

Ultrasensitive detection of hydrogen peroxide at the single-particle nanozyme level

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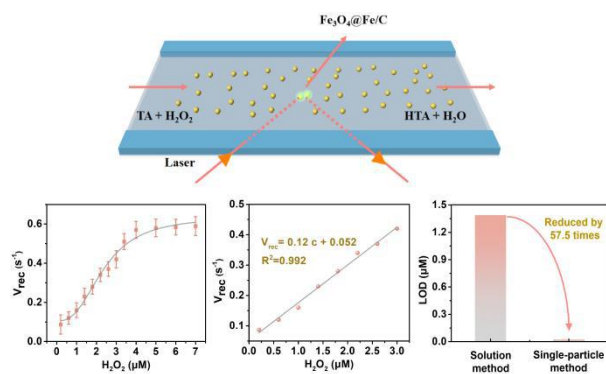
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Single-molecule fluorescence imaging enables a single-particle nanozyme platform that quantifies individual catalytic kinetics and establishes a kinetic parameter-H₂O₂ concentration calibration curve. This strategy achieves a 57.5-fold lower detection limit (24.17 nM) than conventional solution assays (1.39 μM) with a linear range of 0.2-3 μM, demonstrating greatly enhanced sensitivity.

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ABSTRACT: Hydrogen peroxide (H_2O_2) is widely used but highly toxic at elevated concentrations, necessitating ultrasensitive detection for safety and diagnostics. Current peroxidase nanozyme approaches for H_2O_2 detection and kinetic analysis are limited to ensemble measurements, where collective signals mask single-particle heterogeneity, compromise sensitivity, and preclude precise structure-activity correlations. To address this limitation, we herein employ $Fe_3O_4@Fe/C$ as a model single-particle nanozyme and implement single-molecule fluorescence imaging to monitor, in situ, the catalytic oxidation of a non-fluorescent substrate to a highly fluorescent product by H_2O_2 at the single-particle level, thereby constructing an ultrasensitive H_2O_2 detection platform. Through correlating kinetic parameters with H_2O_2 response, we demonstrate that the single-particle assay achieves a limit of detection (LOD) of 24.17 nM with a linear range of 0.2-3 μM , representing a ~58-fold improvement in sensitivity over solution phase ensemble measurements (LOD=1.39 μM). In addition, quantitative analysis of the kinetic parameters reveals pronounced catalytic non-uniformity among individual nanozyme particles. This quantitative analysis system at the single-particle level provides a new method for ultrasensitive detection, and further offers new insights into revealing the microscopic processes of the detection reaction and guiding the study of structure-activity relationships.

KEYWORDS: peroxidase nanozyme, single-particle level, quantitative kinetic parameters, ultrasensitive detection

1 Introduction

Hydrogen peroxide (H_2O_2), as an important chemical raw material, plays a key role in various fields such as food processing, sterilization and disinfection, and environmental protection [1]. However, abnormal levels of H_2O_2 are considered a critical factor leading to a range of diseases [2,3]. Therefore, its trace detection is of great significance for ensuring food safety and public health. Since the first report of peroxidase-like nanozymes in 2007 [4], they have

received extensive attention and achieved remarkable progress [5,6]. Nevertheless, challenges remain, including unclear structure-activity relationships of nanozymes, ambiguous catalytic mechanisms, and low catalytic activity [7,8], which limit the sensitivity of nanozyme-based sensing platforms for H_2O_2 detection and fail to meet the requirements for trace analysis.

Currently, research on the operational processes and mechanisms of nanozyme sensing remains confined to the

macroscopic level, reflecting only the collective properties of nanozymes [9,10]. Such ensemble approaches fail to capture deeper, individualized information from single particles, obscure the true reaction kinetics [11], and make it difficult to establish precise structure-activity relationships, thereby impeding the development of highly active nanozymes. Indeed, at the single-particle level, each particle can be regarded as an independent catalytic unit, yet significant heterogeneity exists among individual particles [12,13]. Consequently, investigations at the single-particle level can eliminate the data interference inherent in ensemble measurements. Moreover, individual particles are more sensitive to their surrounding environment, which can effectively enhance detection sensitivity. In light of this, conducting studies on H_2O_2 detection at the single-particle nanozyme level, deeply exploring the underlying kinetic processes, revealing the heterogeneity among particles, and precisely elucidating structure-activity relationships are essential for achieving highly sensitive H_2O_2 detection and developing nanozymes with superior performance.

Single-molecule fluorescence imaging technology offers ultra-high spatial resolution, excellent detection limits, and nanometer scale localization precision, enabling the investigation of chemical reactions at the single-molecule level and the extraction of information masked in macroscopic measurements [14]. In 2006 [15], researchers first employed single-molecule fluorescence imaging to explore reaction kinetics at the single-particle level, laying a solid foundation for studies on individual nanoparticles. Building upon this, the systematic investigation of catalytic processes in single nanoparticles using single-molecule fluorescence imaging has advanced rapidly [16-20]. For instance, using super-resolution fluorescence microscopy, researchers directly tracked the in situ formation and oxidation of CO_{ads} on individual Pt nanotubes, quantifying for the first time at the single-molecule sub-particle level the influence of surface defects on Pt electrocatalytic performance, and revealing the deep correlation between defect density and catalytic activity under operating conditions [21]. Similarly, based on this technique, in situ probing of the electrocatalytic hydrogen evolution process was accomplished. The spillover of hydrogen from Pd nanocubes to the surrounding substrate was directly observed, and significant heterogeneity of Pd-H both among

and within particles was revealed through Gaussian broadening kinetic analysis [22]. Furthermore, by integrating optical imaging, single-molecule techniques, and single-particle photocurrent measurements, researchers achieved in situ quantitative analysis of charge transfer processes at the single-particle/single-cell scale at microbe semiconductor interfaces, overcoming the limitations of traditional macroscopic characterization methods [23]. Single-molecule fluorescence imaging thus enables the precise determination of single-particle reaction kinetics, the analysis of inter-particle heterogeneity, and the elucidation of structure-activity relationships. Nevertheless, its application to real-time monitoring of catalytic reactions on individual nanozyme particles, accurate quantification of H_2O_2 , and acquisition of critical microscopic information at the single-particle level remains a formidable frontier challenge.

To enable quantitative analysis at the single-particle nanozyme level, we employed $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ as a peroxidase mimicking model and non-fluorescent terephthalic acid (TA) as the substrate. By real-time monitoring of the fluorescent signal from its catalytic oxidation product, highly fluorescent 2-hydroxyterephthalic acid (HTA), we constructed a single-particle detection platform (as shown in Figure 1). Based on the temporal evolution characteristics of single-particle fluorescence trajectories, combined with statistical analysis methods, kinetic parameters of individual nanozyme-catalyzed reactions were quantitatively extracted. A calibration curve between kinetic parameters and H_2O_2 concentration was further established, enabling ultrasensitive detection of H_2O_2 . At the single-particle scale, the detection limit reached as low as 24.17 nM with a linear range of 0.2-3 μM , compared with solution-phase detection, the detection limit was reduced by 57.5-fold, representing a significant enhancement in sensitivity. Furthermore, quantitative analysis of the kinetic parameters unveiled significant inter-particle heterogeneity among the nanozymes.

2 Experimental

2.1. Synthesis of PVP-modified Fe_3O_4

Briefly, 4.0 mmol (649 mg) of FeCl_3 and 0.68 mmol (175.5 mg) of trisodium citrate were dissolved in ethylene glycol (25.0 mL). After complete dissolution, 2 g of PVP ($M_w =$

58,000) and 1.20 g of sodium acetate were added under stirring for 30 min. Next, the mixture was transferred into a 50 mL Teflon-lined stainless steel autoclave, which was sealed and heated at 200 °C for 10 h. After the reaction, an equal volume of absolute ethanol was added to the mixture, followed by centrifugation at 6000 rpm for 5 min to remove the solid precipitate. The supernatant was further centrifuged at 12,000 rpm for 10 min, and the resulting solid was collected. The product was washed 4-5 times with deionized water and ethanol, and then freeze-dried.

2.2 Synthesis of Fe₃O₄@Fe/C

In a typical procedure, 0.5 mmol of PVP-coated Fe₃O₄ was dispersed in 10 mL of deionized water. To this dispersion were added appropriate amounts of Fe(NO₃)₃·9H₂O (0.5 mmol) and glucose (0.8 mmol) sequentially. The resulting mixture was stirred and heated to 80 °C until complete evaporation of water, giving a precursor designated as Fe₃O₄@Fe³⁺/glucose. This precursor was then calcined at 700 °C for 3 h (ramping rate: 5°C min⁻¹) under inert gas protection, affording the target product Fe₃O₄@Fe/C.

2.3 Single-particle nanoenzyme-based device for detecting hydrogen peroxide

TIRF imaging was carried out on an Olympus IX-73 inverted microscope fitted with a commercial TIRF attachment. Excitation was provided by a 295-nm UV-LED, whose output was cleaned by a bandpass filter and then coupled off-axis into the rear focal plane of a 100×oil-immersion objective (NA 1.45). By directing the beam onto the coverslip-sample interface at a super-critical angle, an evanescent field was created to excite fluorophores in the immediate vicinity of the interface. The emitted 444 nm fluorescence was gathered by the same objective, routed through a long-pass dichroic mirror (cut-off ~380 nm) and a bandpass emission filter to reject scattered excitation light, and finally projected by a tube lens onto an EMCCD camera with frame-transfer capability, enabling sensitive low-noise imaging. A 61-ms exposure time per frame was used to improve signal visibility. The entire setup comprised a home-built TIRF microscope and a home-made microfluidic reaction cell.

2.4 Fabrication of a microfluidic reaction cell

In a typical procedure, the Fe₃O₄@Fe/C particles (50 ng mL⁻¹) were deposited onto a coverslip via spin-coating. This coverslip was then incorporated into a flow cell with dimensions of 40 mm × 2 mm × 110 μm, constructed by

sandwiching double-sided adhesive tape between another coverslip. Prior to use, the coverslip was sequentially sonicated in ethanol and ultrapure water for 15 min each, and then functionalized with an aminoalkylsiloxane reagent ((3-aminopropyl) triethoxysilane, APTES). The protonated amine groups render the surface positively charged in aqueous solution, thereby facilitating the electrostatic immobilization of Fe₃O₄@Fe/C onto the substrate. Before measurement, after the mixed solution had fully filled the flow cell, the microfluidic pump was turned off to allow the solution to remain static for a set interval. This step ensured that the active sites of each individual probe particle had already captured H₂O₂ molecules prior to detection. A syringe pump was employed to drive the solution flow, with the flow rate set at 5 μL min⁻¹.

2.5 Solution-based assay for H₂O₂

In a typical assay, 50 μL of Fe₃O₄@Fe/C (0.2 mg·mL⁻¹), 50 μL of TA (20 mM), and H₂O₂ at different concentrations were added in sequence to 300 μL of pH 7.0 phosphate buffer. Ultrapure water was then used to adjust the total volume to 500 μL. Following a 30 minute reaction period, fluorescence spectra were collected with excitation set to 315 nm. The Fe₃O₄@Fe/C nanocomposite was isolated via high-speed centrifugation (12,000 rpm, 3 min), after which the resulting supernatant was subjected to fluorescence spectroscopic analysis.

2.6 Chemical materials and characterization

All the chemical materials and characterization methods employed in this work are provided in the Supporting Information.

3 Results and discussion

3.1. Design the nanocatalyst platform

Fluorogenic reactions provide an essential foundation for real-time single-nanoparticle catalysis studies, enabling the detection of individual catalytic events through single-molecule fluorescence microscopy. In this work, the oxidation of non-fluorescent TA to highly fluorescent HTA by H₂O₂ (Figure S1) was employed as a model reaction to investigate the reaction kinetics on single nanozyme particles. Specifically, Fe₃O₄@Fe/C nanoparticles with Fenton-like activity served as the nanozyme (Figure 1a). These particles catalyze H₂O₂ decomposition to generate highly reactive hydroxyl radicals (·OH), which subsequently

oxidize TA to produce HTA. This fluorogenic transformation offers a direct optical readout of the catalytic process, allowing us to monitor nanozyme activity in real time (Figure 1b). To achieve single-particle resolution, $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ particles were immobilized on a coverslip via spin-coating. Upon introducing the reaction solution, TA molecules are oxidized to HTA at the surface of individual nanoparticles, generating fluorescent bursts under excitation illumination (Figure 1c). Subsequently, we recorded stochastic fluorescence bursts across the surface, and a representative fluorescence intensity time trajectory from one spot exhibits random off-on switching (Figure 1d, and Figure S2, Movie S1), reflecting the stochastic nature of single-molecule catalytic turnovers. Each fluorescent blink reflects the complete sequence of product generation, surface release, and subsequent transport, corresponding to a single catalytic turnover. The dark-state lifetime (τ_{off}) spans from reactant binding to product emergence (formation time), thereby reporting the intrinsic reaction kinetics (ν_{rec}). In contrast, the bright-state (τ_{on}) persistence reports the residence time of individual molecules on the nanozyme surface before their dissociation and migration away from the active site, a collective event we term the product diffusion process, from which the diffusion kinetics are extracted (ν_{dif})^[15-19].

3.2. Morphology and structural characterization

Transmission electron microscopy (TEM) images (Figure 2a and Figure S3a) confirm the successful formation of Fe_3O_4 nanostructures. The Fe_3O_4 surface is completely coated with a carbon layer, which upon thermal reduction yields the final $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ composite structure with a diameter of approximately 200 nm (Figure 2b and Figure S3b). High-resolution TEM (HRTEM) images (Figure 2c) clearly reveal the lattice fringes of $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$. For quantitative analysis, we measured the spacings of ten lattice fringes, obtaining widths of 2.96 and 2.03 nm, which correspond to interplanar spacings of 0.296 and 0.203 nm, respectively. These values are assigned to the (220) plane of Fe_3O_4 (JCPDS No. 75-0033) and the (110) plane of Fe (JCPDS No. 06-0696).

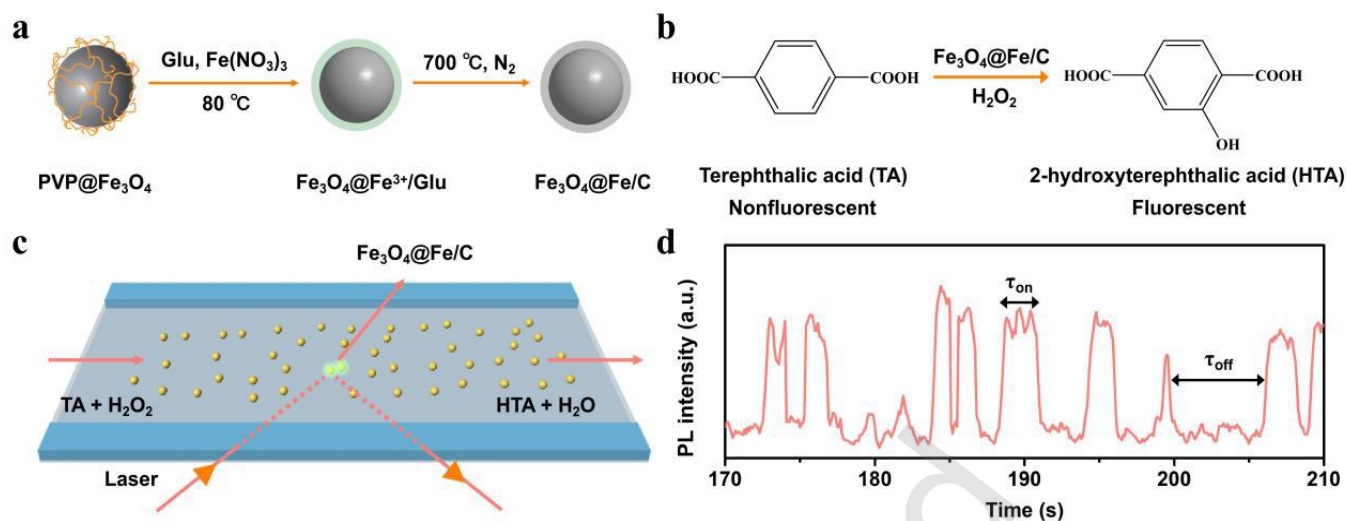


Figure 1. (a) Synthesis pathway of nanozyme Fe₃O₄@Fe/C. (b) Nanozyme-catalyzed oxidation of TA to HTA. (c) Schematic depiction of catalytic events occurring at the level of individual nanoparticles. (d) Part of the intensity trajectory extracted from the glowing region shown in panel (c). Fluorescence signals were integrated over the entire diffraction-limited spot.

In addition, amorphous graphitic carbon can be observed at the outermost layer of Fe₃O₄@Fe/C (indicated by black arrows). To further investigate the elemental composition and spatial distribution, high-angle annular dark field (HAADF) imaging and energy-dispersive X-ray spectroscopy (EDS) elemental mapping were performed on individual Fe₃O₄@Fe/C nanostructures (Figure 2d-f and Figure S3c). The results show that Fe and O are distributed throughout the entire nanoparticle. Notably, the precise localization of C within the shell cannot be unambiguously determined. Furthermore, no distinct core-shell interface is discernible in the HAADF image, which is attributed to the projection overlap of the three-dimensional spherical shell structure onto the two-dimensional TEM image, consistent with previous reports in the literature [24].

To determine the crystal structure and phase composition of the sample, X-ray diffraction (XRD) characterization was carried out. As shown in Figure 2g, the characteristic diffraction peaks located at 30.1°, 35.4°, 43.1°, 53.4°, 57.0°, and 62.6° can be indexed to the (220), (311), (400), (422), (511), and (440) crystal planes of Fe₃O₄, respectively (JCPDS No. 75-0033). In addition, a weak diffraction peak observed at 44.7° corresponds to the (110) plane of metallic Fe (JCPDS No. 06-0696), confirming the presence of an iron phase. These results indicate that, under an inert argon atmosphere, glucose is in-situ converted into carbon while simultaneously promoting the thermal reduction of a

portion of Fe₃O₄, ultimately generating a small amount of metallic Fe [25]. This not only enables the in-situ coating of a carbon shell but also endows the composite with a unique core-shell structure, laying a structural foundation for subsequent catalytic applications. The absence of carbon diffraction peaks in the XRD pattern can be attributed to its amorphous nature, which is consistent with the TEM observations. The surface chemical composition and electronic states of the Fe₃O₄@Fe/C composite were analyzed by XPS. The XPS full scan spectrum shows peaks corresponding to the Fe, O, and C elements, respectively (Figure S4). The high-resolution Fe 2p spectrum (Figure 2h) exhibits distinct peaks, with those at 712.4 and 726.5 eV corresponding to the Fe³⁺ states of Fe 2p_{3/2} and Fe 2p_{1/2}, respectively. Meanwhile, the peaks at 710.7 and 724.2 eV are attributed to Fe²⁺ states of Fe 2p_{3/2} and Fe 2p_{1/2} [26,27]. The peak at 717.9 eV is a satellite feature associated with high-spin iron oxide species [28,29]. Moreover, the peak at 707.2 eV indicates the existence of Fe⁰, providing additional evidence for the existence of elemental iron within the composite material [29]. These observations are consistent with the XRD analysis results. The high-resolution spectrum of C 1s (Figure 2i) contained three peaks, the peak at 284.8 eV arises from the C=C bond, whereas those at 286.4 and 289.1 eV are assigned to the C-O and C=O bonds, respectively [30-32]. Deconvolution of the O 1s spectrum (Figure S5) yields three distinct components at 529.2, 530.1,

and 531.4 eV, which are assigned to lattice oxygen (O_L), respectively [33].
carbonyl ($C=O$) species, and hydroxyl groups ($-OH$),

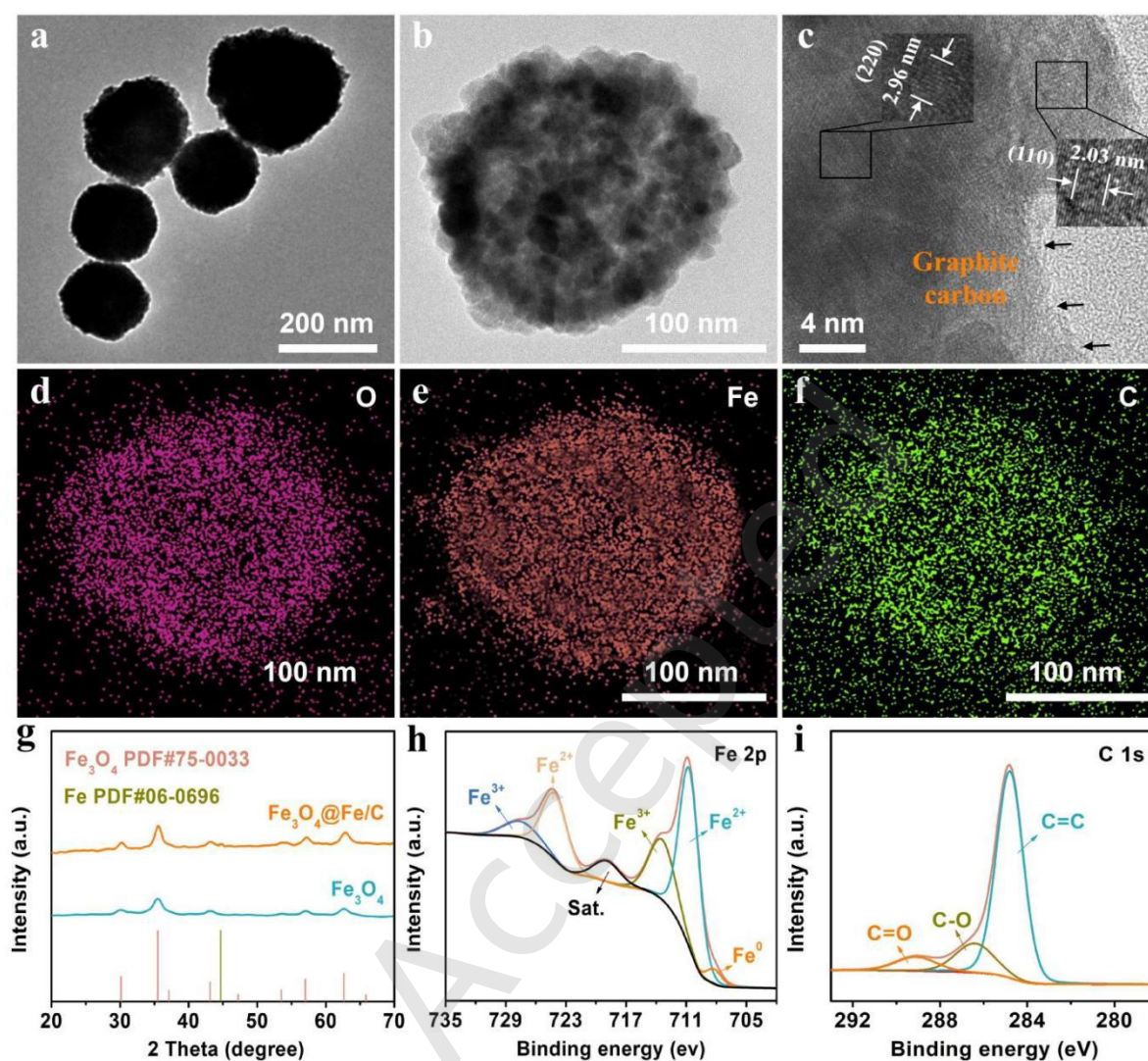


Figure 2. (a) TEM image of Fe_3O_4 . (b) TEM image of $Fe_3O_4@Fe/C$. (c) HRTEM image of $Fe_3O_4@Fe/C$. (d-f) EDS elemental maps of O, Fe, and C, respectively. (g) XRD patterns of the composites. (h) Fe 2p, (i) C 1s high-resolution XPS spectra of $Fe_3O_4@Fe/C$.

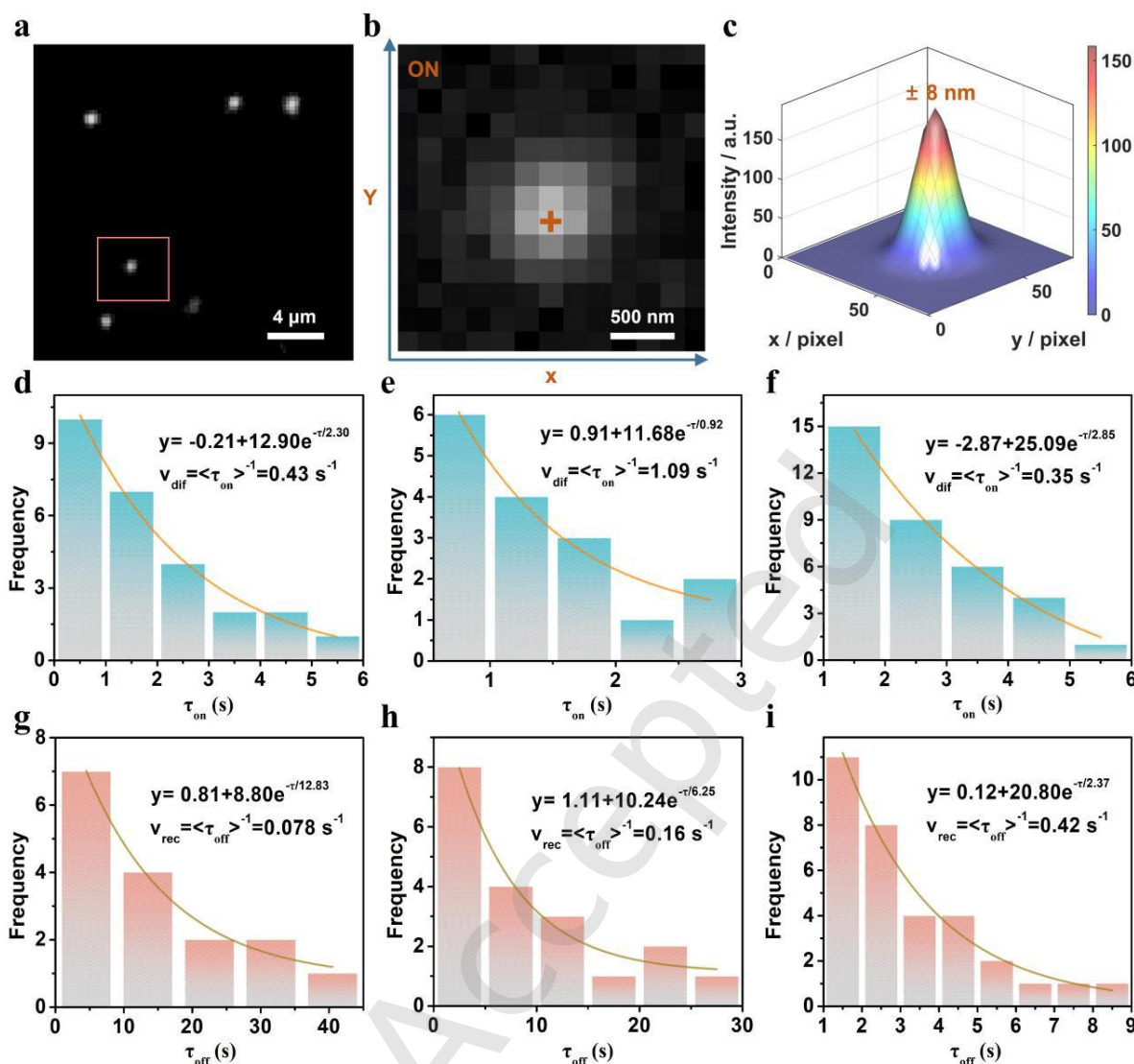


Figure 3. Investigation of catalytic reactions on individual nanozyme particles. (a) Representative fluorescence snapshot of HTA recorded under the catalytic reaction. (b) Fluorescence view of an individual feature highlighted in (a). (c) Three-dimensional depiction of the panel (b) image, the centroid of this point spread function (PSF) can be localized with nanometer precision (± 8 nm) and is denoted by a red cross in (b). (d-f) Distribution histograms of τ_{off} acquired at varying H_2O_2 concentrations. Fitting the data points to a single-exponential decay yields the mean value $\langle \tau_{\text{off}} \rangle$, from which the catalytic reaction rate is derived as $\nu_{\text{rec}} = \langle \tau_{\text{off}} \rangle^{-1}$. (g-i) Corresponding histograms for τ_{on} under different H_2O_2 levels. A single-exponential fit of these data provides the average $\langle \tau_{\text{on}} \rangle$, and the product diffusion rate is consequently obtained as $\nu_{\text{dif}} = \langle \tau_{\text{on}} \rangle^{-1}$.

3.3. Study of fluorescence reactions on single-particle nanozymes

A representative fluorescence image of the catalytic product is shown in Figure 3a. To confirm that the $\text{Fe}_3\text{O}_4/\text{Fe/C}$ nanozyme particles are individually dispersed rather than aggregated, we carried out atomic force microscopy (AFM) characterization on the coverslips with spin-coated samples. The representative AFM image presented in Figure S6 demonstrate that the particles are well isolated and

uniformly distributed, with no signs of agglomeration. Figure 3b displays the emission pattern of a single HTA molecule (marked by the red circle in Figure 3a) captured during one fluorescent burst. Owing to the optical diffraction limit, the fluorescence signal of an individual emitter is distributed across several adjacent camera pixels, appearing as a point-spread function (PSF). By fitting this fluorescence profile with a two-dimensional (2D) Gaussian function, the centroid of the PSF can be localized with nanometer precision (Figure 3c). This super-resolution localization

capability allows us to pinpoint the spatial origin of each HTA molecule generated on a single nanozyme particle during catalysis, thereby enabling direct visualization and quantitative analysis of the nanozyme catalytic kinetics at the single-molecule level. We then introduced the reactant-containing solution (different concentrations of H_2O_2 , 0.5 mM TA, pH=7.0 phosphate buffer) over the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme at a constant flow rate of $5 \mu\text{L min}^{-1}$, delivered by a syringe pump. By means of single-molecule fluorescence microscopy, we recorded the product fluorescence of HTA, affording intensity versus time trajectory. For every recorded trace, the τ_{on} and τ_{off} manifest as stochastic fluctuations (Figure 1d and Figure S2), their ensemble statistics faithfully reflect the intrinsic catalytic kinetics. In the statistical limit, the inverse mean off-time ($\langle\tau_{\text{off}}^{-1}\rangle$) and inverse mean on-time ($\langle\tau_{\text{on}}^{-1}\rangle$) map onto the average rate constants for product generation (v_{rec}) and product release (v_{dif}), respectively, at the single-particle level. To ensure a more reliable signal, the intensity threshold was applied to extract both τ_{off} and τ_{on} intervals from individual $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozymes (Figure S7). Figure 3d-f display the statistical distributions of τ_{on} for individual nanozyme particles at H_2O_2 levels of 0.2 μM , 1 μM , and 3 μM (representative fluorescence trajectories are provided in Figure S8). The corresponding product desorption kinetics, extracted from $\langle\tau_{\text{on}}^{-1}\rangle$, are 0.43 s^{-1} , 1.09 s^{-1} and 0.35 s^{-1} , respectively. Upon raising the H_2O_2 concentration from 0.2 μM to 1 μM , the desorption velocity increases substantially from 0.43 s^{-1} to 1.09 s^{-1} , yet at 3 μM , it drops to 0.35 s^{-1} . This non-monotonic dependence implies surface saturation or product inhibition under elevated substrate levels, thereby extending the surface residence time of generated molecules. In parallel, analysis of τ_{off} affords catalytic reaction rate of 0.078 s^{-1} , 0.16 s^{-1} and 0.42 s^{-1} (Figure 3g-i), displaying a characteristic substrate-dependent trend. Thus, these single-particle kinetic metrics quantitatively elucidate the catalytic properties of individual nanozyme particles and their concentration-responsive behavior.

Notably, single-exponential fitting is applicable to the analysis of single-particle reaction rates and product diffusion rates in the present study. First, the substrate TA was kept saturated and thus not rate-limiting. Second, the product generation rate (v_{rec}) increased with H_2O_2 concentration and leveled off at high levels (Figure 4a),

indicating that only one reactant adsorbs on the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme surface, while the other neither adsorbs nor competes. Hence, the off-state follows a non-competitive Langmuir-Hinshelwood (L-H) mechanism [16], with fast adsorption-desorption equilibrium making product generation the rate-limiting step. In contrast, the product diffusion rate (v_{dif}) showed a different dependence: it rose initially, peaked at 1.8 μM , then fell (Figure S12), characteristic of competitive L-H behavior. Here, competition is between substrate and product for the same adsorption sites, not between two substrates. At low concentrations ($<1.8 \mu\text{M}$), low product coverage renders diffusion gradient-driven, so diffusion is rate-limiting and its rate increases with substrate. At higher concentrations ($>1.8 \mu\text{M}$), excess substrate occupies active sites, forcing product desorption via higher-energy pathways, decreasing the diffusion rate constant and making diffusion again rate-limiting. Therefore, Both reaction and diffusion have a single rate-determining step, justifying single-exponential fitting for each kinetic data.

3.4. Single-particle vs. ensemble detection performance

The intrinsic heterogeneity among individual nanozyme particles is directly manifested in their catalytic kinetic parameters. As exemplified by an independently selected $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme (Figures S9 and S10), its kinetic profile deviates from that shown in Figure 3, even under identical H_2O_2 concentrations. Therefore, comparative investigation of the kinetic behaviors among individual particles provided direct evidence for substantial inter-particle heterogeneity in catalytic performance. We also investigated the compositional heterogeneity among single nanozyme particles. TEM-EDS mapping (Figure S11 and Table S2) showed that the particles differed in size and elemental content. Such structural differences may impact active-site accessibility and electron transfer, leading to disparate catalytic activities. This inherent variability underscores the critical necessity of performing extensive statistical analyses on a sufficiently large population of single-particle measurements to reliably extract representative kinetic parameters. Therefore, to ensure statistical robustness, we therefore performed systematic sampling across a large ensemble of single-particle nanozymes, with each data point representing extensive

statistical averaging. As illustrated in Figure 4a, the catalytic product formation rate (v_{rec}) escalated with increasing H_2O_2 levels, ultimately plateauing to reveal saturation kinetics. The product diffusion rate (v_{dif}) displayed a distinct concentration-dependent profile (Figure S12), initially rising before declining as the H_2O_2 concentration further increased. This anomalous dependence indicates direct H_2O_2 engagement in the liberation of generated molecules from the active site. Figure 4b reveals a good linear dependence of the catalytic rate enhancement on H_2O_2 concentration across the 0.2–3 μM range ($R^2 = 0.992$). The LOD of 24.17 nM ($S/N=3$) for H_2O_2 could be achieved, manifesting remarkable

analytical performance. This value is comparable to, or even better than, those reported in previous studies (Table S3), confirming the competitive performance of our sensing platform. Furthermore, the H_2O_2 response of the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ ensemble was evaluated in the solution phase, using the test conditions optimized as shown in Figure S13. When subjected to incremental oxidant doses, the solution-based system displayed a characteristic emission enhancement (Figure 4c). Notably, the optical signal exhibited a linear dependence on the analyte concentration across the 4–22 μM range

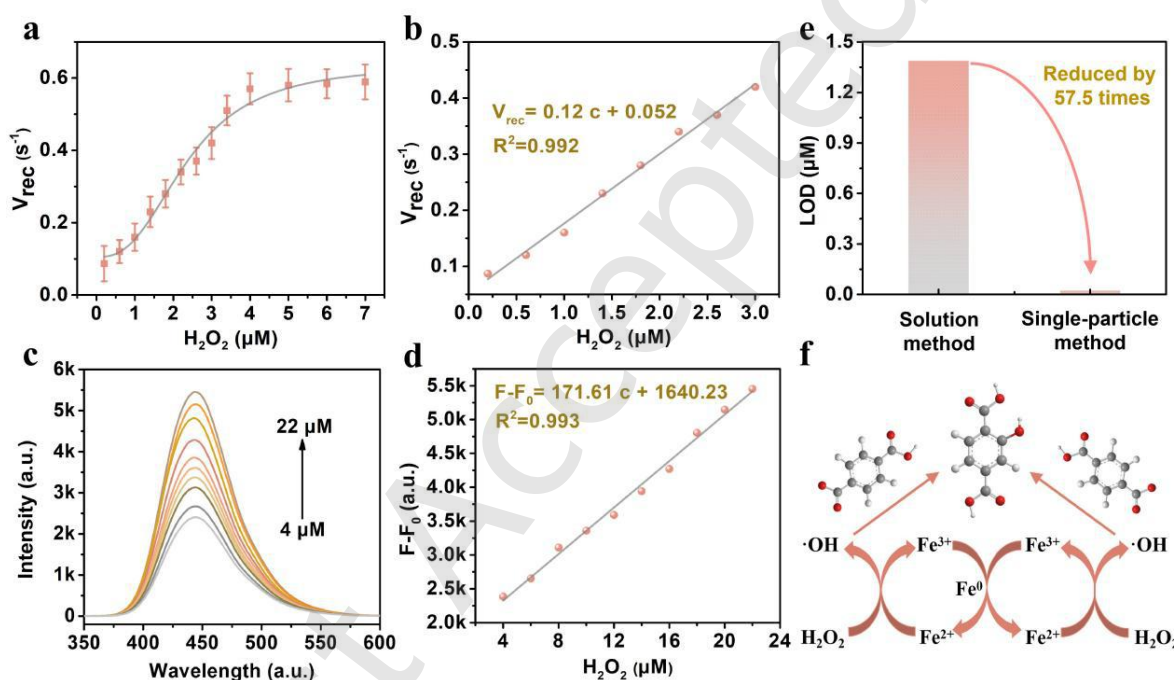


Figure 4. (a) Catalytic reaction rate (v_{rec}) of individual nanozymes at varying H_2O_2 concentrations, each data point represents the average from >20 trajectories. Error bars represent the standard error of the mean (SEM). (b) v_{rec} as a function of H_2O_2 concentration (0.2–3 μM). (c) Fluorescence spectra of the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ + TA system in phosphate buffer (pH 7.0) with H_2O_2 concentrations ranging from 4 to 22 μM . (d) Linear calibration curve for H_2O_2 over the range of 4–22 μM . (e) Comparison of detection limits between the solution-based method and the single-particle method. (f) Possible catalytic mechanism of $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$.

(Figure 4d), affording a detection limit of approximately 1.39 μM ($S/N=3$). Comparative evaluation revealed that the single-nanozyme platform markedly outperforms the bulk-phase counterpart, with the LOD narrowing by almost 58-fold (Figure 4e), highlighting the substantial merits of individual-particle analysis. Additionally, as demonstrated in Figure S14 and Figure S15, the presence of other ions caused negligible interference with the detection of H_2O_2 ,

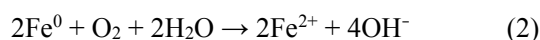
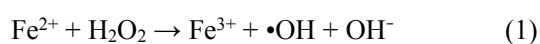
demonstrating the high selectivity and robust anti-interference performance. Ensemble-phase detection averages signals from numerous $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozymes, which cancels individual variations and provides abundant active sites, thus yielding a wide linear range. Conversely, single-particle detection relies on counting individual $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$, where the limited active sites become quickly saturated at higher concentrations, leading to a narrow

linear window, an inherent feature of this detection strategy.

The demonstration of analytical practicality is a vital step in method development. To this end, we performed measurements of H_2O_2 in tap water employing the present sensor, as summarized in Table S4. When various amounts of the analyte were added into tap water, recoveries consistently exceeded 96 %, while RSD below 7 %. Such outcomes clearly indicate that the single-particle sensor can reliably quantify the H_2O_2 in practical sample.

3.5. Possible catalytic mechanism

According to previous reports, the catalytic mechanism of $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ can be described in the following reactions [8,34,35]:



As described above, a synergistic catalytic mechanism for the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme is proposed as follows: 1) The surface-active ferrous sites (Fe^{2+}) on Fe_3O_4 initiate a Fenton-like reaction, decomposing H_2O_2 into highly oxidative $\bullet\text{OH}$ radicals while being oxidized to Fe^{3+} (equation 1); 2) Concurrently, the embedded zero-valent iron (Fe^0) acts as a sacrificial electron donor, spontaneously oxidizing to Fe^{2+} by releasing two electrons, thereby continuously replenishing the Fe^{2+} consumed in step 1 and priming the catalytic cycle (equation 2); 3) More importantly, Fe^0 rapidly reduces the accumulated Fe^{3+} back to Fe^{2+} , which not only circumvents the sluggish kinetics of Fe^{3+} reduction by H_2O_2 inherent to classical Fenton chemistry, but also suppresses the hydrolysis of Fe^{3+} into catalytically inactive ferric hydroxides/passivation layers. Ultimately, these potent radicals oxidize TA to produce highly fluorescent HTA, as mechanistically depicted in Figure 4f. Based on the potential mechanism of nanozyme-catalyzed generation of free $\bullet\text{OH}$ radicals, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was employed as a radical trapping agent to verify this hypothesis. DMPO can rapidly react with short-lived $\bullet\text{OH}$ to form stable spin adducts exhibiting characteristic electron spin resonance (ESR) spectra, thereby providing a reliable basis for the qualitative detection of radicals. The ESR spectroscopy results are presented in Figure S16. In the

complete catalytic system containing $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$, DMPO, and H_2O_2 , a typical ESR signal of the $\text{DMPO}/\bullet\text{OH}$ spin adduct was clearly observed, with hyperfine coupling constants matching the characteristic 1:2:2:1 relative intensity distribution, which is in excellent agreement with the reported spectral features of $\bullet\text{OH}$ adducts in the literature [36]. In contrast, no discernible $\text{DMPO}/\bullet\text{OH}$ spin adduct ESR signals were detected in either control system lacking $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme or H_2O_2 substrate. These results conclusively demonstrating that $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ nanozyme, through its peroxidase-like activity, can efficiently catalyze the decomposition of H_2O_2 to generate free $\bullet\text{OH}$ radicals, a process that follows a typical Fenton-like reaction pathway. We have supplemented the XPS valence state analysis of the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ sample after the catalytic cycle, and the peak-fitting results of the Fe 2p high-resolution spectra are presented in Figure S17. Through quantitative analysis of the spectra before and after the reaction, we obtained the relative contents of Fe^0 , Fe^{2+} and Fe^{3+} (Table S5 and Table S6). The results show that, compared with the fresh sample, the Fe^0 content decreased markedly after the reaction, while the proportion of Fe^{3+} increased significantly and that of Fe^{2+} slightly decreased. This valence evolution trend directly evidences a well-defined electron-transfer pathway during the reaction, which is in excellent agreement with our proposed catalytic cycle mechanism.

4 Conclusions

In summary, this study developed a new strategy for single-particle nanozyme catalytic analysis based on single-molecule fluorescence imaging technology. By real-time monitoring the process of H_2O_2 oxidation of TA catalyzed by the peroxidase-like nanozyme $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ to generate the highly fluorescent product HTA, we captured the stochastic on-off switching behavior of fluorescence emission signals at the single-particle level. Through statistical analysis of a large number of single-particle fluorescence time trajectories, we extracted the distributions of τ_{on} and τ_{off} dwell times, which correspond to the product diffusion and catalytic reaction event, respectively, thereby achieving quantitative determination of catalytic kinetic parameters. Based on the linear correlation between v_{rec} and H_2O_2 concentration, we constructed an ultrasensitive quantitative detection platform with a detection limit as low

as 24.17 nM and a linear range of 0.2-3 μM . Compared with solution-phase (ensemble) measurements, the single-particle strategy reduced the detection limit by nearly 58-fold (from 1.39 μM to 24.17 nM), fully highlighting the significant advantages of single-particle analysis in eliminating ensemble averaging effects and uncovering individual catalytic activity. Apart from enabling ultrasensitive detection, this methodology is expected to serve as a versatile platform for probing nanozyme structure-activity relationships, offering a promising avenue toward the development of high-performance nanozymes.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

Acknowledgements

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author Contribution Statement

Wei Fu: Writing-original draft, Data curation, Conceptualization. Wanjun Xu: Methodology. Zihao Ma: Methodology. Xingqin Fu: Supervision, Methodology. Yang Li: Formal analysis. Lei Bao: Supervision. Wanjun Zhao: Methodology. Zirui Qiao: Writing-review & editing,

Supervision, Methodology. Zhifang Liu: Writing-review & editing, Supervision, Methodology. Juncheng Jin: Writing-review & editing, Supervision, Methodology, Funding acquisition.

Use of AI statement

None

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Electronic Supplementary Material

Ultrasensitive detection of hydrogen peroxide at the single-particle nanozyme level

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1. Characterization techniques

The morphologies and Energy-dispersive X-ray spectroscopy (EDS) of $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ were performed on a field-emission TEM (FE-TEM; FEI Tecnai G2 F20). X-ray photoelectron spectroscopy (XPS) data were acquired with a Thermo Escalab 250XI spectrometer using an aluminum excitation source. X-ray diffraction (XRD) patterns were collected on a D2 PHASER diffractometer (Bruker AXS, Germany). Fluorescence spectra were recorded with an FS5 spectrophotometer (UK).

2. Materials

Materials used in the experiments include terephthalic acid (TA), ethanol ($\text{CH}_3\text{CH}_2\text{OH}$), hydrogen peroxide (H_2O_2) were acquired from Aladdin Reagent Company (Shanghai, China). Polyvinyl Pyrrolidone ($\text{Mw}=58000$), ferric trichloride (FeCl_3), trisodium citrate, ethylene glycol (EG), sodium acetate, Ferric nitrate nonahydrate, glucose (Glu) were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). The deionized water used throughout the experiment was home-made. All anions employed in the analytical detection were in the form of their corresponding sodium salts, and all cations were in the form of their corresponding chlorides, both obtained from Shanghai Titan Scientific Co., Ltd. All reagents were of analytical grade and were used without further purification.

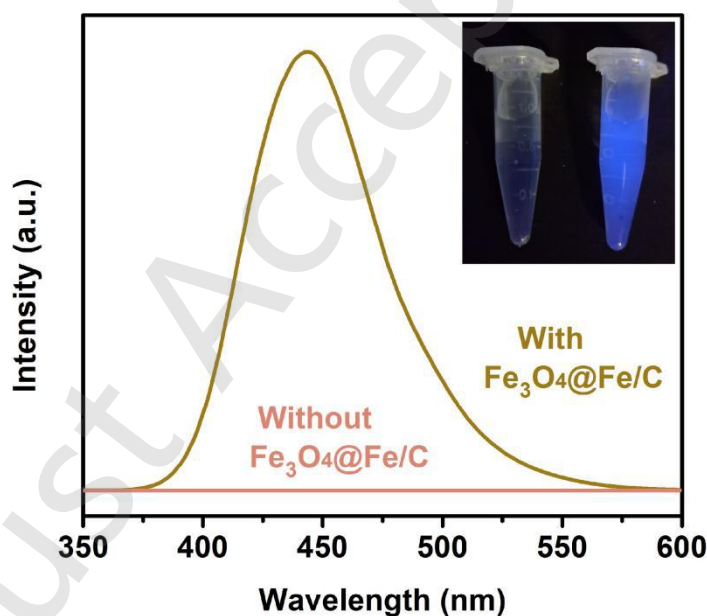


Figure S1. PL spectra of $\text{H}_2\text{O}_2+\text{TA}$ mixture (red line) and $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}+\text{H}_2\text{O}_2+\text{TA}$ system (brown trace). Inset: digital photographs of these two corresponding solutions under 305 nm excitation.

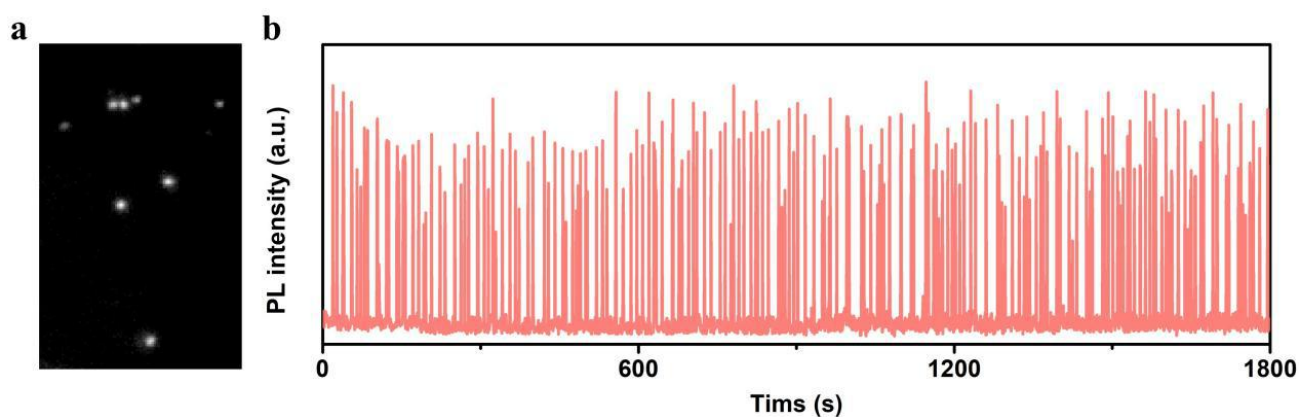


Figure S2. a) A typical fluorescence image of catalytic products. b) Fluorescence intensity time trajectory of a representative spot in (a).

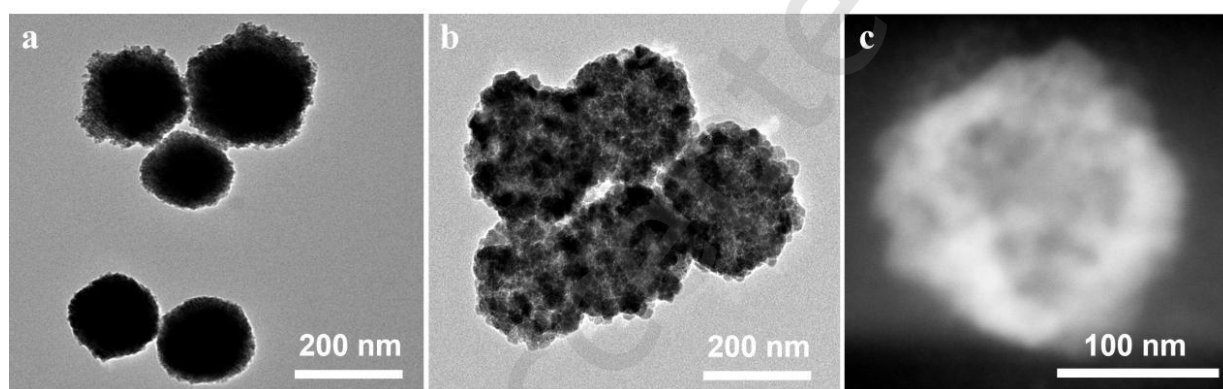


Figure S3. (a) TEM image of Fe_3O_4 . (b) TEM image of $\text{Fe}_3\text{O}_4@\text{Fe/C}$. (c) HAADF-TEM image of $\text{Fe}_3\text{O}_4@\text{Fe/C}$.

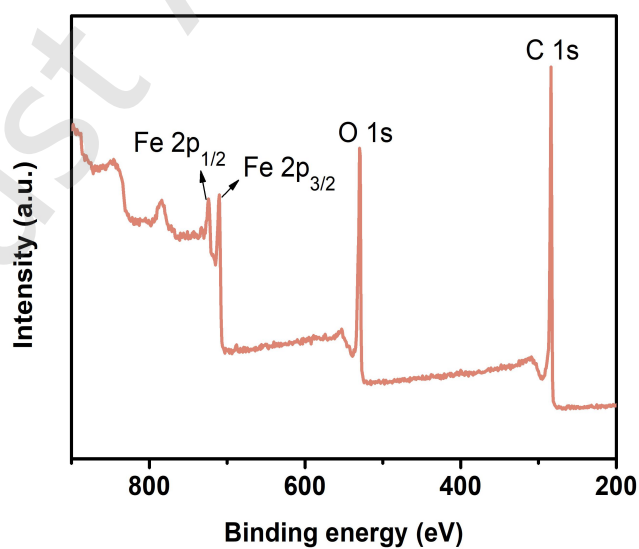


Figure S4. XPS survey of $\text{Fe}_3\text{O}_4@\text{Fe/C}$

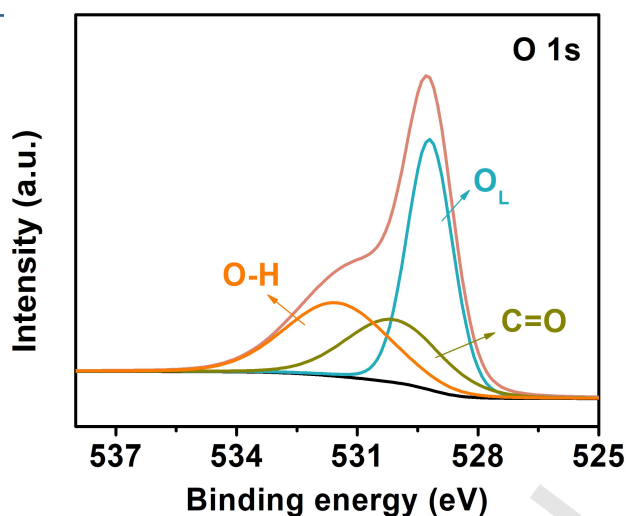


Figure S5. O 1s high-resolution XPS spectra of $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$

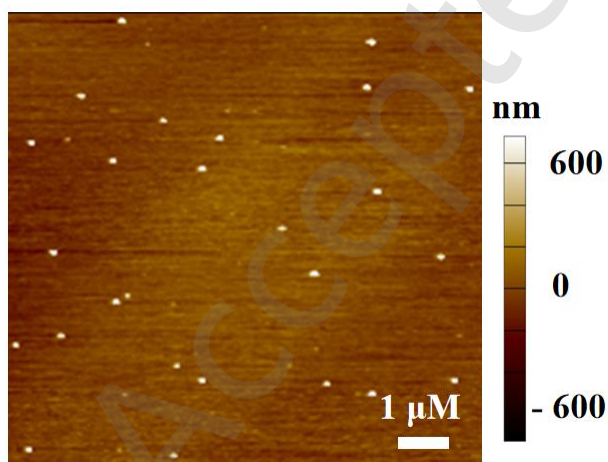


Figure S6. AFM images of the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ on spin-coated coverslip substrates.

To pinpoint the centroid of the fluorescence PSF, a 13×13 pixel region ($\sim 2.1 \times 2.1 \mu\text{m}^2$) extracted from the $\text{Fe}_3\text{O}_4@\text{Fe}/\text{C}$ fluorescence image (fluorescence originates from the product) in Figure 3b was analyzed via two-dimensional Gaussian fitting. For enhanced accuracy, the image data were fitted using the following model [1,2]:

$$I(x, y) = A + Bx + Cy + I_0 \int_{x-\delta}^{x+\delta} dX \int_{y-\delta}^{y+\delta} dY \exp \left[-\frac{1}{2} \left(\frac{X - x_0}{\sigma_x} \right)^2 - \frac{1}{2} \left(\frac{Y - y_0}{\sigma_y} \right)^2 \right]^2$$

$I(x, y)$ — the scattering intensity for a given coordinate (x, y) ;

$A + Bx + Cy$ — the background in the fitting;

I_0 — the intensity of the Gaussian function;

δ — the half of pixel size;

(x_0, y_0) — the centroid of the Gaussian function;

σ_x — the standard deviations in x ;

σ_y — the standard deviations in y .

The parameters in fitting the data in Figure 3b are summarized in Table S1.

Table S1. Parameter fitting results.

A	B	C	σ_x (nm)
0.034	0.058	94.25	267.10
σ_y (nm)	x_0 (nm)	y_0 (nm)	
212.51	493.09	298.67	

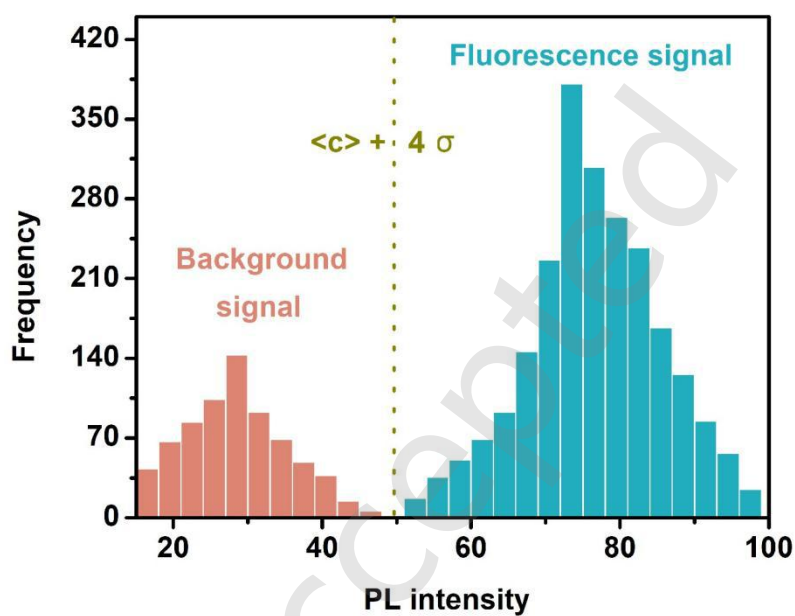


Figure S7. Discrimination between fluorescence and background signals. To improve data reliability, a threshold set at $\langle c \rangle + 4\sigma$ (depicted by the dark yellow dashed line) was applied to differentiate product fluorescence from background noise [3-5], where $\langle c \rangle$ denotes the mean background intensity and σ its standard deviation. Intensities exceeding this threshold are classified as fluorescence signals, whereas those falling below are considered background.

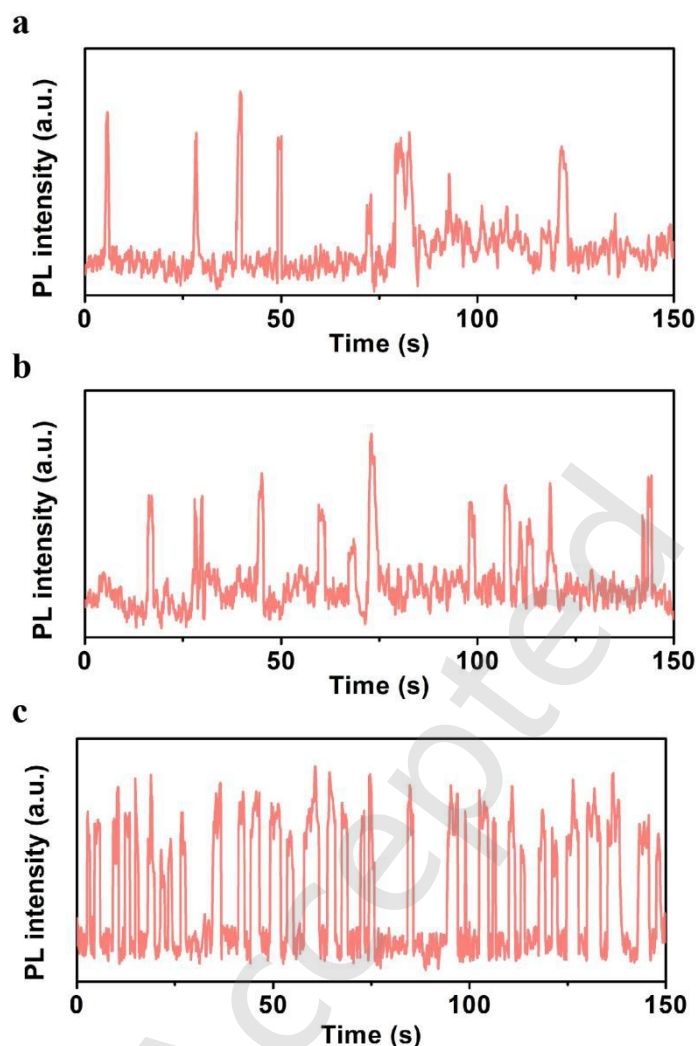


Figure S8. Time-dependent PL intensity traces recorded at the single-nanozyme level during catalysis, measured under varying H_2O_2 concentrations. (a) $0.2 \mu\text{M}$; (b) $1 \mu\text{M}$; (c) $3 \mu\text{M}$. Given the sustained catalytic activity observed in Figure S2b, we are convinced that both the intrinsic decomposition of H_2O_2 and catalyst deactivation can be effectively avoided over the 150 s measurement period. Therefore, the fluorescence signals acquired within the 150 s window are considered reliable for quantitative data analysis.

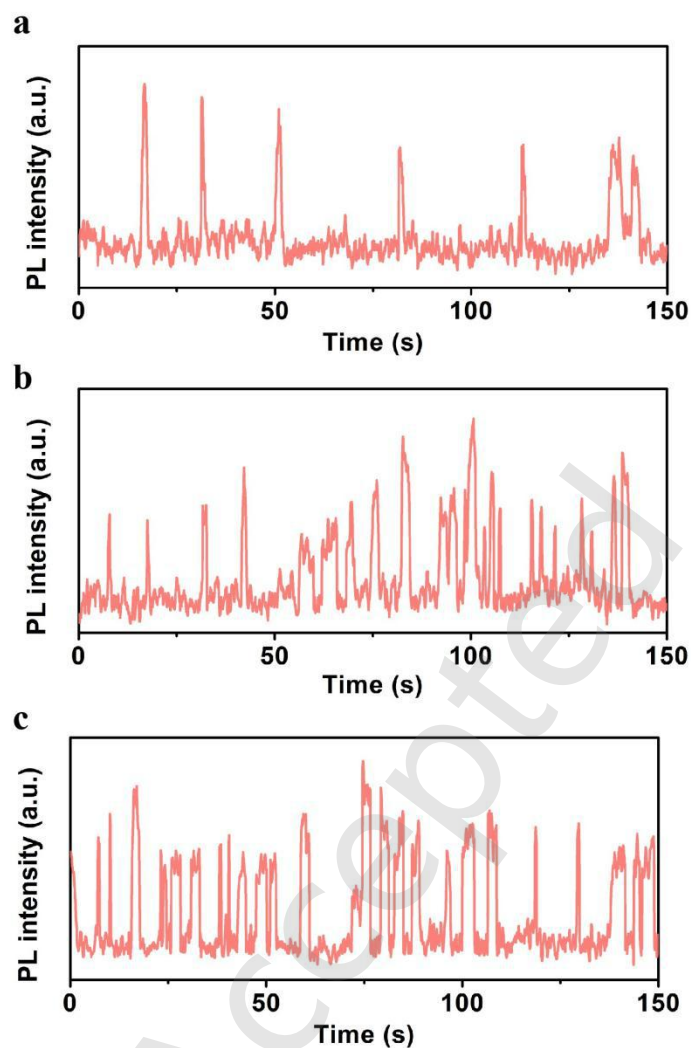


Figure S9. Time-dependent PL intensity traces recorded at the single-nanozyme level during catalysis, measured under varying H_2O_2 concentrations. (a) $0.2\ \mu\text{M}$; (b) $1\ \mu\text{M}$; (c) $3\ \mu\text{M}$.

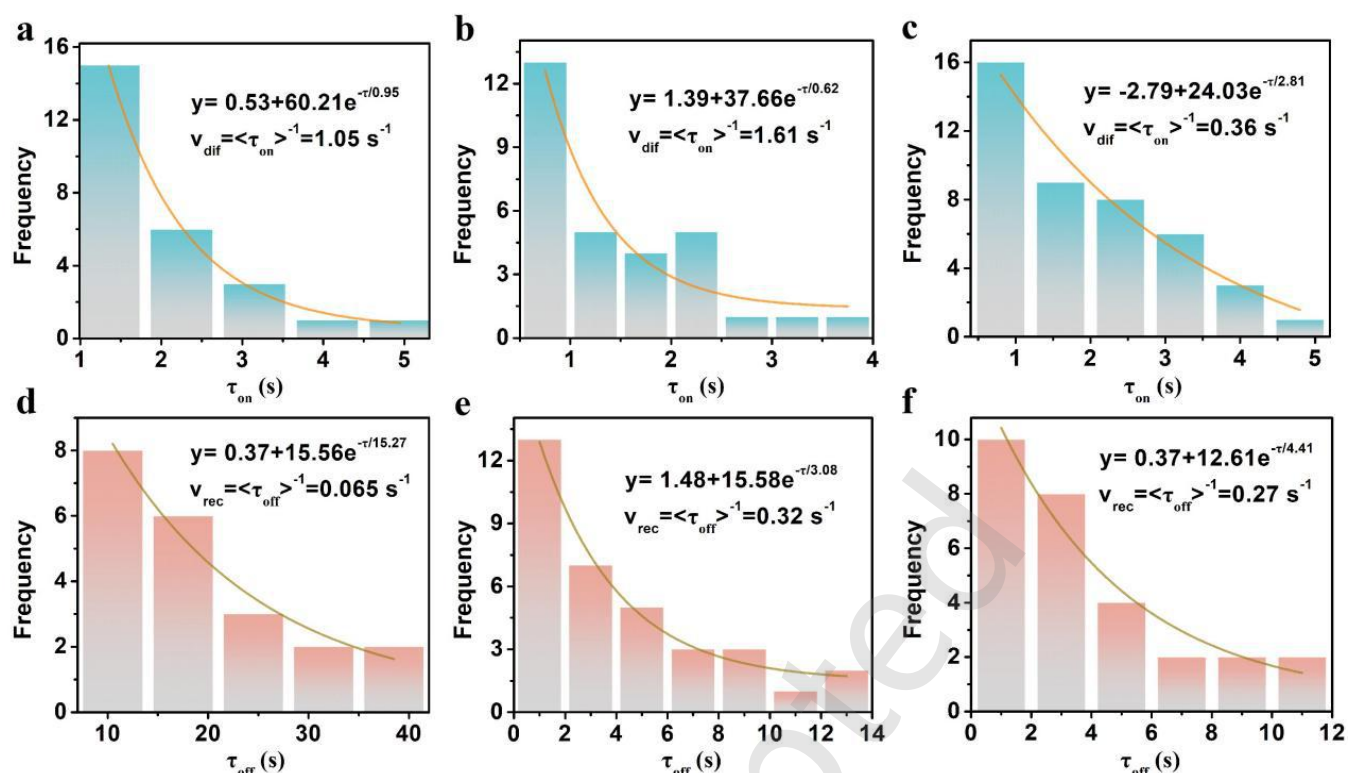


Figure S10. Time-dependent PL intensity traces recorded at the single-nanozyme level during catalysis, measured under varying H_2O_2 concentrations. (a, d) 0.2 μM ; (b, e) 1 μM ; (c, f) 3 μM .

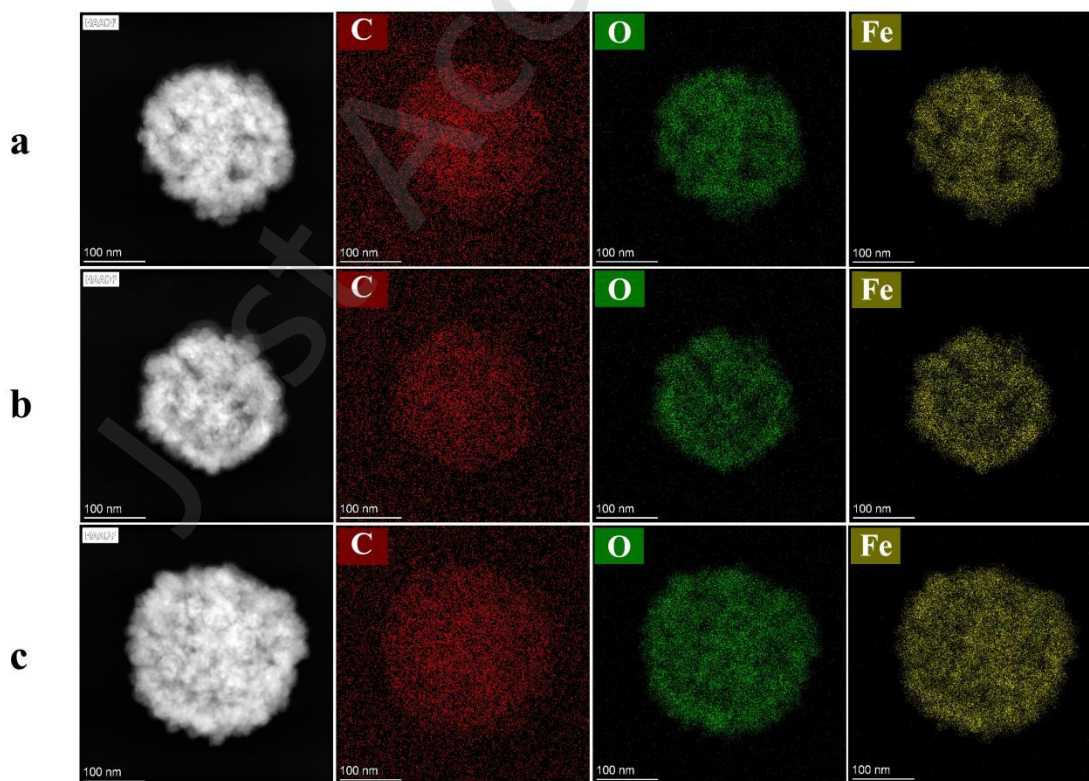


Figure S11 TEM-EDX elemental mapping of three randomly selected $Fe_3O_4@Fe/C$ nanozyme particles.

(a) Particle 1; (b) Particle 2; (c) Particle 3.

Table S2. Elemental contents of C, O, and Fe for three randomly selected Fe₃O₄@Fe/C particles, as determined by TEM-EDX.

Sample	Element	Mass Fraction (%)
Particle 1	C	12.27
	O	31.76
	Fe	55.97
Particle 2	C	14.31
	O	30.64
	Fe	55.05
Particle 3	C	15.49
	O	34.76
	Fe	49.75

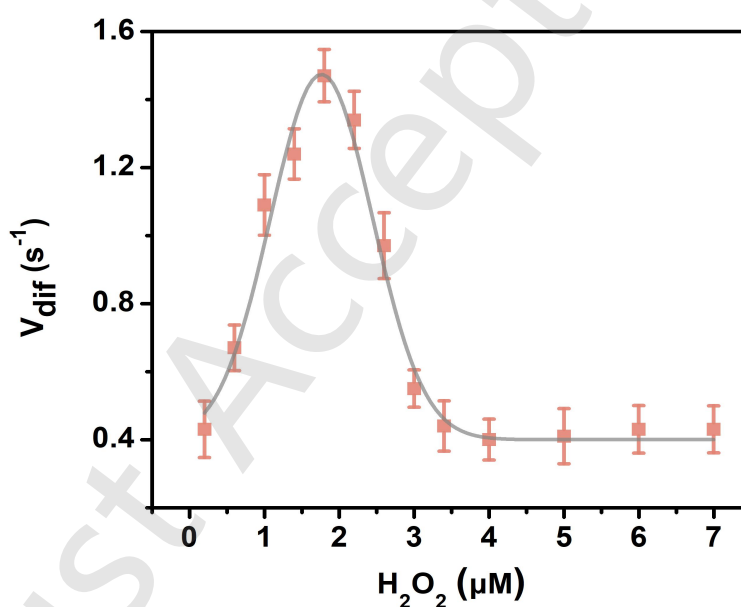


Figure S12. Product diffusion rate (v_{dif}) at varying H₂O₂ concentrations, each data point represents the average from >20 trajectories.

Table S3. Comparison of detection limits for H₂O₂ sensors based on nanozymes.

Materials	Linear range (μM)	pH	LOD (μM)	Ref.
Cyt c@PCN-222 NP	0-2	7.4	0.13	6
FeN ₄ -SAzyme	10-1000	4	7.97	7
FeCo-CDs	200-1500	4	0.77	8
Pt/MoO _x	0.5- 2665.5	7.4	0.26	9
FeCu-NZs	0.1-3800	5	0.06	10
Fe-SNC	3-1000	7	1.3	11
Fe _{0.8} Ni _{0.2} S ₂ /ZIF-67	0.25-10	4	0.039	12
CuFeS ₂	50-9000	4	22	13
Pd/Pt-HNPs	0.25-100	4	1.14	14
GQDs-Fe	2-10	7.0	0.02	15
Fe ₃ O ₄ @Fe/C (Solution phase)	4-22	7.0	1.39	This work
Fe ₃ O ₄ @Fe/C (Single-particle level)	0.2-3	7.0	0.024	This work

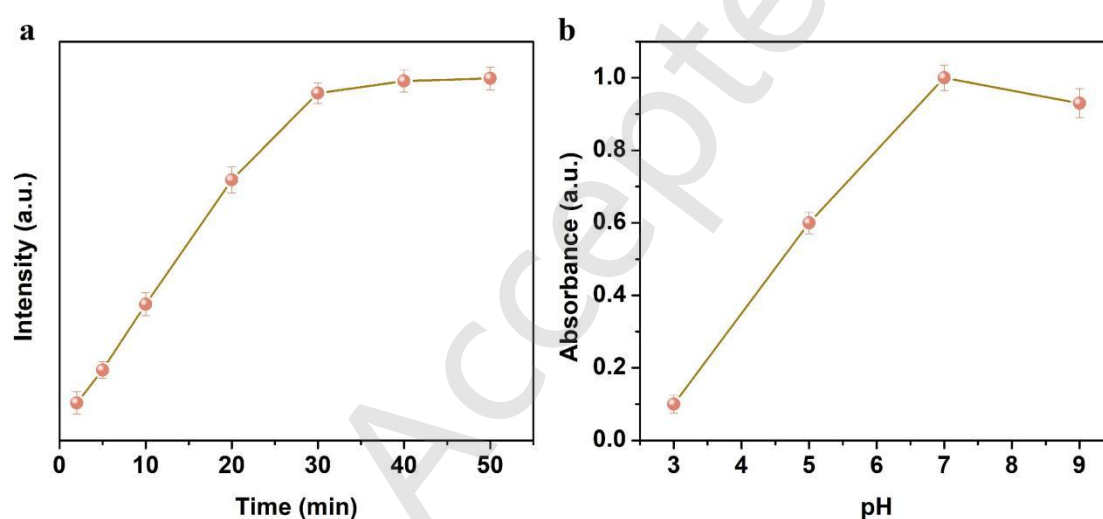


Figure S13. UV-vis absorption spectra for (a) effect of pH, (b) reaction time on peroxidase-mimicking activity of Fe₃O₄@Fe/C nanozyme. The optimal reaction conditions for the ensemble tests, as determined from the condition optimization experiments, were a reaction time of 30 min and a pH of 7.0.

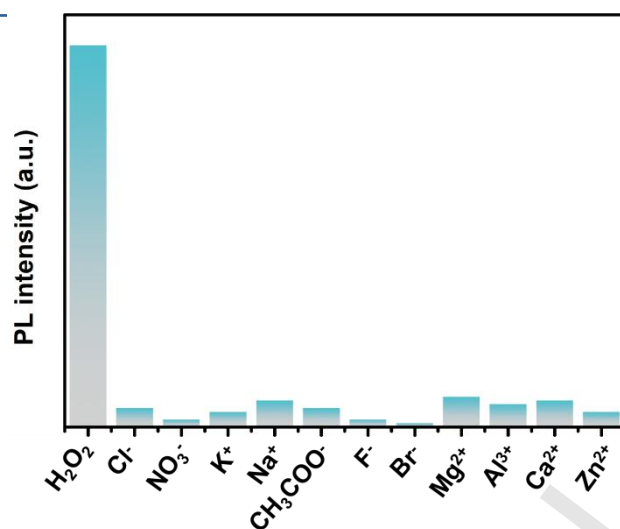


Figure S14. Selectivity assays for H_2O_2 detection. The concentrations of each substance was 100 μM .

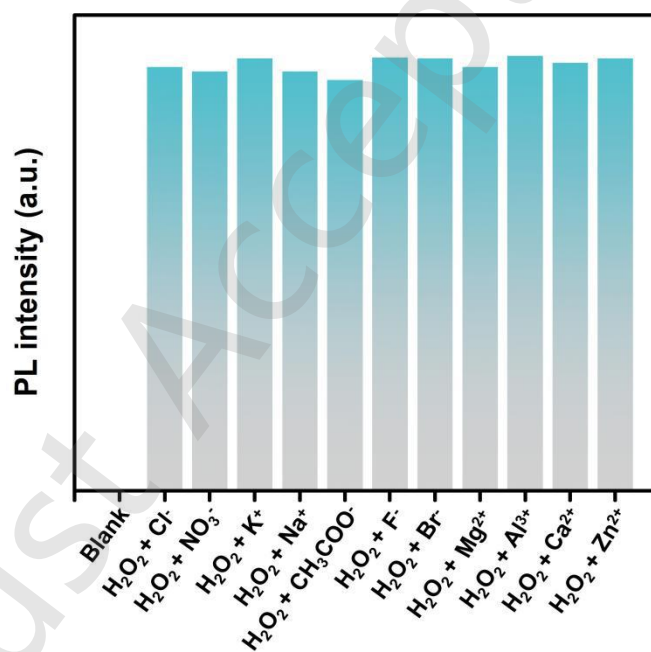


Figure S15. Interference testing histogram for H_2O_2 detection. The concentration of H_2O_2 is 100 μM , and that of other ions are 300 μM .

Table S4. Detection of H₂O₂ in water samples using a single-particle sensing platform

Sample	Added (μM)	Detected (μM)	Recovery (%)	RSD (%)
Tap Water	0	ND	-	-
	1	1.06	103.8	4.85
	2	1.94	98.7	6.04
	2.5	2.57	96.1	4.17

ND: no detected

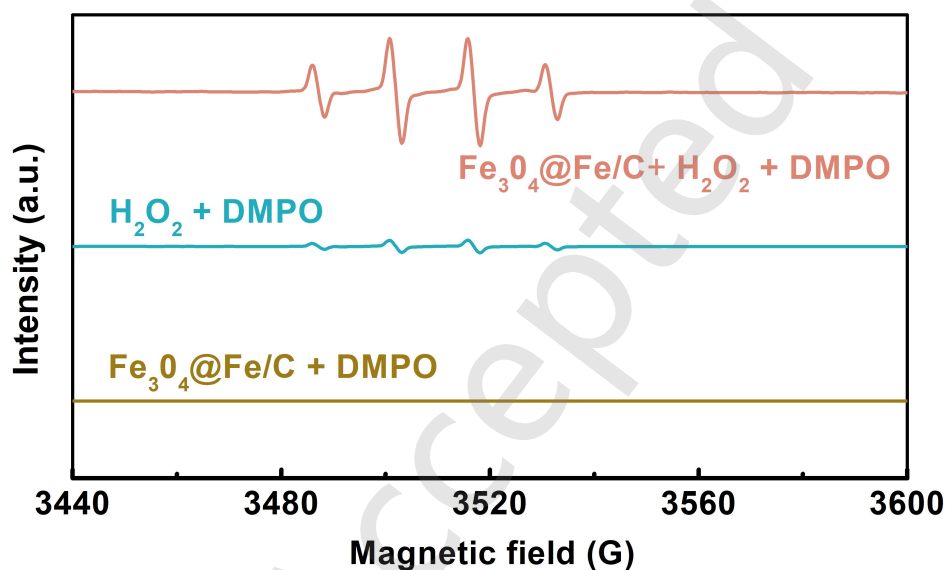
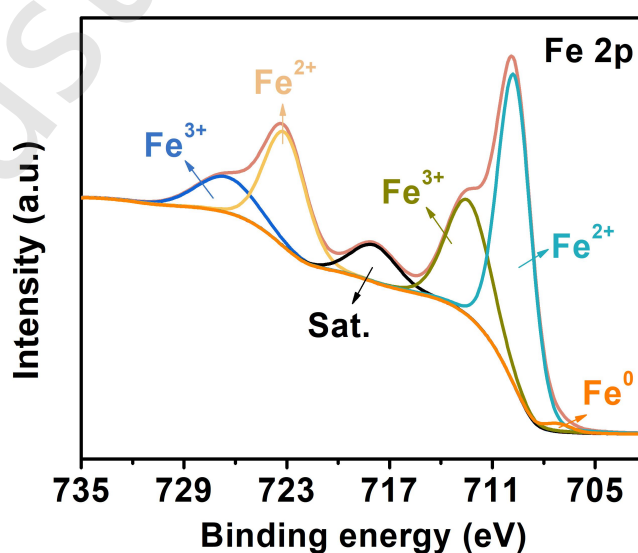
**Figure S16.** ESR spectra of various systems. Reaction condition: 1 mg/mL Fe₃O₄@Fe/C, 50 mM DMPO, or 18 mM H₂O₂ for 5 min under ambient condition.**Figure S17.** Fe 2p high-resolution XPS spectra of Fe₃O₄@Fe/C after the reaction

Table S5. Fe 2p spilt and fitting results of Fe₃O₄@Fe/C before the reaction (derived from Figure 2h).

Parameters	Fe 2p _{3/2} (Fe ³⁺)	Fe 2p _{1/2} (Fe ³⁺)	Fe 2p _{3/2} (Fe ²⁺)	Fe 2p _{1/2} (Fe ²⁺)	Fe ⁰	Sat.
Peak	712.4 eV	726.5 eV	710.7 eV	724.2 eV	707.2 eV	717.9 eV
Integral Area	21972.4	7849.397	49339.41	22723.84	3335.371	7856.903
Proportion	19.43 %	6.94 %	43.63 %	20.10 %	2.95 %	6.95 %

Table S6. Fe 2p spilt and fitting results of Fe₃O₄@Fe/C after the reaction.

Parameters	Fe 2p _{3/2} (Fe ³⁺)	Fe 2p _{1/2} (Fe ³⁺)	Fe 2p _{3/2} (Fe ²⁺)	Fe 2p _{1/2} (Fe ²⁺)	Fe ⁰	Sat.
Peak	712.4 eV	726.5 eV	710.7 eV	724.2 eV	707.2 eV	717.9 eV
Integral Area	19546.2	8160.87	39601.44	18261.93	849.672	8220.21
Proportion	21.03 %	8.78 %	42.61 %	19.66 %	0.91 %	7.02 %

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