe-N_{DCDA2}-C and Fe-N_{MELA3}-C.

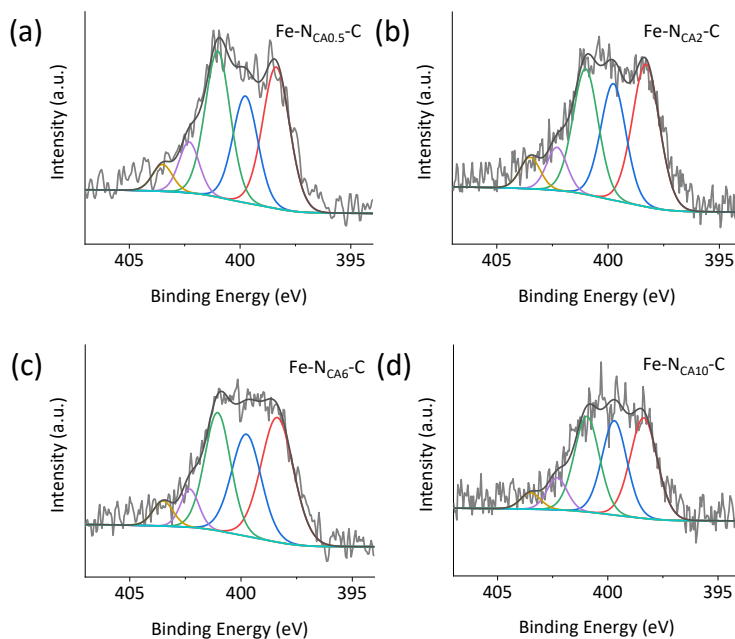


Figure S7. Deconvoluted high-resolution N 1s spectra of the Fe-N_{CA}-C synthesized with altered CA concentration.

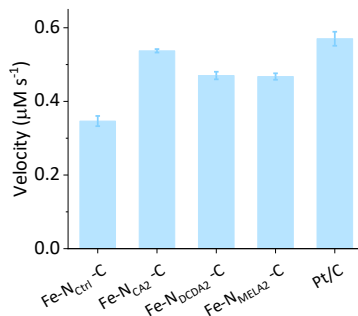


Figure S8. Initial oxidation velocity of 1 mM TMB catalyzed by 20 µg mL⁻¹ Fe-N_{Ctrl}-C, Fe-N_{CA2}-C, Fe-N_{DCDA2}-C, Fe-N_{MELA2}-C and Pt/C nanozyme in air-saturated 0.1 M HAc-NaAc (pH 3.6), respectively.

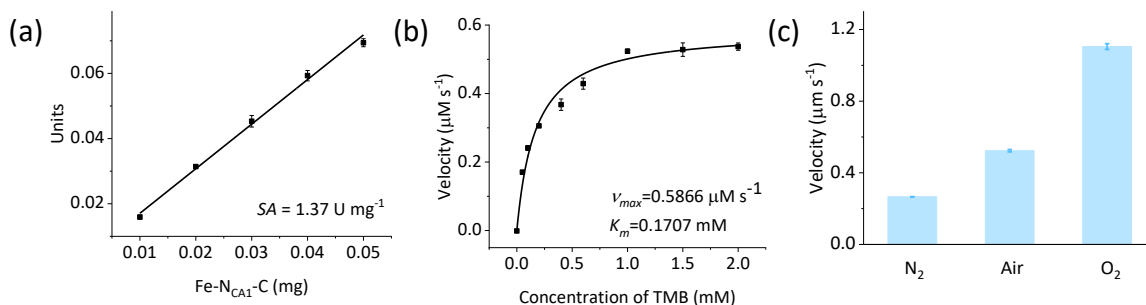


Figure S9. (a) Specific activity of Fe-N_{CA1}-C in air-saturated 0.1 M HAC–NaAc (pH 3.6). (b) Typical Michaelis-Menten curves of 20 μg mL⁻¹ Fe-N_{CA1}-C in air-saturated 0.1 M HAC–NaAc (pH 3.6). (c) Initial oxidation velocity of 1 mM TMB catalyzed by 20 μg mL⁻¹ Fe-N_{CA}-C nanozyme in N₂, air and O₂-saturated 0.1 M HAC–NaAc (pH 3.6), respectively.

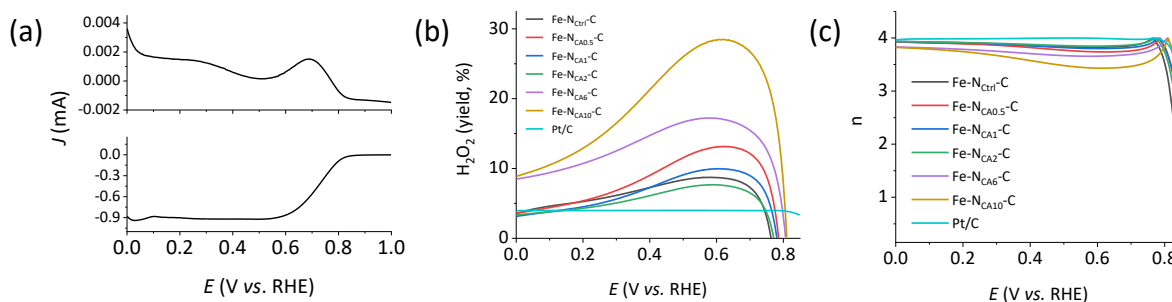


Figure S10. (a) Linear sweep O₂ activation voltammograms (bottom) and the corresponding platinum ring current (top) of Pt/C in O₂-saturated 0.1 M HClO₄ electrolyte. Sweep rate: 10 mV/s, rotation rate: 1600 rpm. Yield of H₂O₂ (b) and the electron transfer number (n, c) of the Fe-N_{Ctrl}-C, all the Fe-N_{CA}-C samples, and Pt/C catalyzed ORR in 0.1 M HClO₄.

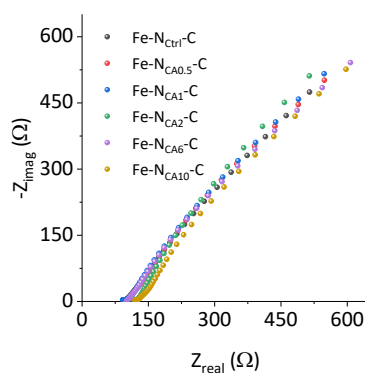


Figure S11. Nyquist plots of the Fe-N_{Ctrl}-C and all the Fe-N_{CA}-C.

Table S1. The physical properties of cyanamide (CA), dicyandiamide (DCDA), and melamine (MELA).

| Sample | Mol. Form. ^a | Mol. Wt. ^b | N/C ^c (wt %) | M. P. ^d (°C) | B. P. ^e (°C) |
|--------|--|-----------------------|-------------------------|-------------------------|-------------------------|
| CA | CN ₂ H ₂ | 42.05 | 66.6 | 45.56 | 140 |
| DCDA | C ₂ N ₄ H ₄ | 84.09 | 66.6 | 211 | 252 |
| MELA | C ₃ N ₆ H ₆ | 126.14 | 66.6 | 345 | Sub |

^aMolecular formula. ^bMolecular weight. ^cN/C weight ratio. ^dMelting point. ^eBoiling point. M. P. and B. P. of CA and MELA, and the M. P. of DCDA was obtained from *CRC Handbook of Chemistry* (<https://hbcpc.chemnetbase.com/faces/contents/ContentsSearch.xhtml>). A simply entry “sub” indicates the solid has a significant sublimation pressure at ambient temperatures. The B. P. of DCDA was obtained from *National Center for Biotechnology Information* (<https://www.ncbi.nlm.nih.gov/>).

Table S2. Specific surface area, weight percentage of iron, atom percentage of nitrogen and pyrolysis yield of different Fe-N-C.

| Sample | SSA ^a (m ² g ⁻¹) | Fe ^b (wt %) | N ^c (atom %) | Yield ^d (%) |
|--------------------------|--|------------------------|-------------------------|------------------------|
| Fe-N _{Ctrl} -C | 489.7 | 1.05 | 3.88 | 28.05 |
| Fe-N _{CA0.5} -C | 561.0 | 1.16 | 4.07 | 23.81 |
| Fe-N _{CA1} -C | 571.6 | 1.42 | 5.17 | 21.95 |
| Fe-N _{CA2} -C | 586.0 | 1.46 | 5.18 | 18.67 |
| Fe-N _{CA6} -C | 884.2 | 0.80 | 4.73 | 11.65 |
| Fe-N _{CA10} -C | 1097.5 | 0.39 | 3.88 | 8.44 |
| Fe-N _{DCDA2} -C | 505.8 | 1.20 | 5.00 | 23.10 |
| Fe-N _{MELA2} -C | 590.0 | 1.24 | 6.77 | 24.98 |

^aThe specific surface area calculated by the Brunauer-Emmett-Teller method. ^bThe iron content determined by inductively coupled plasma mass spectrometry (ICP-MS). ^cThe atom percentage of N obtained from the X-ray photoelectron spectroscopy (XPS). ^dThe pyrolysis yield of Fe-N-C.