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The Depletable Peroxidase-like Activity of Fe₃O₄ Nanozymes Accompanied with Phase Transformation Triggered by Separate Migration of Electrons and Iron Ions

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Abstract

As the pioneering Fe_3O_4 nanozymes, their explicit peroxidase (POD)-like catalytic mechanism remains elusive. Although many studies have proposed the surface Fe^{2+} -induced Fenton-like reactions accounting for their POD-like activity, few focus on the internal atomic changes and their contribution to the catalytic reaction. Here we report that Fe^{2+} within Fe_3O_4 transfers electrons to the surface via the $\text{Fe}^{2+}\text{-O-Fe}^{3+}$ chain, regenerating the surface Fe^{2+} and enabling a sustained POD-like catalytic reaction. This process occurs with the outward migration of excess oxidized Fe^{3+} from the lattice, which is a rate-limiting step. After prolonged catalysis, Fe_3O_4 nanozymes suffer the phase transformation to $\gamma\text{-Fe}_2\text{O}_3$ with a depletable POD-like activity. This self-depleting characteristic of nanozymes with internal atoms involved in electrons transfer and ion migration is well-validated on lithium iron phosphate nanoparticles. We reveal a key yet ever ignored issue concerning the necessity of considering both surface and internal atoms when designing, modulating, and applying nanozymes.

Introduction

Since the first discovery of Fe₃O₄ nanoparticles (NPs) with intrinsic peroxidase (POD)-like activity in 2007, nanomaterial-based artificial enzymes (nanozymes) and their extensive applications have rapidly attracted attention over the past decade.¹⁻¹⁰ Recently, the research efforts on nanozymes have gradually shifted from application-oriented to mechanism-oriented, as a well-defined mechanism is the key to design, modulate, and apply the catalytic properties of nanozymes.¹¹⁻¹⁵ Given the intricate structure-activity relationships and restricted characterization techniques, however, fewer breakthroughs have been made in understanding the explicit mechanism of most nanozymes.^{3,4}

Despite being a pioneering nanozyme, research on the catalytic mechanism of the POD-like activity of Fe₃O₄ NPs is still limited.^{10,14-19} To date, it is generally accepted that high-reactive hydroxyl radicals ($\cdot\text{OH}$) generated by Fenton-like reactions (Equation 1-2) involving the surface Fe²⁺ under acid conditions contributes to the POD-like activity of Fe₃O₄ NPs.^{18,19} Similar to natural horseradish peroxidase (HRP), Fe₃O₄ nanozymes follow the ping-pong mechanism and Michaelis-Menten kinetics.¹⁰ Besides, their catalytic performances are influenced by particles size, morphology, lattice structure, doping, surface modification, substrates used, as well as the catalytic environment exposed, all of which could affect the surface active sites by altering the surface chemistry.^{10,16,17,20-23} Other individual studies have investigated the absorption, activation, and desorption processes of substrates (e.g. H₂O₂ and TMB) on the surface of Fe₃O₄ at the atomic level based on density functional theory and developed some descriptors to predict their POD-like activity.^{14,15}

The above mechanistic studies share a theoretical premise: only the surface-active sites play a decisive role in the enzymatic-like property of nanozymes since catalysis occurs mainly on the particle surface or interface. This view is now widely recognized and works for most types of nanozymes.^{1,2,4,11,23,24} For example, in a recent controversial question regarding how to define “nanozyme concentration”, Liu et al. argued that considering the whole particle or all atomic units within a particle as an enzyme unit would overestimate and underestimate the catalytic activity of nanozymes, respectively, because it is the surface atoms that are really the catalytic active sites.²⁴ However, in the Fenton-like reactions triggered by Fe₃O₄ nanozymes, we noticed that the reaction rate constant of Equation (1) is much higher than that of Equation (2), which implies that the surface-active Fe²⁺ is hardly recovered after being oxidized. This irreversible oxidation of surface Fe²⁺ prompts us to ponder if only the surface atoms of the nanozymes, particularly for metal oxide nanozymes, act in enzymatic-like catalysis, would these active sites be exhausted after long-term catalysis, rendering the nanozymes inactive? So far, nevertheless, no relevant studies can conclusively answer this crucial question.

Here we propose a key yet ever ignored issue regarding the POD-like mechanism of nanozymes by characterizing the chemical composition and catalytic activity of the recycled Fe₃O₄ NPs participating in cyclic POD-like catalysis. Both surface and interior Fe²⁺ were found to impart POD-like property to Fe₃O₄ nanozymes. Namely, Fe²⁺ inside the particle transfers its electron to the surface layer, regenerating the surface Fe²⁺ and sustaining the catalytic reaction. This process is coupled with the outward migration of excess oxidized Fe³⁺,

which is a rate-limiting step. As the catalysis continues, Fe_3O_4 is slowly oxidized into $\gamma\text{-Fe}_2\text{O}_3$ accompanying the depleted enzyme-like activity, similar to the conventional low-temperature oxidation of magnetite, only with different electron receptors. This self-depleting characteristic of nanozymes with internal atoms involved in electrons transfer and ion migration is further demonstrated by a typical model material, LiFePO_4 , which contains the redox-active metal sites and mobile lithium ions (Li^+) encapsulated in a rigid phosphate network. This paper reveals that internal atoms may also contribute to the nanozyme-catalyzed reactions even though these reactions occur on the surface of NPs, which is thought-provoking when designing, regulating, and applying nanozymes.

Results

Synthesis and Characterization of IONPs. Near-spherical magnetite nanoparticles (Fe_3O_4 NPs) with an average diameter of 10.16 ± 0.12 nm (Supplementary Fig. 1a) were synthesized using the chemical co-precipitation method.¹⁸ Maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and hematite ($\alpha\text{-Fe}_2\text{O}_3$) NPs were derived by calcining the Fe_3O_4 NPs powder at 200 °C and 650 °C for 2 hours, respectively (Fig. 1a). XRD and Raman spectra (Supplementary Fig. 1b-c) show the successful synthesis of these three iron oxide NPs (IONPs). These IONPs were uniformly dispersed in an aqueous solution at pH of 3 by ultrasonication (Supplementary Fig. 1d). To avoid affecting the enzymatic-like activity, all particles were free of the surface coating. Their POD-like activities were assessed using different colorimetric substrates, including TMB, ABTS, and OPD, in the presence of H_2O_2 . The results show that their catalytic activity followed the order of Fe_3O_4 NPs $\gg \gamma\text{-Fe}_2\text{O}_3$ NPs $> \alpha\text{-Fe}_2\text{O}_3$ NPs (Supplementary Fig. 2). To

better quantify their POD-like activity, we calculated their specific activity (a_{nano}) according to the specified method,^{26,27} which were 1.79, 0.44, and 0.03 U·mg⁻¹, respectively (Fig. 1b). As previously reported,^{10,18} the higher catalytic ability of Fe₃O₄ NPs originates from the Fenton-like reaction triggered by the surface Fe²⁺ (Supplementary Fig. 3). The negligible a_{nano} of α -Fe₂O₃ NPs compared with γ -Fe₂O₃ NPs is ascribed to the change of the inverse spinel structure due to the higher calcination temperature.

Fig. 1 The synthesis of IONPs and cyclic POD-like catalysis. (a) Illustration of the synthesis process of IONPs. (b) The specific activity (a_{nano}) of these three IONPs with TMB as colorimetric substrates. (c) Diagram of the cyclic catalysis assay. (d) Kinetic study of a_{nano} values of Fe₃O₄ NPs with the days of cyclic catalytic reaction. (e) The fitted Fe2p XPS spectra and (f) Raman spectra of Fe₃O₄ NPs recycled after catalysis on days 0, 1, 3, and 5. (g) The Fe L-edge

NEXAFS spectra of Fe₃O₄ NPs and recycled Fe₃O₄ NPs after 5 days of catalysis. Dotted lines represent the reference spectra of FeSO₄ and Fe₂O₃ powder. (h) TEM and HRTEM images of Fe₃O₄ NPs and recycled Fe₃O₄ NPs after 5 days of catalysis. Inset: the corresponding SAED pattern.

Cyclic POD-like catalysis of Fe₃O₄ NPs. To investigate whether the surface Fe²⁺ of Fe₃O₄ NPs is depleted after participating in prolonged catalysis, we continuously increased the amount of substrate TMB under sufficient H₂O₂ with as-synthesized three IONPs as "continuous catalysts", and monitored the absorbance changes of TMB oxidation products at 650 nm within 12 h. From Supplementary Fig. 4, even though the TMB was increased from 0.087 mM to 0.52 mM, the Fe₃O₄ NPs were still able to continuously and rapidly engage in the catalytic reaction for a long duration (≥ 12 h) without showing signs of depletion. We speculated two reasons: 1) the amount of substrate is still too low to completely consume the surface-active Fe²⁺; 2) The Fe²⁺ within Fe₃O₄ NPs provides the impetus for the continuous catalysis.

Cyclic POD-like catalytic assays (Fig. 1c) were carried out as validation, which could provide sufficient substrates for Fe₃O₄ NPs to keep exerting their POD-like capacity. We evaluated the a_{nano} of the recycled Fe₃O₄ NPs within five days. The results show that the catalytic ability of Fe₃O₄ NPs decreased to a level comparable to that of γ -Fe₂O₃ NPs after five days of cyclic catalysis, while the changes of γ -Fe₂O₃ NPs were negligible (Fig. 1d and Supplementary Fig. 5). It pushed us to wonder how the surface-active Fe²⁺ of Fe₃O₄ NPs alone could sustain the TMB oxidation up to 100 hours? Conceivably, if only the surface-active sites are responsible for the enzyme-like performance, nanozymes will

deactivate when the surface-active sites are exhausted.

To reveal the potential reasons for the sustained catalytic capacity of Fe_3O_4 NPs, we characterized the physicochemical properties of the recycled Fe_3O_4 NPs using different methodologies. The chemical states of Fe atoms in Fe_3O_4 NPs recycled from catalysis at days 0, 1, 3, and 5 were first analyzed by XPS technology. The X-ray penetration depth of the analyzed sample ranges from 2 to 10 nm. Since the average diameter of as-synthesized Fe_3O_4 NPs is around 10 nm, the Fe valence state obtained from the Fe2p fitting analysis can be approximated as the oxidation state of individual Fe_3O_4 NPs. As shown in Fig. 1e, the Fe^{2+} in Fe_3O_4 NPs decreased from the original 30.9% to 0% with the increase of cyclic catalytic days, indicating that the interior Fe^{2+} was also oxidized to Fe^{3+} in the successive POD-like reactions. Furthermore, in the Raman spectra of the recycled Fe_3O_4 NPs, the feature of the A_{1g} mode band shifted from 660 cm^{-1} to 700 cm^{-1} , corresponding to a transition from magnetite to maghemite (Fig. 1f).^{28,29} Besides, this phase transformation was also confirmed by the NEXAFS spectroscopy. Figure 1g shows the Fe L-edge NEXAFS spectra of the control Fe_3O_4 NPs and the recycled Fe_3O_4 NPs after 5 days of catalysis, in comparison with two reference spectra of FeSO_4 and Fe_2O_3 . The increased splitting L_3 peak near 708 eV for the recycled Fe_3O_4 NPs coincides with reference Fe_2O_3 .³⁰⁻³² Additionally, TEM images (Fig. 1h) and XRD pattern (Supplementary Fig. 6) show that the influence of this transformation on the particle morphology, size, and lattice structure is negligible. Based on these characterization results, we conclude that both surface and internal Fe^{2+} can be oxidized into Fe^{3+} accompanied by a gradual phase transformation to $\gamma\text{-Fe}_2\text{O}_3$ during Fe_3O_4 nanozymes exerting their POD-like activity.

Aeration oxidation kinetics of Fe₃O₄ NPs. We assume that the oxidation of Fe₃O₄ nanozymes induced by POD-like catalysis is comparable to the traditional low-temperature (< 200 °C) air oxidation of magnetite since the crystal structure of both remains unchanged during the oxidation process.³³ Both magnetite and maghemite contain 32 O atoms per unit cell. The difference is that the former contains 24 Fe atoms (16 Fe³⁺ and 8 Fe²⁺), while the latter has only 21.33 Fe atoms (all Fe³⁺). Namely, once 8 Fe²⁺ in magnetite are oxidized to 8 Fe³⁺ releasing 8 electrons, a charge imbalance will occur (Equation 3). To maintain electroneutrality, 2.67 Fe³⁺ have to migrate to the crystal surface, leaving the cation vacancy (Equation 4).³⁴ The outward moving Fe³⁺ will coordinate with the surface absorbed O²⁻ which is ionized by the electrons generated by the oxidation of Fe²⁺. Therefore, the phase transformation of Fe₃O₄ to γ-Fe₂O₃ is a single-phase topological reaction accompanied with the separate migration of electrons and excess Fe³⁺.³⁴

Lattice defects have been reported to facilitate the outward migration of excess iron ions, thereby accelerating the oxidation process of magnetite.³⁵ As verification, we compared the aeration oxidation kinetics of Fe₃O₄ NPs synthesized by two methods with different levels of lattice defects. One was prepared by the chemical co-precipitation method as described above (Fig. 1a), which is considered to possess more lattice defects (named cc-Fe₃O₄ NPs). The other was prepared by the thermal decomposition method (Supplementary Fig. 7) with a relatively complete lattice structure (named TD-Fe₃O₄ NPs).³⁶ Both Fe₃O₄ NPs have a similar

average particle size (~ 10 nm) without surface coating. Their aqueous solutions were stirred under the same aeration rate (with air) for 12 h at 120 °C. For a better comparison, the oxidation system of cc-Fe₃O₄ NPs (total 170 mL, 3.6 mg Fe/mL) was much larger than that of TD-Fe₃O₄ NPs (total 30 mL, 0.45 mg Fe/mL). This implies that individual TD-Fe₃O₄ could gain more oxygen than cc-Fe₃O₄ to keep it oxidized. From Fig. 2a-b, both Fe₃O₄ NPs exhibited electronic transitions in the visible and NIR region due to intervalence charge transfer between Fe²⁺ and Fe³⁺,³⁷ which decreased gradually with oxidation time. At the end of aeration oxidation, little absorption beyond 700 nm was observed, indicating a phase transformation from Fe₃O₄ NPs to γ -Fe₂O₃ NPs.³⁷ Besides, the color of both suspensions gradually changed from dark-brown to reddish-brown. Notably, despite the less oxygen exposure for individual cc-Fe₃O₄ NP, its NIR absorption decreased faster than that of TD-Fe₃O₄ NP, especially during the initial oxidation phase (within three hours). These results confirm that more lattice defects favor the oxidation reaction of Fe₃O₄ NPs due to the faster electron and ion transfer.

Analogous to aerated oxidation, the rapid electron and ion migration also facilitates the POD-like catalysis of Fe₃O₄ NPs, with the only difference that the electron receptor changed from O₂ in aerated oxidation reaction to H₂O₂ in POD-like reaction. To prove this, the POD-like activity of cc-Fe₃O₄ NPs and TD-Fe₃O₄ NPs as well as their variation with aerated oxidation time were investigated. As seen in Supplementary Fig. 8, the POD-like activity of cc-Fe₃O₄ NPs was higher (2.8 folds) than that of TD-Fe₃O₄ NPs, despite TD-Fe₃O₄ NPs having a smaller hydrodynamic diameter and negative surface potential contributing to a strong affinity with TMB. Aeration oxidation kinetic studies show that the POD-like activity

of both Fe_3O_4 NPs decreased with oxidation time (Fig. 2c), along with slight fluctuations in hydrodynamic size and surface potential (Supplementary Fig. 9). However, the decline rate of cc- Fe_3O_4 NPs was faster than TD- Fe_3O_4 NPs, particularly in the initial oxidation stage. This phenomenon is consistent with the changes of NIR spectra shown in Fig. 2a-b. These results further confirm that the more lattice defects of Fe_3O_4 NPs, the easier the migration of excess Fe ions, and thus the higher the POD-like activity. It also means that Fe_3O_4 NPs with more defect sites are easier to be depleted when involved in a POD-like reaction due to their excellent catalytic capability.

Fig. 2 The aeration oxidation kinetics of Fe_3O_4 NPs. Variation of UV-vis-NIR absorption of (a) cc- Fe_3O_4 NPs and (b) TD- Fe_3O_4 NPs with aeration oxidation time. Insets are photos of the suspensions corresponding to oxidation times at 0, 0.5, 1, 3, 5, 8, 10, and 12 h. All spectra and photos were obtained at the same Fe concentration. (c) Changes in a_{nano} of the oxidized cc- Fe_3O_4 NPs and TD- Fe_3O_4 NPs during the aeration oxidation.

Catalytic mechanism of the POD-like for Fe_3O_4 NPs. Taken together, the catalytic mechanism of the POD-like activity for Fe_3O_4 NPs can be summarized as follows (Fig. 3): 1) *Fenton-like reaction on the surface*. Firstly, H_2O_2 adsorbed on the surface of particles accept electrons from the surface Fe^{2+} , and then dissociates into highly active $\cdot\text{OH}$ to oxidize the substrates. The surface Fe^{2+} is oxidized to Fe^{3+} ; 2) *Internal electrons transfer*. Then, the adjacent Fe^{2+} inside the surface transfers its electron to the surface Fe^{3+} via the $\text{Fe}^{2+}\text{-O-Fe}^{3+}$

chain, retrieving the surface Fe^{2+} and providing the dynamics for the sustained catalytic reaction; 3) *Excess Fe ions outward migration*. With the in-situ oxidation of internal Fe^{2+} , to maintain electroneutrality, the excess Fe^{3+} in the lattice migrate outward to the surface, leaving cation vacancies; 4) *Chemical composition change*. With the continuous POD-like catalytic reaction, Fe_3O_4 NPs are oxidized from the surface to the interior and finally transformed into $\gamma\text{-Fe}_2\text{O}_3$ NPs. This enzymatic-like reaction-triggered oxidation process of Fe_3O_4 NPs is analogous to the conventional low-temperature air oxidation of magnetite, in which iron ion migration is the rate-limiting step.

Fig. 3 Schematic diagram of the catalytic mechanism of the POD-like activity for Fe_3O_4 NPs.

LiFePO_4 NPs as an ideal verification model. To test the above mechanism, we found an ideal model material, LiFePO_4 , which is commonly applied as cathode material for lithium-ion batteries.³⁸⁻⁴¹ LiFePO_4 undergoes redox reactions along with the lithium insertion/extraction during the charge-discharge process (Equation 5-6) without changing its ordered-olivine structure (Fig. 4a).³⁸ We speculate the charging process of LiFePO_4 is

resembling the oxidation process of Fe₃O₄, both of which involve the oxidation of Fe²⁺ and the migration of internal ions, which motivated us to focus on whether LiFePO₄ NPs also have the POD-like catalytic ability.

Rod-like LiFePO₄ NPs with an average length of 321.9 nm and width of 172.2 nm (Fig. 4b) were successfully synthesized using the solvothermal method³⁸ and characterized via various methodologies (Supplementary Fig. 10 and Table S1-2). As expected, the POD-like activity of LiFePO₄ NPs was demonstrated with different chromogenic substrates including TMB, ABTS, and OPD (Fig. 4c and Supplementary Fig. 11). Also, they follow pH, temperature as well as NPs concentration dependence and the Michaelis-Menten kinetics (Supplementary Fig. 12-13). The optimal pH is about 4.0. The ESR spectra show that ·OH was produced from the decomposition of H₂O₂ catalyzed by LiFePO₄ NPs in a time-dependent manner (Fig. 4d), which is similar to Fe₃O₄ NPs. We then compared the POD-like activity of LiFePO₄ NPs and cc-Fe₃O₄ NPs using two oppositely charged substrates (TMB and ABTS) at pH 3.6. The results consistently show that LiFePO₄ NPs had higher catalytic ability than cc-Fe₃O₄ NPs (Supplementary Fig. 14), and the a_{nano} of LiFePO₄ NPs was approximately four times that of cc-Fe₃O₄ NPs, despite their larger particle size (Fig. 4e). These results imply that LiFePO₄ NPs may share a similar POD-like catalytic mechanism with Fe₃O₄ NPs, differing in that the rapid Li⁺ migration in the lattice of LiFePO₄ NPs confers them a superior POD-like catalytic activity (Fig. 4f).

Fig. 4 LiFePO₄ NPs as verification materials and their POD-like activity. (a) The crystal structure of LiFePO₄ and FePO₄ viewed along the *a*, *b*, *c*-axis. The olivine structure is maintained during Li-ions insertion and extraction. (b) The SEM image of as-synthesized LiFePO₄ NPs. Inset is a photo of LiFePO₄ NPs aqueous solution. (c) The POD-like activity of LiFePO₄ NPs (6.25 μg Fe/mL) with TMB (1.7 mM) as colorimetric substrates under the presence of H₂O₂ (0.8 M) in 0.2 M acetate buffer (pH = 3.6). (d) ESR spectra of spin adducts DMPO/ $\cdot\text{OH}$ produced by LiFePO₄ NPs (10 $\mu\text{g/mL}$) in the presence or absence of H₂O₂ (0.165 M) in 0.2 M acetate buffer (pH = 3.6). (e) Comparison of the a_{nano} of as-synthesized LiFePO₄ NPs and cc-Fe₃O₄ NPs. (f) Diagram of the POD-like catalytic reaction process of LiFePO₄ NPs and Fe₃O₄ NPs.

Phase transformation of LiFePO₄ NPs caused by catalyzing POD-like reaction. The recycled LiFePO₄ NPs from three cycles of POD-like catalysis were proven to suffer a phase

transformation to FePO_4 via multiple characterization techniques. Specifically, the XPS $\text{Fe}2p$ peaks of the recycled LiFePO_4 NPs were shifted toward the higher binding energy (Fig. 5a), indicating the oxidation of Fe^{2+} within the NPs. In the XRD pattern (Fig. 5b), the residual LiFePO_4 phase (marked with “o” in the yellow pattern) in the recycled NPs was negligible, proving that almost all LiFePO_4 were delithiated and oxidized into FePO_4 (marked with “+”) after cyclic POD-like catalysis. This result was further confirmed by ICP analysis that the Li element content in recycled NPs was almost 0 (Table 1). Moreover, the electrochemical property of the recycled NPs was examined using cyclic voltammetry (CV)⁴³ at various scan rates in the voltage range of 0.2 to 0.5 V (Supplementary Fig. 15). The redox peak currents of the recycled NPs were dramatically reduced due to the absence of Li^+ in their lattice (Fig. 5c). This phase transformation, as expected, severely impaired the POD-like activity of the recycled LiFePO_4 NPs (Fig. 5d), in agreement with the self-depleting characteristic of the Fe_3O_4 NPs described above.

Fig. 5 Catalytic mechanism of the POD-like activity for LiFePO₄ NPs. (a) The Fe2p XPS spectra, (b) XRD patterns, (c) CV curves at 2 mV·s⁻¹, and (d) POD-like activities of the control LiFePO₄ NPs and the recycled LiFePO₄ NPs from three cycles of POD-like reaction. The POD-like activity of (e) NaLiFePO₄ NPs (0.4 μg Fe/mL) with different Na-doping levels, and (f) commercial LiFePO₄, Fe₃(PO₄)₂, and FePO₄ (1.25 μg Fe/mL) using TMB (1.7 mM) as substrates in the presence of H₂O₂ (0.8 M) at pH 3.6.

Table 1. ICP analysis of the control and recycled LiFePO₄ NPs.

Element	Concentration (μg/mL)	
	Control LiFePO ₄ NPs	Recycled LiFePO ₄ NPs
Li	15.38	0.66
Fe	120.50	126.54
P	71.02	63.00
Molar ratio of Li: Fe: P	0.97: 0.94: 1	0.05: 1.11: 1

Mobile Li-ions as the limiting factor for LiFePO₄ NPs-catalyzed POD-like reaction. In the field of sodium (Na)-ion batteries, the charge transfer resistances and lattice volume change upon Na⁺ migration are larger for NaFePO₄ electrodes, compared with their Li equivalents due to the larger ionic radius of Na (1.02 Å) than Li (0.76 Å).⁴⁴ Inspired by this, We partially replaced Li with Na in the lattice of LiFePO₄ NPs to explore the potential effect of Na-doping on their POD-like activity. Concretely, three NaLiFePO₄ NPs with similar physicochemical properties but different Na-doping amounts were successfully synthesized (Supplementary Fig. 16 and Table S3). We then compared their POD-like activities under the same reaction conditions and found that the more Na doping, the lower the POD-like activity (Fig. 5e), indicating that the large Na⁺ radius hinders the free migration of Na⁺ and Li⁺ in the crystal, thereby impairing the electron transfer rate. We attempted to use K-doped LiFePO₄ NPs as further proof, however, the large ionic radius of K (1.38 Å) makes it difficult to embed into the electrode materials (Supplementary Fig. 17 and Table S3), which is a common issue in K-ion batteries.⁴⁵

To further prove the decisive role of mobile Li⁺, we measured the POD-like activity of commercially available LiFePO₄, Fe₃(PO₄)₂, and FePO₄ materials with similar hydrodynamic dimensions and surface negative potentials (Supplementary Fig. 18). The results show that their POD-like activity follows LiFePO₄ >> Fe₃(PO₄)₂ > FePO₄ (Fig. 5f), directly confirming that the presence of Fe²⁺ alone in Fe₃(PO₄)₂ cannot ensure the superior catalytic performance, but the transportable Li⁺ contributes to the outstanding POD-like activity of LiFePO₄.

Conclusion

In summary, a detailed mechanism of the POD-like activity of Fe_3O_4 nanozymes is elucidated by characterizing the chemical composition and catalytic activity of the Fe_3O_4 NPs recycled from the long-term POD-like catalysis. These studies demonstrate that all Fe^{2+} in Fe_3O_4 nanozymes contribute to their POD-like activity. The Fe^{2+} inside the particle transfers electrons to the surface, regenerating the surface Fe^{2+} that is directly involved in the sustained catalytic reaction. This process is accompanied by the outward migration of excess oxidized Fe^{3+} from the interior of the crystal, which is a rate-limiting step. Analogous to the low-temperature oxidation of magnetite, Fe_3O_4 NPs participated in the POD-like reaction are eventually oxidized to $\gamma\text{-Fe}_2\text{O}_3$ NPs with a reduced POD-like capacity. Furthermore, this mechanism is well-validated on LiFePO_4 NPs. This work reveals the depletable characteristic of Fe_3O_4 nanozymes that differ from natural enzymes and highlights the potential contribution of internal metal atoms in nanozymes-catalyzed reactions. Meanwhile, these findings bring new thoughts for the mechanistic study and rational design of nanozymes.

Materials and Methods

Chemicals. Ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), dimethyl sulfoxide (DMSO), 3, 3', 5, 5'-tetramethylbenzidine (TMB, $\geq 99\%$), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 98%), o-Phenylenediamine (OPD, 98%), ferric oxide (Fe_2O_3), iron acetylacetonate [$\text{Fe}(\text{acac})_3$], oleic acid (OA), dibenzyl ether, lithium hydroxide ($\text{LiOH} \cdot \text{H}_2\text{O}$), ferric phosphate (FePO_4), rhodamine B (RhB), terephthalic acid (TA), sodium carbonate (Na_2CO_3), and sodium acetate (CH_3COONa) were purchased from Aladdin (Shanghai, China). Tetramethylammonium hydroxide (TMAOH) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Sigma Aldrich. Hydrochloric acid (HCl, 36.0%-38.0%), nitric acid (HNO_3), phosphoric acid (H_3PO_4 , 85% wt), acetic acid (CH_3COOH), hydrogen peroxide (H_2O_2 , 30%), ethyl alcohol, ethylene glycol (EG), and potassium hydroxide (KOH) were purchased from Sinopharm Chemical Reagent Co., Ltd. Ferrous phosphate [$\text{Fe}_3(\text{PO}_4)_2$] was purchased from Shanghai Maclin Biochemical Technology Co., Ltd. Lithium iron phosphate (LiFePO_4) was purchased from Shanghai Xushuo Biological Technology Co., Ltd. All chemicals were used as received without further purification. Deionized water was used throughout the experiments.

Characterization. The particle size and morphology were observed by scanning electron microscopy (SEM, Ultra Plus, Carl Zeiss, Germany). Transmission electron microscopy, selective area electron diffraction (SAED), and high-resolution TEM (HRTEM) were taken using transmission electron microscopy (TEM, JEOL JEM-2100F, Japan). The phase and crystalline structure were explored by X-ray diffraction analyzer (XRD, Bruker D8 Advance,

Germany) using Cu K α radiation. The element composition, chemical structures, and properties of nanoparticles were analyzed by SEM energy dispersive spectroscopy (EDS), ICP-OES (Agilent ICPOES730), Fourier transform infrared spectroscopy (FTIR, Nicolet IS 10, Thermo, USA), Raman spectrometer (Renishaw Invia, UK) and X-ray photoelectron spectroscopy (XPS, Thermo ESCALAB 250Xi, USA). The chemical speciation of Fe in IONPs was determined by X-ray absorption near-edge fine structure (NEXAFS) at the beamline of 08U1A in the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China. The NEXAFS spectra of the references including FeSO₄ and Fe₂O₃ were also measured. Hydrodynamic diameters and Zeta potential of particles were measured by the dynamic light scattering (DLS, Nano-ZS90, Malvern, England). The UV-Vis-NIR absorption spectra were measured using UV3600 (Shimadzu, Japan) and a matched quartz cuvette with 1 cm optical path. Cyclic voltammetry (CV) of LiFePO₄ was measured on Electrochemical Workstation (CHI760e). The production of $\cdot$ OH was detected by the electron spin resonance spectrometer (ESR, Bruker EMXplus, Germany) at ambient temperature. Error bars shown in this paper represent the standard error derived from three repeated measurements.

Synthesis of Fe₃O₄ NPs, γ -Fe₂O₃ NPs, and α -Fe₂O₃ NPs by chemical coprecipitation

method. The naked magnetite nanoparticles were synthesized by a modified chemical coprecipitation method, namely, cc-Fe₃O₄ NPs.¹⁸ In brief, 2.7025 g of FeCl₃·6H₂O and 1.39 g FeSO₄·7H₂O were added to a 100 mL three-necked flask with 10 mL of 2 M HCl reaction solvent. The mixture was stirred at 400 rpm for 10 min under a nitrogen gas (N₂) atmosphere. Then, 62.5 mL of 12.5% TMAOH was quickly poured into the above flask and stirred at 700

rpm for 1 h at room temperature. N_2 was introduced throughout the experiment to remove O_2 from the reaction system. The reaction solution was transferred to a beaker and washed with deoxygenated water three times by magnetic decantation to remove the residual reagents. Part of obtained precipitate was dispersed into deionized water with a pH of 3 to obtain the cc- Fe_3O_4 NPs suspensions, which were stored at 4 °C for subsequent experiments.

The remaining cc- Fe_3O_4 NPs precipitates were dried into black Fe_3O_4 NPs powder under vacuum, and then calcined at 200 °C and 650 °C for 2h to obtain γ - Fe_2O_3 NPs and α - Fe_2O_3 NPs powder, respectively.²⁵ Finally, these three powders were dissolved in a pH of 3 aqueous solutions by ultrasound and stored at 4 °C for subsequent experiments.

Synthesis of Fe_3O_4 NPs by thermal decomposition method. Firstly, the OA-coated Fe_3O_4 NPs were synthesized according to the thermal decomposition method we previously reported (denoted as TD- Fe_3O_4 @OA NPs).³⁶ Then, 4 mL of TD- Fe_3O_4 @OA NPs (1.627 mg Fe/mL) was used to remove the solvent chloroform by rotary evaporation. Next, The remaining precipitates were ultrasonically dissolved in 15 mL of deionized water containing 0.5% TMAOH under vigorous stirring.⁴⁶ Then, the reaction system was transferred to 60 mL of the separatory funnel, and 45 mL of chloroform was added. The mixture was thoroughly mixed. After standing for 15 min, the underlying oil phase liquid was discarded. This extraction procedure was repeated three times to fully remove the free OA that was replaced from the surface of particles by TMAOH. Finally, the obtained Fe_3O_4 NPs aqueous solution (denoted as TD- Fe_3O_4 NPs) was filtered through a 0.22 μ m filter and stored at 4 °C for subsequent experiments.

Synthesis of LiFePO₄ NPs and Na or K-doped LiFePO₄ NPs. LiFePO₄ NPs were successfully synthesized by glycol-based solvothermal method as previously reported.⁴² In a typical route, 0.27 mL H₃PO₄ (85% wt) was dropped into 9 mL of ethylene glycol (EG) containing 0.365 g of LiOH·H₂O. The mixture was stirred thoroughly until the neutralization reaction was complete, showing a milk-white suspension. Then 9 mL of EG dissolved 0.890 g FeSO₄·7H₂O was added into the suspension under vigorous stirring. The mixtures slowly turned grayish-green. After stirring for 30 min, the reaction solution was transferred into a sealed hydrothermal reactor, heated at 180 °C for 10 h. At the end of the reaction, the final products were cooled down to room temperature and washed with deionized water three times.

For Na or K-doped LiFePO₄ NPs, the Li element content (8.69 mmol) of 1%, 5%, and 10% in the above method was replaced with Na element in Na₂CO₃ or K element in KOH. The rest of the synthesis steps remained unchanged. Briefly, for Na-doped LiFePO₄ NPs, 0.365 g of LiOH·H₂O was replaced with 4.6 mg of Na₂CO₃ and 0.361 g of LiOH·H₂O (denoted as NaLiFePO₄-1), 23.0 mg of Na₂CO₃ and 0.346 g of LiOH·H₂O (denoted as NaLiFePO₄-5), and 46.1 mg of Na₂CO₃ and 0.328 g of LiOH·H₂O (denoted as NaLiFePO₄-10), respectively. For K-doped LiFePO₄ NPs, 0.365 g of LiOH·H₂O was replaced with 4.9 mg of KOH and 0.361 g of LiOH·H₂O (denoted as KLiFePO₄-1), 24.3 mg of KOH and 0.346 g of LiOH·H₂O (denoted as KLiFePO₄-5), and 48.7 mg of KOH and 0.328 g of LiOH·H₂O (denoted as KLiFePO₄-10), respectively.

The POD-like activity of NPs. The POD-like activity of NPs was measured by different colorimetric substrates, including TMB, ABTS, and OPD, in the presence of H₂O₂ under different reaction conditions. The absorbance of the colored oxidation products at the corresponding wavelength (TMB_{ox}: 650 nm; ABTS_{ox}: 415 nm; OPD_{ox}: 492 nm) was monitored by UV-Vis spectroscopy. The effects of pH (2.5-11), temperature (10-65 °C) and LiFePO₄ NPs concentration (0-6.25 µg Fe/mL) on POD-like activity were also studied.

Measurement of the specific activity (a_{nano}) of NPs. The POD-like specific activity of NPs synthesized in this work was determined according to the modified method specified in the national standard of China (GB/T 37966-2019).²⁶ The following provided the general procedures: a. Monitor the temperature inside the quartz cuvette and incubate all reagents and samples to 25 °C; b. Add 2.000 mL of 0.2 M acetate buffer solution (pH = 3.6) to a reaction container; c. Add 0.100 mL of different concentrations of NPs and 0.100 mL of 10 mg/mL of TMB in order, mix completely and incubate for 60 s at 25 °C; d. Add 0.100 mL of 30% H₂O₂ and mix completely; e. Immediately transfer the appropriate amount of reaction solution to the cuvette and monitor the changes of absorbance at 650 nm within the specified times. The initial change rate of absorbance (min⁻¹) is obtained from the slope of the early phase of time course; f. Measurement of reagent blank rate 1 and reagent blank rate 2: 30% H₂O₂ and NPs are respectively replaced by deionized water. The measurement procedure is as described in Step a-e above; g. After deducting the reagent blank rate 1 and 2, the POD-like catalytic activity units (b_{nano}) of NPs can be calculated according to Equation (7):

$$b_{\text{nano}} = \frac{V \times (\Delta A / \Delta t)}{\varepsilon \times l} \quad (7)$$

where b_{nano} is the POD-like catalytic activity units of NPs (U); V is the total volume of reaction solution (μL); $\Delta A/\Delta t$ is the slope of the initial liner portion of absorbance changing over time after correcting with reagent blank rate 1 and 2 (min^{-1}); ε is the molar absorption coefficient of TMB derivative ($39000 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$); l is the optical path of the cuvette (cm).

The specific activity of NPs (a_{nano}) can be calculated according to Equation (8):

$$a_{\text{nano}} = \frac{\Delta b_{\text{nano}}}{\Delta m_{\text{Fe}}} \quad (8)$$

where a_{nano} is the specific activity of NPs ($\text{U} \cdot \text{mg}^{-1}$); m_{Fe} is the total Fe element mass contained in added NPs (mg); $\frac{\Delta b_{\text{nano}}}{\Delta m_{\text{Fe}}}$ is the slope of the curve plotting the b_{nano} against different masses of NPs (m_{Fe}).

ESR measurement. The production of $\cdot\text{OH}$ was detected by adding $10 \mu\text{g Fe/mL}$ of cc- Fe_3O_4 NPs or LiFePO_4 NPs and 30 mM of DMPO into $300 \mu\text{L}$ of acetate buffer ($\text{pH}=3.6$) with or without 0.5% H_2O_2 . The signal of the spin adduct (DMPO/ $\cdot\text{OH}$) was recorded at ambient temperature by ESR spectrum at the 1st, 5th, and 10th min after all reagents were mixed. The experimental parameters were as follows: 1 G modulation amplitude, 100 kHz modulation frequency, 6.325 mW microwave power, 9.829 GHz resonance frequency.

Cyclic POD-like catalysis of NPs. For IONPs, 0.1 g of Fe_3O_4 NPs or $\gamma\text{-Fe}_2\text{O}_3$ NPs powder was added to a 400 mL of acetate buffer ($\text{pH } 3.6$) containing 20 mL TMB (10 mg/mL) and 40 mL of $30\% \text{ H}_2\text{O}_2$. This catalytic reaction system lasted for one day (20 h) at $25 \text{ }^\circ\text{C}$. After catalysis, Fe_3O_4 NPs or $\gamma\text{-Fe}_2\text{O}_3$ NPs in reaction solution were recycled by magnetic separation, and then ultrasonic washed with deionized water several times. The recycled NPs

were re-added into a new reaction system mentioned above to catalyze a new round of POD-like reactions. Totally, 5 rounds (or days) of catalysis were carried out, and each round lasted for 20 h at 25 °C. The Fe₃O₄ NPs recycled on days 0, 1, 3, and 5 were taken for further characterization.

For LiFePO₄ NPs, 1 mL of LiFePO₄ NPs (8.4 mg Fe/mL) was added to a 20 mL of acetate buffer (pH 3.6) containing 1 mL TMB (10 mg/mL) and 2 mL of 30% H₂O₂. This catalytic reaction system lasted for one hour at 25 °C. After catalysis, LiFePO₄ NPs in the reaction solution were recycled by centrifugation, and then ultrasonic washed with deionized water several times. The recycled LiFePO₄ NPs were re-added into a new reaction system mentioned above to catalyze a new round of POD-like reactions. Totally, 3 rounds of catalysis were carried out, and each round lasted for 1 h at 25 °C. The LiFePO₄ NPs recycled from the 0 and 3 rounds were taken for further characterization.

Aerated oxidation of Fe₃O₄ NPs. 170 mL of the as-synthesized cc-Fe₃O₄ NPs suspensions (3.57 mg Fe/mL) were transferred into a 250 mL three-necked flask and heated from room temperature to 120 °C. Air was pumped into the suspension at a constant rate (9 L/min) for 12 h under stirring (350 rpm). Condensation reflux was kept throughout the aeration oxidation. Samples oxidized at 0, 0.5, 1, 3, 5, 8 10, and 12 h were used for subsequent characterization.

As for TD-Fe₃O₄ NPs, the only difference was that 30 mL of TD-Fe₃O₄ NPs (0.45 mg Fe/mL) were transferred into a 100 mL three-necked flask. Other steps were consistent with the aeration oxidation of cc-Fe₃O₄ NPs.

Electrochemical performance of LiFePO₄ NPs. Cyclic voltammetry (CV) was used to evaluate the electrochemical performance of LiFePO₄ NPs and recycled LiFePO₄ NPs in a three-electrode cell.⁴³ Saturated calomel was used as the reference electrode and platinum wire as the counter electrode. NPs were mixed with 0.5% Nafion solution in a 1:9 volume ratio and dropped into the polished glassy carbon electrode, which was dried and used as the working electrode. The electrolyte solution was 0.2 M acetate buffer (pH=3.6). CV was recorded between 0.2 and 0.5 V at voltage scan rate (v) ranging from 0.2 to 2 mV·s⁻¹.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary Information files. All other relevant source data are available from the corresponding authors upon reasonable request.

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Competing interests

The authors declare no competing interests.

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