Fe-N-C Artificial Enzyme: Activation of Oxygen for Dehydrogenation and Monoxygenation of Organic Substrates under Mild Condition and Cancer Therapeutic Application

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Developing highly efficient artificial enzymes that directly employ O₂ as terminal oxidant has long been pursued but has rarely achieved yet. We report Fe-N-C has unusual enzyme-like activity in both dehydrogenation and monoxygenation of organic substrates with ~100% selectivity by direct using O₂.

The natural enzymes catalyzed oxidation of organic substrates with O₂ is crucial for the metabolism of living organisms.¹⁻² A remarkable example of aerobic oxygenation is mediated by the cytochrome P450, which could reductively activate O₂ to dehydrogenate and monoxygenate a variety of exogenous and endogenous substrates under very mild conditions.³ However, problems such as sensitivity of catalytic activity to environmental conditions, and the high costs largely limit applications of natural enzymes. A solution to this problem may be offered by constructing artificial structures as natural enzyme mimics. Recently a great deal of landmark works for biomimetic homogeneous catalysts such as metalloporphyrins,⁴⁻⁶ metal complexes,⁷⁻⁹ and heterogeneous “artificial enzyme” such as Au and Fe₃O₄ nanoparticles¹⁰⁻¹⁷ and graphene oxide¹⁸ have been explored to mimic enzyme-like catalytic activity, but most of them cannot directly use O₂ and have to use organic/inorganic
peroxides\textsuperscript{19} such as H\textsubscript{2}O\textsubscript{2} as artificial oxygen donors.\textsuperscript{20} Moreover, natural enzymes and few existing biomimetic catalysts, which can directly use O\textsubscript{2}, are soluble in a homogeneous system, making them difficult to be separated and regenerated. Thus, exploring heterogeneous biomimetic catalysts with enzyme-like activity that could directly utilize O\textsubscript{2} under mild conditions is fascinating but still remains challenging.

Platinum group metal-free (PGM-free) metal-nitrogen-carbon catalysts (M-N-C) prepared by pyrolysis of M/N/C-containing precursors recently shows excellent electrocatalytic O\textsubscript{2} reduction activities at low overpotential.\textsuperscript{23-25} Pioneering works in microscopic and spectra investigation such as high-angle annular dark-field scanning transmission electron microscope (HAADF-STEM) and synchrotron extended X-ray absorption fine structure spectra (EXAFS) have comprehensively disclosed that the key structural motif of M-N-C responsible for the O\textsubscript{2} reduction. They generally contain N-coordinated metal (M-N\textsubscript{x}) embedded in basal planes of carbon or bridging two graphene planes at edges,\textsuperscript{23-24, 26} very similar to the active sites of some natural enzymes such as cytochrome P450. These facts hint the great potential of M-N-C in O\textsubscript{2} activation and their potential as natural enzyme-like biomimetic catalysts.
Herein, we demonstrate that the Fe-N-C materials could act as a heterogeneous enzyme-like biomimetic catalyst, which dehydrogenate and monoxygenate a number of substrates of high selectivity by the direct reductive activation of O$_2$ at Fe-N$_x$ active sites in aqueous solutions at room temperature. Moreover, the Fe-N-C artificial enzyme had excellent stability, ease of separation and recyclability, and uncompromisingly high catalytic activities in organic media, high temperature, extreme pH, and even physiological conditions.

Figure 1a showed the general way to prepare Fe-N-C. Briefly, the as prepared Fe-containing ionic liquid precursor was pyrolyzed in N$_2$ and the product was leached in HCl to remove the inactive iron particles and other iron species from agglomeration during pyrolysis if any. The microstructure of Fe-N-C, evaluated by scanning electron microscope (SEM, Figure 1b) and bright-field scanning
transmission electron microscope (BF-STEM, Figure 1c), was sheet-like. The high-angle annular dark-field scanning TEM (HAADF-STEM) images and EDS mapping (Figure 1d) demonstrated a uniform distribution of Fe, N and C in Fe-N-C. X-ray diffraction (XRD, Figure S1) showed the as-prepared Fe-N-C comprised typical graphitic carbon. Of note, few Fe-related clusters or nanoparticles were observed in the above high resolution microscopic and diffraction characterization, most presumably suggesting a uniform atomic distribution of Fe in Fe-N-C.

To understand the detailed chemical/electronic structures of Fe and N in the surface of Fe-N-C artificial enzyme, X-ray photoelectron spectroscopy (XPS) was further performed. The Fe2p XPS spectrum (Figure 1e) showed five peaks, corresponding to Fe$^{2+}$ 2p$_{3/2}$ (710.8 eV), Fe$^{3+}$ 2p$_{3/2}$ (714.3 eV), Fe$^{2+}$ 2p$_{1/2}$ (723.3 eV), Fe$^{3+}$ 2p$_{1/2}$ (727.9 eV) and the satellite peak (719.8 eV), agreeing with the reported result. The Fe content in Fe-N-C was further quantified to be 0.71 wt.% by inductively coupled plasma mass spectrometry (ICP-MS). The N1s XPS spectrum (Figure 1f) could be deconvoluted into pyridinic N (398.4 eV), N$_x$-Fe (399.7 eV), pyrrolic N (400.2 eV), graphitic N (401.2 eV), and oxidized N (403.0 eV) (Figure 1g and Table S1). Therefore, the as-prepared Fe-N-C consisted of Fe-/N-dopants, and graphitic carbon, and Fe was most presumably dispersed in the form of single atom by complexing with aromatic N.

To evaluate the Fe-N-C catalytic activity in dehydrogenation, Hantzsch 1,4-dihydropyridines (1,4-DHP) was used as model molecule, which was an important class of drug for cardiovascular diseases and could be dehydrogenized into diethyl 2,6-dimethyl-3,5-pyridine-dicarboxylate (DDPD) (Figure 2a) by natural enzymes. The catalytic reactions were carried out in air-saturated phosphate buffer solution (pH=7.4) at room temperature. As a control, the catalytic activity of metalloporphyrins, such as 5,10,15,20-tetraphenyl-21H,23H-porphine iron(III) chloride (FeTPPCl), and typical artificial enzymes, such as nano-Fe$_3$O$_4$ were also evaluated (Figure 2b). The O$_2$-dependent oxidative dehydrogenation of 1, 4-DHP catalyzed by Fe-N-C was
firstly evaluated by UV-vis absorption spectroscopy. A new absorption peak at 272 nm, typically ascribing to pyridine-based derives DDPD, was observed (Figure S2) for the product, an evident proof of the occurrence of the catalytic oxidative dehydrogenation reaction. High-performance liquid chromatography (HPLC) gave further evidences of the pyridine-based product by referring to a standard (Figure S3). Moreover, by using a dissolved oxygen electrode, the consumption of O₂ during catalysis was verified (Figure S4). These results collectively verified that Fe-N-C could dehydrogenate substrates by using O₂ under mild conditions.
Figure 2 (a) Chemical reaction of dehydrogenation of 1, 4-DHP by P450 or Fe-N-C to the corresponding DDPD product. (b) Lineweaver-Burk plot of the 1, 4-DHP dehydrogenation catalyzed by Fe-N-C and controls. (c) Arrhenius plot for the dependence of the rate constant (K) for dehydrogenation of 1, 4-DHP by Fe-N-C on temperature (T). Relative dehydrogenation activity of Fe-N-C in different temperature (d), pH (e) and solvents (f). The recovery and recyclability of Fe-N-C (g). The room temperature (RT) was around 20 °C.

To get more kinetic insights, the catalytic activity of Fe-N-C artificial enzyme for dehydrogenation by directly using O₂ was studied by enzyme kinetics theory
and methods. The typical kinetic parameters such as the catalytic constant ($K_{\text{cat}}$), Michaelis-Menten constant ($K_m$), Maximal velocity ($V_{\text{max}}$) and the catalytic efficiency ($K_{\text{cat}}/K_m$) are listed in Table S2. It was found the $K_m$ and $K_{\text{cat}}/K_m$ of Fe-N-C, were comparable to that of some cytochrome P450 enzymes for dehydrogenation of N-heterocycles (Table S3). The activation energy ($E_a$) of Fe-N-C was further calculated to be 45.4 kJ/mol (Figure 2c), comparable to that of some enzymes ranging from 30 to 80 kJ/mol for hydrogen abstraction, verifying Fe-N-C could be developed as a biomimetic dehydrogenation catalyst directly using O$_2$ under mild conditions. As a control, the dehydrogenation activity of FeTPP(Cl and nano-Fe$_3$O$_4$ were also evaluated (Figure 2b). In contrast, although both FeTPP(Cl and nano-Fe$_3$O$_4$ could dehydrogenate substrate by employing H$_2$O$_2$ as an oxygen donor (Figure S5), they could not directly utilize O$_2$ to dehydrogenate 1, 4-DHP (Figure 2b, S5 and Table S2).

Another two Fe-N-C artificial enzymes containing similar Fe-N$_x$ center, i.e. Fe-N-C (Ben) and Fe-N-C (Ppy) were also prepared by using other Fe-containing precursors (See Experimental section in SI). Interestingly, both of them demonstrated the apparent dehydrogenation activity (Figure 2b and Table S2), indicating the general catalytic character of Fe-N-C in direct utilizing O$_2$ to dehydrogenate substrate under mild condition. Moreover, the modulated kinetics (Table S2) suggested the dehydrogenation activity could be further boosted in principle by optimizing the electronic structure of Fe-N$_x$ via engineering the “solid ligand” (N-doped carbon).

Most natural enzymes would be denaturized and become less active in organic media, high temperature and extreme pH. As advantages, Fe-N-C exhibited an enhanced catalytic activity in elevated temperature up to 70 °C (Figure 2d) and no deactivation at extreme pH and different polar organic media (e.g. n-Hexene, CH$_2$Cl$_2$ and CH$_3$CN) (Figure 2e and 2f), indicating its higher catalytic stability, which was essentially ascribed to the robust structure of Fe-N-C. Moreover, most natural enzymes were difficult to separate and regenerate as well as other homogeneous artificial enzymes that have been developed
recently, such as Mn complex and Cu/TEMPO. The separation and recyclability of Fe-N-C artificial enzyme was thus further examined by a simple centrifugation and washing with CH$_2$Cl$_2$ and diethyl ether to remove the product. As shown in Figure 2g, Fe-N-C exhibited negligible loss of activity after five cycles, indicating the excellent stability and recyclability. In these regards, Fe-N-C was superior to natural enzymes that only work in mild conditions and not easy to be separated, many heterogeneous artificial enzymes that cannot directly use O$_2$, and homogeneous artificial enzymes that are hard to be separated and recycled in biomimetic catalysis (Table S4).

**Table 1** Fe-N-C catalyzed dehydrogenation and monoxygenation of different substrates by direct using oxygen in aqueous solution.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Selectivity (%)</th>
<th>Yield (%)</th>
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The selective oxidation of N, P-containing compounds is one of the most important organic transformations for construction of key intermediates in the fine chemical industry. Except for 1, 4-DHP, the Fe-N-C artificial enzyme was also applicable for selective dehydrogenization of a broad scope of N, P-containing substrates by direct using O$_2$ under room temperature in aqueous solutions (Table S5 and Figure S6, S7, S8 and S9). For example, Fe-N-C could dehydrogenize diphenylhydrazine into azobenzene in 75.3% yield. Moreover, Fe-N-C can be extended to monoxygenate triphenylphosphine into triphenylphosphine oxide in 21.6% yield (Table S5). The selectivity for all of the corresponding products was nearly 100%. These results indicated the potential
of Fe-N-C artificial enzyme as low-cost and sustainable heterogeneous catalyst for the selective oxidation in industrial processes.

The intermediate species of O₂ activation (¹O₂, O₂•− and H₂O₂, instead of strongly aggressive OH•− that often leads to non-selective oxidation) during Fe-N-C catalyzed oxidation were then further monitored and verified by trapping methods (Figure S10a and S10b). A kinetic isotope effect was observed obviously by increasing the content of deuterons (Figure S10c), suggesting that protons were transferred at the rate-determining step. Moreover, based on SCN⁻ poisoning experiment, it was confirmed that the active site of O₂ activation catalyzed by Fe-N-C should contain Fe (Figure S10d). In these regards, these specific intermediate reactive oxygen species (ROS) at Fe-N₄ center (Figure S10d) were similar to that of the some natural enzymes, providing a platform to mimic enzymes for metabolizing exogenous or endogenous substrates in vitro. However, the Fe-N-C catalyzed O₂ activation with formation of ¹O₂, O₂•− and H₂O₂ was rarely reported by other artificial enzyme mimics, because most of them cannot directly use O₂ and have to use artificial oxygen donors such H₂O₂. It was noted that activation of O₂ at Fe-N site was supposed to be attributed to strong interactions between catalytic site and N-doped carbon support, which changed the electronic structure of Fe-N site. During O₂ activation, the Fe-N center probably bound and activated ³O₂ to form ¹O₂, and then was converted to HO₂•− by obtaining proton and electron from substrates. Subsequently, the generated HO₂•− picked up electron to produce HO₂⁻, which further performed dehydrogenation or monooxygenation of organic substrates. In this regard, except for biomimetic catalytic reaction for molecules conversion, Fe-N-C is also promising for applications that require specific ROS. Cancer cells were vulnerable to damage by an excessive level of ROS that is incompatible with cellular survival. Thus using Fe-N-C as an exogenous ROS-modulating material is likely to increase ROS generation and cause elevation of ROS above a cellular tolerability threshold, leading to cancer cell death. For this, we further investigated the capability of Fe-N-C artificial enzyme in reducing the viability of lung cancer cells.
Figure 3. (a) Fluorescence microscopic images of lung cancer cells treated with and without CB and Fe-N-C for 24 h. (b) MTT assay of lung cell viability incubated with and without CB and Fe-N-C. (c) Proliferation rate of CB and Fe-N-C incubated lung cancer cells determined by cell counting.

Fluorescence microscopic images in Figure 3a disclosed Fe-N-C artificial enzyme could significantly decrease the number of proliferative lung cancer cells compared with that of control carbon black (CB) sample. Moreover, based on the statistical result of cell count (Figure 3b), it was observed that the proliferative lung cancer cells was killed by 88% after adding Fe-N-C, which was remarkably superior to CB. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) arrays result (Figure 3c) further indicated the lung cancer cell survival rate was reduced by approximately 70% under 24 h normal oxygen conditions after subjecting the cells to Fe-N-C, agreeing with the statistical result of cell counting. In contrast, the CB only reduced the 22% lung cancer cells (Figure 4c). These results clearly implied the potential of Fe-N-C for cancer therapy via ROS production. It is noted that photodynamic therapy (PDT) is
widely utilized clinically to treat tumors by using ROS, which is triggered from photosensitizer and oxygen under light illumination. However, the primary downsides of PDT are low light utilization efficiency and its inability to treat tumors located deep under the skin due to absorption, scattering and the short penetration depth of light in tissues. Compared to PDT, Fe-N-C could activate the dioxygen and generate ROS without any light illumination. Thus, Fe-N-C artificial enzyme might be developed as an effective ROS-modulating material and constitute a potential therapeutic strategy in a smart system for cancer treatment, even for deeper layers of tumor tissues when recognition unit and reversible on/off switch for regulating ROS release was constructed.

In summary, we reported the general catalytic character of Fe-N-C artificial enzyme in direct utilizing O2 to oxidize substrates of nearly 100% selectivity under mild condition. The corresponding catalytic activity of Fe-N-C could be tuned by modulating the type of precursors for pyrolysis. Fe-N-C had several striking superior features with respect to classical heterogeneous artificial enzymes, and homogeneous artificial enzymes in durability of working in harsh conditions (extreme pH and high temperature), ease of separation and recycling, and direct use of O2 without sacrificial oxygen donors (e.g. H2O2). This work demonstrated the enzyme-like activity of Fe-N-C could be used in a wide range of new potential applications in biomimetic catalysis, industrial oxidation processes and biomedicine. It should be aware that the yield of catalytic hydrogenation and monooxygenation was not very high. Work focused on the structural modulation of Fe-N-C for higher activity and more challenged reactions is ongoing.

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References


