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Protocol update

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Optimizing the standardized assays for determining the catalytic activity and kinetics of peroxidase-like nanozymes

Jia-Jia Zheng[1,2,3], Feiyan Zhu[1,3], Ningning Song[1], Fang Deng[1], Qi Chen[1], Chen Chen[1], Jiuyang He[1] ✉, Xingfa Gao[2] ✉ & Minmin Liang[1] ✉

Abstract

Nanozymes are nanomaterials with enzyme-like catalytic properties. They are attractive reagents because they do not have the same limitations of natural enzymes (e.g., high cost, low stability and difficult storage). To test, optimize and compare nanozymes, it is important to establish fundamental principles and systematic standards to fully characterize their catalytic performance. Our 2018 protocol describes how to characterize the catalytic activity and kinetics of peroxidase nanozymes, the most widely used type of nanozyme. This approach was based on Michaelis–Menten enzyme kinetics and is now updated to take into account the unique physicochemical properties of nanomaterials that determine the catalytic kinetics of nanozymes. The updated procedure describes how to determine the number of active sites as well as other physicochemical properties such as surface area, shape and size. It also outlines how to calculate the hydroxyl adsorption energy from the crystal structure using the density functional theory method. The calculations now incorporate these measurements and computations to better characterize the catalytic kinetics of peroxidase nanozymes that have different shapes, sizes and compositions. This updated protocol better describes the catalytic performance of nanozymes and benefits the development of nanozyme research since further nanozyme development requires precise control of activity by engineering the electronic, geometric structure and atomic configuration of the catalytic sites of nanozymes. The characterization of the catalytic activity of peroxidase nanozymes and the evaluation of their kinetics can be performed in 4 h. The procedure is suitable for users with expertise in nano- and materials technology.

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[1]Experimental Center of Advanced Materials, School of Materials Science & Engineering, Beijing Institute of Technology, Beijing, China. [2]Laboratory of Theoretical and Computational Nanoscience, National Center for Nanoscience and Technology of China, Beijing, China. [3]These authors contributed equally: Jia-Jia Zheng, Feiyan Zhu. ✉e-mail: hejiuyang@bit.edu.cn; gaoxf@nanoctr.cn; mmliang@bit.edu.cn

Key points

- Nanozymes are nanoparticles designed to have catalytic properties similar to those of natural enzymes. Design and optimization of nanozyme properties require analytical methods to characterize their physical properties as well as their catalytic activity and kinetics.
- This is an updated protocol for measuring catalytic behavior that incorporates data from measured physical properties unique to each nanoparticle as well as density functional theory calculations into the Michaelis–Menten approach.

Key references

Jiang, B. et al. *Nat. Protoc.* **13**, 1506–1520 (2018): https://doi.org/10.1038/s41596-018-0001-1

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Introduction

In recent decades, nanozymes have been reported as promising alternatives to natural enzymes owing to their high stability, low cost and convenient storage[1,2]. By combining the unique physicochemical properties and enzyme-like catalytic properties, artificial nanozymes have been used in a wide range of applications from in vitro biological detection[3,4] to in vivo disease diagnosis[5–7] and biomedicine development[8–12]. So far, there have been more than 200 research institutions and groups worldwide working on nanozyme research, and hundreds of nanozymes have been developed, covering thousands of nanomaterials[13–17]. Along with the substantial advances and broader applications of nanozymes as a main surrogate of natural enzymes, the fundamental principles, basic concept definitions and systematic standards should be strictly established to fully characterize their catalytic performance. Especially with the emergence of 'nanozymology'[18], it is urgent to develop nanozymes in unique nanozymological methods to drive the scientific development of nanozyme research.

In 2018, we reported the first protocol to characterize the catalytic activity and kinetics of peroxidase nanozymes[19]. We showed the dependency of the reaction rate on the concentrations of the substrates of peroxidase nanozymes, making it possible to detect the kinetic activities of peroxidase nanozymes. However, the unique physicochemical properties of nanomaterials that ultimately determine the catalytic activity and kinetics of artificial nanozymes are not involved in the kinetic equation. In this protocol, we provide an improved approach that better describes the kinetic activities of peroxidase nanozymes with various shapes, sizes and compositions by combining both the enzyme-based Michaelis–Menten kinetics and the physicochemical properties of nanozymes. The approach facilitates the appropriate evaluation of catalytic performance among different nanozymes and will benefit the future development of nanozymology, from basic research to practical applications.

Overview of the procedure

This protocol provides a step-by-step procedure to characterize the catalytic activity and kinetics of peroxidase nanozymes (Fig. 1). The calculations take into account measurable physicochemical properties:

- The number of active sites can be determined by measuring the adsorption of CO molecules on the surface of nanozymes, using the temperature-programmed CO desorption method (Part 1, Steps 1–8, Table 1) (Supplementary Fig. 1).
- Other physicochemical properties such as surface area, shape, and size can be characterized using conventional Brunauer–Emmett–Teller (BET) and transmission electron microscope (TEM) methods (Part 1, Steps 9–10).

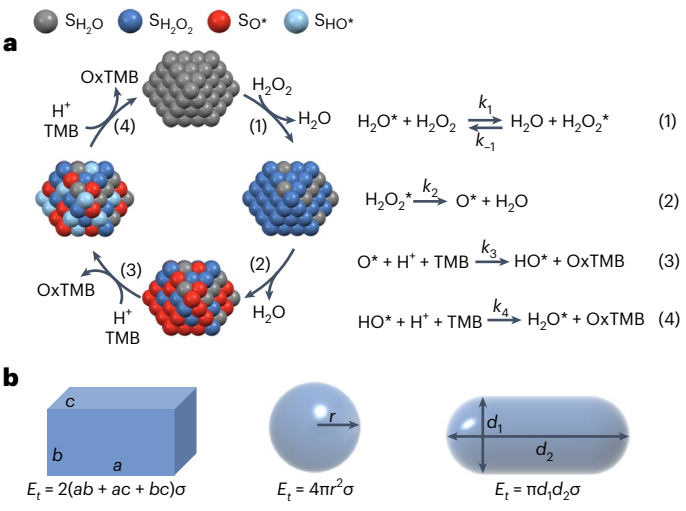


Fig. 1 | Reaction pathways for peroxidase nanozymes of different physicochemical properties. a, Elementary steps for the typical TMB oxidation reaction catalyzed by peroxidase nanozyme, where H_2O_2 serves as the oxidant. S_{H2O}, S_{H2O2}, S_O, and S_{HO}, represent the active sites occupied by H_2O, H_2O_2, O radical and OH radical, respectively. **b**, A schematic representation of nanozymes with different sizes and shapes. σ represents the number of catalytic sites per unit area (mol per m^2). E_t represents the total number of catalytic sites on the surface of nanozymes (mol).

Table 1 | General guideline for determining surface active sites

Methods	Advantages	Reference
CO adsorption measurement	Widely used technique to characterize active sites of nanozymes	25–27
	Each CO molecule occupies one catalytic site via chemisorption	
	Measure CO adsorption amount to estimate total catalytic sites on the surface of nanozymes	
Inductively coupled plasma optical emission spectrometry	Provide quantitative information on metal active sites of nanozymes	28
	Effective when all surface metal atoms are active and participate in the reaction	
XPS	This technique is useful for carbon-based nanozymes to analyze the chemical state and composition of the surface. This analysis can further estimate the number of catalytic sites	29
X-ray absorption near-edge structure/extended X-ray absorption fine structure	For single-atom nanozymes, these techniques offer insights into local atomic structure and coordination environment around specific atoms. These methods help identifying and quantifying single atom active sites	29
Mathematical counting method	Estimate active sites based on structure, composition, and reactivity of specific sites	30

The peroxidase-like catalytic activity of peroxidase nanozymes is tested by monitoring the oxidation of peroxidase substrates, such as 3,3,5,5-tetramethylbenzidine (TMB), 3,3′-diaminobenzidine(DAB), *o*-phenylenediamine (OPD) and 2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), in the presence of H_2O_2, resulting in unique color reactions (Part 2, Steps 11–17 and the 'Troubleshooting' section) (Fig. 2a–c).

These data are used to determine the specific activity (SA) of peroxidase nanozymes (Part 2, Steps 17 and 18) (Fig. 2d) and to characterize their catalytic kinetics (Part 3, Steps 20–28) (Fig. 3) using the nanozyme-catalyzed colorimetric reaction; in contrast with the 2018 protocol, these kinetic equations are adjusted to incorporate the physicochemical measurements where meaningful.

Three representative peroxidase nanozymes, including Fe_3O_4 nanoparticles (NPs), Pt NPs and Pt-atom centered single-atom nanozyme (Pt-SAzyme), are used as examples to present how to perform the procedure.

The last section of the procedure describes how computational methods performed on crystal structure data can be used to access the catalytic kinetics of nanozymes, which are useful for understanding the peroxidase-like catalytic activity of nanomaterials and designing better peroxidase-like nanozymes (Part 4, Steps 29–35) (Fig. 4 and Table 2).

Development of the protocol

Nanomaterials display peroxidase-like activity by catalyzing the oxidation of peroxidase substrates (e.g., TMB, DAB or OPD) with H_2O_2 to produce colorimetric reactions. As shown in Fig. 1a, colorimetric reactions catalyzed by peroxidase nanozymes are heterogeneous reactions occurring on the surface of nanomaterials. The microkinetic reaction pathway takes into account the following considerations:

1. The surface of nanozymes is covered by H_2O molecules before the reaction in an aqueous solution.
2. H_2O_2 molecules are adsorbed onto the surface of nanozymes by replacing H_2O molecules along with the addition of H_2O_2. This reaction reaches equilibrium with an equilibrium constant K_1.
3. The adsorbed H_2O_2 molecules are decomposed into O radicals (O*).
4. Radicals react with H^+ and TMB forming product OxTMB.

In the steady-state approximation[20,21], reactions 2–4 exhibit the rate constants of k_2, k_3 and k_4, respectively. The reaction rate (v_0) catalyzed by peroxidase nanozymes can be then represented by equation 1.

$$v_0 = \frac{C_1[H_2O_2][TMB][E_t]}{C_2[H_2O_2] + C_3[TMB] + C_4[H_2O_2][TMB]} \quad (1)$$

where $C_1 = K_1k_2k_3k_4[H^+]$, $C_2 = K_1k_2(k_3 + k_4)$, $C_3 = k_3k_4[H_2O][H^+]$ and $C_4 = K_1k_3k_4[H^+]$; $[H_2O]$, $[H^+]$, $[H_2O_2]$ and [TMB] are concentrations of substrates H_2O, H^+, H_2O_2 and TMB, respectively; $[E_t]$ represents

the total number of catalytic sites on the surface of the nanozymes (see Supplementary Note 1 for details). In an aqueous solution, the concentration of H_2O ($[H_2O]$) is almost constant during the reaction. In addition, pH may influence the stability of nanozymes, but it is difficult to be considered in deriving the microkinetics. Therefore, $[H_2O]$ and $[H^+]$ are taken as constants in equation 1.

Equation 1 shows the same form as that derived for an enzyme-catalyzed reaction with the ping-pong mechanism, confirming that the reaction catalyzed by peroxidase nanozymes occurs with the same mechanism[22]. Specifically, by fixing the concentration of one substrate ($[H_2O_2]$ or [TMB]), one can obtain the dependency of the reaction rate on the other one in the form of the Michaelis–Menten equation, as represented by equations 2 and 3.

$$\upsilon_0\,(H_2O_2) = \frac{\frac{C_1[TMB][E_t]}{C_2+C_4[TMB]}[H_2O_2]}{\frac{C_3[TMB]}{C_2+C_4[TMB]}+[H_2O_2]} = \frac{\upsilon_{max}^{H_2O_2}[H_2O_2]}{K_m^{H_2O_2}+[H_2O_2]} \quad (2)$$

$$\upsilon_0(TMB) = \frac{\frac{C_1[H_2O_2][E_t]}{C_3+C_4[H_2O_2]}[TMB]}{\frac{C_2[H_2O_2]}{C_3+C_4[H_2O_2]}+[TMB]} = \frac{\upsilon_{max}^{TMB}[TMB]}{K_m^{TMB}+[TMB]} \quad (3)$$

These equations show the fundamental reasons why the Michaelis–Menten equation should be employed to describe the kinetics of peroxidase nanozymes for their substrates, H_2O_2 and TMB.

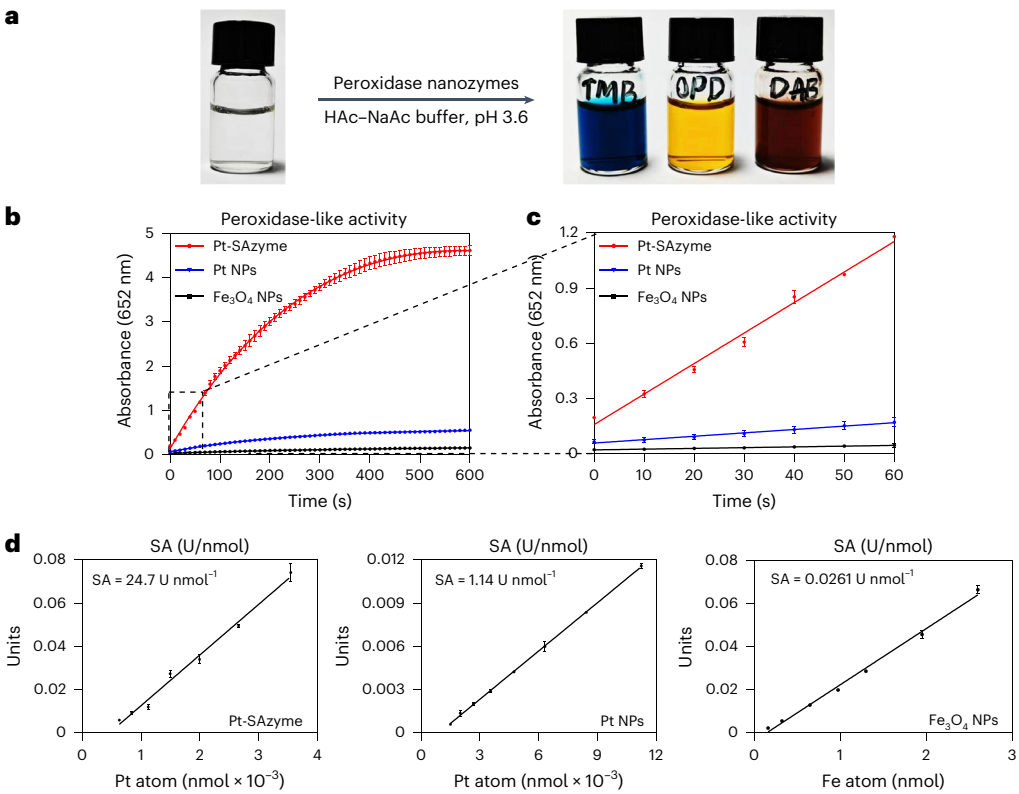
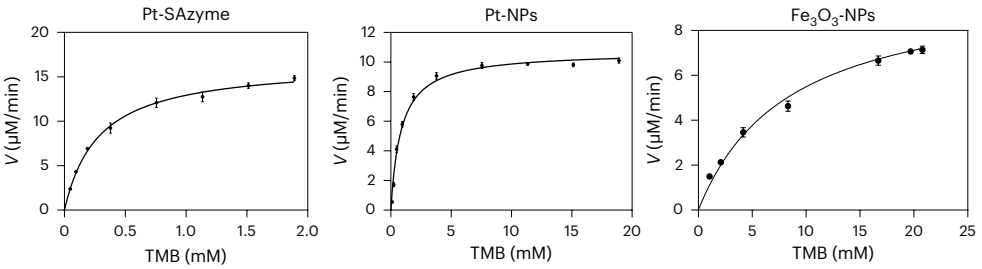


Fig. 2 | Peroxidase-like catalytic activity characterization using this updated protocol. a, Fe_3O_4 NPs, Pt NPs or Pt-SAzyme show the typical peroxidase-like activity by catalyzing the oxidation of peroxidase substrates (TMB, DAB and OPD) to produce the unique color reactions. **b**, The reaction–time curves of TMB color reaction catalyzed by peroxidase Fe_3O_4 NPs, Pt NPs or Pt-SAzyme. **c**, The magnified initial linear portion of the reaction–time curves. A length of 60 s was chosen for the initial rate period because the R^2 coefficients were close to 1 during this period after a linear-regression analysis. Absorbance values are measured in arbitrary units. **d**, Specific activities (U/nmol active site) of the measured nanozymes. $n = 3$ independent measurements for all bars in **b**–**d**, error bars represent mean ± s.d.).



Nanozymes	[E/active site]	K_m	v_{max}	k_{cat}	k_{cat}/K_m
	(M)	(mM)	($M\ s^{-1}$)	(s^{-1})	($M^{-1}\ s^{-1}$)
Pt-SAzyme	8.50×10^{-10}	0.282	2.76×10^{-7}	325	1.15×10^{6}
Pt NPs	2.00×10^{-8}	0.847	1.78×10^{-7}	8.90	1.05×10^{4}
Fe_3O_4 NPs	1.15×10^{-7}	0.841	1.68×10^{-7}	1.46	1.74×10^{3}

Fig. 3 | Catalytic kinetics characterization of the peroxidase Fe_3O_4 NPs, Pt NPs and Pt-SAzyme using this protocol. The initial reaction velocity (v) was measured in 0.2 M NaAc/HAc buffer pH 3.6 at 37 °C. The concentration of H_2O_2 was fixed at 1.0 M, which is excessive to ensure that the tested nanozymes in the reaction solution are saturated with H_2O_2 substrate. TMB concentrations were varied ($n = 3$ independent measurements, error bars represent mean ± s.d.). [E] is the concentration of active sites on the tested nanozymes. The molar concentration of active sites is determined by the CO adsorption method. K_m is the Michaelis constant, v_{max} is the maximal reaction velocity and k_{cat} is the catalytic constant, where $k_{cat}=v_{max}/[E]$ and the k_{cat}/K_m value indicates the peroxidase-like catalytic efficiency of the tested nanozymes.

In the 2018 protocol[19], the kinetic parameters (v_{max} and K_m) for the nanozymes were determined by measuring the reaction rates against the concentrations of the substrates using the NP concentration as nanozyme concentration[19]. This approach determines the overall kinetics of nanozymes without considering the influence of their physicochemical properties. This is not true, and their physicochemical properties can vary greatly. This means that the Michaelis–Menten equation derived originally for natural enzymes is not adequate for studying nanozyme kinetics without considering their physicochemical properties.

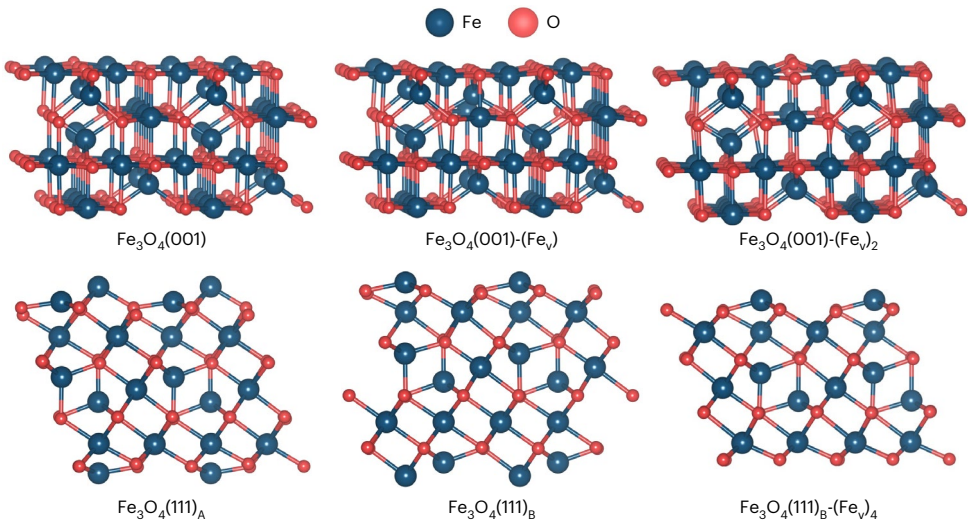


Fig. 4 | Slab models of Fe_3O_4 nanozymes. Two different exposed surfaces of Fe_3O_4 NPs were considered. For the Fe_3O_4 (111) surface, there are two possible surface structures (Fe_3O_4 (111)$_A$ and Fe_3O_4 (111)$_B$), with different numbers of exposed iron sites. As iron vacancy may exist in the synthesized Fe_3O_4 NPs, surface structures with different numbers of iron vacancies were also considered. The coordinates of these structures are given in Supplementary Data 1.

Table 2 | Adsorption energies of H_2O_2 and OH on different exposed surfaces of Fe_3O_4 nanozymes

	$E_{ads,H2O2}$ (eV)	$E_{ads,OH}$ (eV)
$Fe_3O_4(001)$	−1.22	−3.28
$Fe_3O_4(001)$-(Fe_v)	−0.77	−2.41
$Fe_3O_4(001)$-$(Fe_v)_2$	−0.93	−1.96
$Fe_3O_4(111)_A$	Dissociation[a]	−3.78
$Fe_3O_4(111)_B$	−1.19	−3.42
$Fe_3O_4(111)_B$-$(Fe_v)_4$	−1.36	−3.15

[a]Dissociation means that the H_2O_2 molecule cannot form a stable adsorption structure on $Fe_3O_4(111)_A$ and undergone the dissociation reaction to form two adsorbed OH groups once approaching the nanozyme surface.

Therefore, further efforts were made in this work to establish a connection between the physicochemical properties of nanozymes and their catalytic kinetics. Taking H_2O_2 as the substrate, for example, the initial reaction rate (v_0) can be represented by equation 4, and the v_{max} and K_m for H_2O_2 can thus be represented by equations 5 and 6 (refs. 23,24) (see Supplementary Note 2 for details).

$$v_0(H_2O_2) = ms\sigma \frac{A_1 e^{\frac{-\Delta G^{\neq}}{RT}}[H_2O_2]}{A_2 e^{\frac{\Delta G_1}{RT}} + [H_2O_2]} \quad (4)$$

$$v_{max}^{H_2O_2} = ms\sigma A_1 e^{\frac{-\Delta G^{\neq}}{RT}} \quad (5)$$

$$K_m^{H_2O_2} = A_2 e^{\frac{\Delta G_1}{RT}} \quad (6)$$

where m and s are the mass and specific surface area of nanozymes, σ is the average number of catalytic sites per surface area ($[E_t] = ms\sigma$), ΔG_1 is the Gibbs free energy change for H_2O_2 adsorption on the nanozyme surface, $\Delta G^{\neq}$ is the activation energy of the rate-determining step, R and T are the ideal gas constant and temperature, A_1 and A_2 are quantities whose formulation depends on the rate-determining step. Similarly, the Michaelis–Menten kinetics, v_{max} and K_m for TMB as the substrate can be represented by equations 7, 8 and 9.

$$v_0(TMB) = ms\sigma \frac{A_3 e^{\frac{-\Delta G^{\neq}}{RT}}[TMB]}{A_4 e^{\frac{\Delta G_1}{RT}} + [TMB]} \quad (7)$$

$$v_{max}^{TMB} = ms\sigma A_3 e^{\frac{-\Delta G^{\neq}}{RT}} \quad (8)$$

$$K_m^{TMB} = A_4 e^{\frac{\Delta G_1}{RT}} \quad (9)$$

Equations 4–9 demonstrate that the kinetic parameters of nanozymes, usually experimentally determined[1,16,19], are actually influenced by both their physical properties (m, s and σ) and the abilities (ΔG_1 and $\Delta G^{\neq}$) to catalyze chemical reactions. Therefore, in this updated protocol, not only the enzyme-based Michaelis–Menten kinetics but also the physicochemical properties of nanozymes are used to characterize the catalytic kinetics of peroxidase nanozymes with various shapes, sizes and compositions.

The apparent catalytic kinetics (v_{max} and K_m) can be characterized as previously described[19]. However, to incorporate the effects of physicochemical properties of nanozymes on their catalytic kinetics, the mass, specific surface area and the average number of catalytic sites per surface area, contributing to the total number of catalytically active sites ($[E_t]$), should also be characterized. For example, the CO uptake and desorption analysis[25,26] can be conducted to determine the Carbon monoxide (CO) adsorption amount, which is closely related to the $[E_t]$ of nanozymes. More specifically, the mass and specific surface area can be routinely

characterized. With these physical parameters, the average number of catalytic sites per surface area (σ) can be easily determined. As σ could be a constant for one nanomaterial with a specific shape, a database of σ for different nanozymes can be constructed. With the database of σ, one may roughly estimate [E_t] of a nanozyme by its shape and size, as shown in Fig. 1b. Therefore, it is anticipated that the implementation of this updated protocol will benefit the establishment of structure–activity relationships in nanozymes and improve the development of nanozyme research. This updated protocol is mainly applicable to all the peroxidase nanozymes with a determinable number of active sites.

Applications of the protocol

The method presented here describes the peroxidase-like catalytic activities of nanozymes by measuring the kinetics for a single site. Peroxidase-like activities have been found in hundreds of nanomaterials, including metals, metal oxides and carbon.

An important application of this updated approach is to re-evaluate nanozymes where the kinetics were measured using methods that assumed that all NP catalytic sites had the same properties; the results obtained using these methods may not represent their intrinsic catalytic abilities as peroxidase mimics.

Further development of nanozymes requires precise control of their activity by engineering the electronic, geometric structure and atomic configuration of the active atoms; thus, determining the activity of a single site becomes essential. As the methods to determine the catalytic activity have been previously described[19] and the measurement of the number of active sites and surface areas are straightforward, this protocol will be easily and broadly applied in this field. In addition, our method can be used to determine the density of catalytic sites of σ on nanozymes. A database of σ for different nanomaterials can be constructed with the broad application of the present method. This will simplify the procedures to determine the kinetics of a single site and promote fundamental research on the structure–activity relationship in both experiment and theory.

Limitations of the protocol

This updated standardized method has similar limitations to the previous technique[19], including the applicability and possible interference of NPs themselves in assessing catalytic kinetics while using the nanozyme-catalyzed colorimetric reaction. First, this updated technique has been specially developed for peroxidase nanozymes, and is thus not applicable to other nonperoxidase nanozymes, such as oxidase nanozyme, superoxide dismutase nanozyme, catalase nanozyme, hydrolase nanozyme, haloperoxidase nanozyme and many others that have been reported. Instead, it is a standardized technique to characterize the catalytic kinetics of a single site of peroxidase nanozymes, which can be extended to other types of nanozymes to establish standardized methods. Secondly, the absorption spectrum of some NPs may overlap with that of substrates. To overcome such interference of NPs in determining catalytic kinetics, two methods have been suggested previously: (i) subtracting the background absorbance from the NPs and (ii) using other colorimetric substrates with no overlapping absorption.

Another possible limitation of this updated technique is the determination of the number of catalytic sites, which is evaluated through the measurement of CO molecule adsorption on the NPs (CO adsorption and temperature-programmed CO desorption). This standardized method is not universally applicable to all nanozymes, but principally applicable to metal-based nanozymes, such as metal, metal oxide and single-/dual-atom nanozymes, which have high affinities to CO molecules. Alternatively, the direct temperature-programmed desorption (TPD) technique can be used to determine the number of catalytic sites on carbon nanozymes with low CO affinity, by decomposing the carbon nanozymes and measuring the quantity of probe molecules.

Experimental design

Characterization of the number of active sites on nanozymes

CO adsorption measurement is a widely used technique to characterize the number of active sites of NPs, which is also applicable for nanozymes. In an ideal case, each CO molecule will

occupy one catalytic site via chemisorption. Therefore, the total number of catalytic sites of nanozymes (E_t) can be determined by measuring the CO adsorption amount (n_{CO}, mol/g) on the surface of nanozymes, conducted in a U-shaped quartz reactor inside a CO-TPD instrument equipped with a thermal conductivity detector (TCD) and mass spectrometer[25,26]. The synthesized nanozyme samples (100–150 mg) can be subjected to sequential cleaning (thermal annealing), CO uptake (CO pulse chemisorption at −80 °C), and desorption (temperature-programmed CO desorption) cycles. These three phases can be repeated for each annealing temperature up to a maximum of 800 °C. CO adsorption amounts (n_{CO}, mol/g) can be determined by integral areas of TPD peak, which will steadily grow with the annealing temperature and eventually become constant. The number of active sites can then be obtained according to E_t [site] = $m \times n_{CO}$ [mol g^{-1}] × N_A [site mol^{-1}], where m is the mass of the nanozyme and N_A represents the Avogadro's constant. This method is adaptable to metal-containing nanozymes, such as metal, metal oxide and single-/dual-atom nanozymes with a high affinity to CO molecules. On the other hand, nanozymes with low affinity to CO molecules, such as carbon nanomaterials cannot be measured by this method, because the active sites of carbon nanozymes are usually heteroatoms or functional groups, which can be decomposed into some heteroatom-containing species such as CO, CO_2, CN, HCN, CS and SO_2 upon heating. In such cases, the aforementioned third step, namely TPD, can be adapted to determine the number of active sites by integral areas TPD peaks for heteroatom-containing species, such as CO and CO_2 (for O sites, CN and HCN for N sites, as well as CS and SO_2 for S sites[27].

Many other methods can be employed to determine these active sites. For example, inductively coupled plasma optical emission spectrometry can provide quantitative information on the metal active sites present in nanozymes when all the surface metal atoms are active and participate in the reaction[28]. For carbon-based nanozymes, X-ray photoelectron spectroscopy (XPS) can also provide information about the chemical state and composition of the nanomaterial's surface. By further analyzing the XPS spectra, the number of catalytic sites can be estimated. For single-atom nanozymes, X-ray absorption spectroscopy techniques, such as X-ray absorption near-edge structure or extended X-ray absorption fine structure, can provide valuable information on the local atomic structure and coordination environment around specific atoms. This can help in identifying and quantifying single-atom active sites[29]. In addition, the number of active sites can also be roughly estimated using a mathematical counting method based on the nanozyme's structure, composition and known reactivity of specific sites[30]. The choice of method usually depends on the nature of the nanozymes, the accessibility of active sites and the desired level of accuracy and sensitivity required for the measurement. Generally, a combination of techniques is usually used to ensure a more reliable determination of the active sites on nanozymes (Table 1).

Characterization of the catalytic activity and kinetics of peroxidase nanozymes

As recommended in our previous protocol[19], the measurement of catalytic activity and kinetics is performed at 37 °C, where the natural horseradish peroxidase has an optimal activity. The kinetic constants can be characterized by measuring the reaction rate against the concentration of the substrates according to the Michaelis–Menten equation, as shown in equations 2 and 3. The steps used to perform the peroxidase nanozyme-catalyzed reactions and calculate their catalytic activity are the same as those described in the 2018 protocol[19].

The reaction velocities are recorded by fixing the concentration of one substrate while varying that of the other one. Then, the kinetic constants can be obtained by the fitted Michaelis–Menten equation for the reaction velocity and the initial substrate concentration (refer to the 'Development of the protocol' section). Equations 2 and 3 indicate that K_m is independent of the number of active sites (E_t), but v_{max} depends on E_t. We therefore define the catalytic rate constant as $k_{cat} = v_{max}/[E_t]$.

The SA is thus defined as the activity units (U) per active site of nanozyme (rather than per unit mass of nanozyme, as in the 2018 protocol). The catalytic efficiency per single site can then be compared for different nanozymes.

Computational kinetics for a single site of peroxidase nanozymes

According to equations 4 and 7, the experimentally measured K_m and υ_{max} values of nanozymes for both substrates depend on the adsorption energy of H_2O_2 ($E_{ads,H2O2}$) and the activation energy (E_a) for the elementary step (equations 18–19 and 21–22 in Supplementary Note 2), as well as the unique physicochemical properties (m, s and σ) of nanozymes.

Our previous density functional theory (DFT) studies have demonstrated that the value of E_a decreases and then increases as the value of $E_{ads,OH}$ increases, with a minimum at an $E_{ads,OH}$ value of −2.6 eV, indicating an inverted volcano relationship between E_a and $E_{ads,OH}$ (refs. 31,32). $E_{ads,H2O2}$ and $E_{ads,OH}$ can thus be used to infer the kinetics for a single site of peroxidase nanozymes, which can be experimentally confirmed using this updated protocol. Therefore, these calculated DFT values and the intrinsic physicochemical properties of nanozymes can be used together with the experimentally measured Michaelis–Menten kinetics to characterize the catalytic performance of peroxidase nanozymes. The crystal structures are necessary to calculate these energy values.

The Vienna Ab initio Simulation Package (VASP 5.4.4)[33,34] can be employed to calculate $E_{ads,OH}$ for nanozymes from their crystal structures using the following computational settings:

(1) The Perdew–Burke–Ernzerhof functional[35] with Grimme's semi-empirical 'D3BJ' dispersion correction[36,37].
(2) The plane wave basis sets[38] with an energy cut-off of 450 eV for valence electrons.
(3) The projector-augmented wave pseudopotentials[39] for core electrons.
(4) The convergence criteria for energy and atomic force of 10^{-5} eV and 0.02 eV/Å, respectively.
(5) The Brillouin zone sampled via a 3 × 3 × 1 Monkhorst-Pack[40] k-point mesh.

The adsorption energy of molecular adsorbates (H_2O_2 and HO) can be obtained using equation (10) after performing geometry optimizations using the aforementioned computational settings:

$$E_{ads,mol} = E_{mol@surface} - E_{surface} - E_{mol} \quad (10)$$

where $E_{mol@surface}$, $E_{surface}$ and E_{mol} are energies of the surface with molecular adsorbate, separated surface and molecular adsorbate, respectively. A workflow to perform such calculations is available at https://github.com/xingfagao/PyPOD.

Materials

Reagents

▲ **CAUTION** Always handle potentially harmful reagents in a fume hood while wearing appropriate protective equipment (e.g., wear gloves, protective eyewear and a laboratory coat).

- Acetic acid (HAc) (Adamas-beta, cat. no. 64-19-7)
- NaAc (Merck, cat. no. 127-09-3)
- KCl (HUSHI, cat. no. 7447-40-7)
- NaCl (HUSHI, cat. no. 7647-14-5)
- Na_2HPO_4 (HAWN, cat. no. 7558-79-4)
- KH_2PO_4 (Aladdin, cat. no. 7778-77-0)
- NaOH (Sigma-Aldrich, cat. no. S8045)
- Chloroplatinic acid hexahydrate ($H_2PtCl_6{\cdot}6H_2O$) (Sigma-Aldrich, cat. no. 18497-13-7)
- Poly (*N*-vinyl-2-pyrrolidone) (PVP, M_W = 30,000) (Thermo Scientific, cat. no.9003-39-8)
- Acetone (TGREAG, cat. no.67-64-1)
- Methanol (TGREAG, cat. no. 67-56-1)
- PtO_2 (Macklin, cat. no. 1314-15-4)
- TMB (Aladdin, cat. no. 54827-17-7)
  ▲ **CAUTION** TMB is flammable and toxic by inhalation, in contact with skin and if swallowed. Avoid prolonged or repeated exposure.

- DAB (J&K Scientific, cat. no. 868272-85-9)
  ▲ **CAUTION** DAB is flammable and toxic by inhalation, in contact with skin and if swallowed. Avoid prolonged or repeated exposure.
- OPD (J&K Scientific, cat. no. 95-54-5)
  ▲ **CAUTION** OPD is toxic for inhalation, ingestion and skin contact. Avoid prolonged or repeated exposure. Wash thoroughly after handling.
- Hydrogen peroxide 30% (wt/vol) aqueous solution (Sinopharm chemical reagent Beijing, cat. no. 7722-84-1)
  ▲ **CAUTION** Hydrogen peroxide is highly irritant to eyes, skin and upon ingestion.
- HNO_3 (Sinopharm Chemical Reagents, cat. no. 7697-37-2)
  ▲ **CAUTION** Causes severe burns. Do not inhale the vapor. Avoid contact with eyes, skin and clothing. Avoid prolonged or repeated exposure.
- Dimethyl sulfoxide (DMSO) (HAWN, cat. no. 67-68-5)
  ▲ **CAUTION** DMSO is harmful if inhaled or absorbed through the skin. It is flammable.
- HCl (TGREAG, cat. no. 7647-01-0)
  ▲ **CAUTION** Causes severe burns. Do not inhale the vapor. Avoid contact with eyes, skin and clothing. Avoid prolonged or repeated exposure.
- Helium (He) gas (Air Liquide, 0523-He-01)
- Carbon monoxide (CO) in He (1.985% (vol /vol)), (Air Liquide, L214506029)
  ▲ **CAUTION** Careful handling of the gas cylinder is necessary due to the toxic and potentially flammable nature of CO gas. Adhere to appropriate safety protocols while working with CO gas, including proper ventilation and approved gas handling procedures.

NPs

- 100 nm Fe_3O_4 NPs (Nanjing Nanoeast Biotech, cat. no. Mag9000)
- In-house made Pt NPs, see 'Reagent setup'
- In-house made Pt single atom NPs, see 'Reagent setup'

Equipment

- UV–visible spectrometer (Hitachi, model U-3900)
- CO-TPD instrument (Micromeritics, model Autochem II 2920)
- Thermostatic water bath (LICHEN, model no. HH-2)
- TEM (JEOL, model JEM-1400)
- Forced-air drying oven (Tianjin Taisite Instruments, model 101-2A)
- Avanti J-E centrifuge (Beckman Coulter, model no. 369001)
- Thermometer (Beijing Dihui Technology)
- Biochemistry Cultivation Cabinet (Shanghai Bluepard Instruments)
- 96-well 'U'-bottom plate (Corning, cat. no.2797)
- Immunochramato Reader HR201 (Shenzhen Highcreation Technology, model HR201)
- IsoFlow Dispenser (Imagene Technology)
- Color reader (Shanghai Kinbio Tech., Model DT2050)
- 10-1,000 µL pipette tips (Thermo Fisher Scientific)
- 10-1,000 µL mechanical pipette (Eppendorf)
- Microplate shaker (Thermo Fisher Scientific, cat. no. 88-861-023)
- Software (the Vienna Ab initio Simulation Package, VASP 5.4.4; Materials Studio Visualizer; Visualization for Electronic and STructural Analysis, VESTA)
- Hardware (high-performance computers, Sogon, cat. no. X86-2H3)
- BET surface area analyzer (Quantachrome, Model Nova 3200e)

Reagent setup

5% (wt/vol) BSA–PBS solution

Dissolve 5 mg of BSA powder in 100 mL PBS. This can be stored at 4 °C for a maximum of 3 months.

Preparation of Pt-SAzyme

1. Prepare the precursor powders by mixing 50 mg of PtO_2 NPs with 1 g of aluminum alloy powders using a ball milling process.
2. Add 1 g of the prepared precursor powders to 10 mL of a 4 % (wt/vol) saline solution and allow it to react for 2 h with gentle stirring.
3. Centrifuge the resulting suspension at 5,000*g* for 10 min at 4 °C and wash them five times with deionized water.
4. Collect the prepared Pt-SAzymes through freeze drying and suspend them in 1 mL of 5% (wt/vol) BSA–PBS solution. Store the Pt-SAzyme at 4 °C for a maximum of 3 months.

Preparation of Pt NPs

1. Dissolve 49 mg of $H_2PtCl_6 \cdot 6H_2O$ and 133 mg of PVP (Mw = 30,000) in 180 mL of methanol in a 500 mL mouth flask and then reflux at 80 °C for 3 h.
2. After natural cooling to room temperature (20–30 °C), precipitate the Pt NPs by acetone overnight.
3. Collect Pt NPs by centrifugation at 6,000*g* for 10 min.
4. Disperse Pt NPs in methanol to form a dispersion solution of 1 mg/mL. Store the Pt NPs at 4 °C for a maximum of 3 months.

TMB/OPD/DAB solution

Dissolve TMB, OPD or DAB in DMSO to a concentration of 10 mg/mL.

The prepared colorimetric solutions are light sensitive and should be handled in the dark and used only on the day of preparation.

NaAc–HAc buffer

Prepare a reaction solution containing 0.2 M HAc and 0.2 M NaAc in 250 mL deionized water and adjust the final pH to 3.6. The buffer can be stored at 4 °C for up to 3 months.

Equipment setup

CO-TPD instrument

The Autochem II 2920 is used to measure the active sites of nanozymes. Pretreat the nanozyme sample at 300 °C for 30 min, followed by cooling to 30 °C and purging in He gas for 30 min. The furnace temperature is then cooled to −50 °C, and a gas flow of 2% (vol/vol) CO/He is introduced. Record the mass spectrometry (MS) signals for $m/z = 2$, $m/z = 28$ and $m/z = 44$. Allow the nanozyme sample to adsorb CO for ~30 min, then turn off CO and purge the samples in He for ~30 min. As the temperature ramps up from −50 to 300 °C, the CO signal is measured using a TCD.

Procedure

Part 1: characterization of active site using CO-TPD technique, surface area using BET technique, and shape and size of nanozymes using TEM

● **TIMING** ~28 h

1. Load ~18 mg of the NP sample to be tested onto an appropriate sample holder.
   ▲ **CRITICAL STEP** Ensure that the samples are thoroughly cleaned and free from any contaminants before loading. Contaminants can adsorb to the surface, either competing with CO or altering the surface properties of the tested samples.
2. Pretreat the sample by treating in Ar gas at 300 °C for 30 min (temperature ramping from room temperature from 20 to 30 °C to 300 °C at 10 °C/min).
3. Cool down to 30 °C, purge the sample in He for 30 min, and then further cool down to −50 °C for ~30 min.
   ▲ **CRITICAL STEP** Ensuring sufficient cooling time is important to achieve accurate, reliable and reproducible results. Insufficient cooling can lead to thermal fluctuations

that destabilize the baseline, making it difficult to distinguish between the background signal and the actual sample peaks.

4. Change the gas flow to 1.985% (vol/vol) CO/He and start recording the MS signal of $m/z = 28$, which represents the CO signal. Collect the data on temperature, gas flow rates and desorption profiles throughout the experiment.
5. Allow the sample to adsorb CO for ~30 min. Then, switch off the supply of CO and subsequently purge the sample with He gas for ~30 min.
   ▲ **CRITICAL STEP** Dose the CO gas onto the sample surface while maintaining a constant carrier gas flow. A constant flow of carrier gas ensures that the baseline thermal conductivity, which is critical for comparisons, remains stable. Fluctuations in carrier gas flow can lead to variations in baseline readings, which can skew the detector's response to the presence of CO gas and other components. known gas flow.
6. As the temperature ramps up from −50 to 300 °C, measure the CO signal by TCD.
7. Using the calculated CO amount in Step 4 corresponding to the peak of CO in He gas, calculate the CO amount corresponding to the desorption peak area (refer to Supplementary Fig. 1).
8. Calculate the number of active sites by the amount of CO desorbed according to the following equation:

$$E_t\,[\text{site}] = m \times n_{CO}\,[\text{mol}/g^{-1}] \times N_A$$

   where m is the mass of nanozyme, N_A represents the Avogadro's constant and n_{CO} is the desorbed amount of CO per mass of nanozyme (mol g^{-1}).
9. Determine the surface area of the tested nanozymes using the conventional BET method. To do this:
   - degas the sample in a flow of inert gas at a high temperature to ensure it is free of adsorbed gases and moisture.
   - cool the sample to liquid nitrogen temperature.
   - introduce an inert adsorbate gas, such as nitrogen, to the sample.
   - incrementally increase the pressure of the gas to formulate an adsorption isotherm.
   - at each pressure increment, allow the system to reach equilibrium where no more gas is adsorbed.
   - record the volume of gas adsorbed at each pressure step.
   - calculate the specific surface area of the sample using the volume of gas obtained from the BET plot.
10. Determine the shape and size of the tested nanozymes using the conventional TEM method. To do this:
    - place a small droplet of the NP suspension on a carbon-coated copper grid and dry at room temperature before imaging.
    - analyze the nanozyme using TEM operating at an accelerating voltage of 100–300 kV.
    - capture images at various magnifications to visualize the size and shape of the NPs.

Part 2: characterization of the peroxidase-like activity of nanozymes

● **TIMING ~2 h**

▲ **CRITICAL** This section of the procedure is analogous to Steps 1–8 of the 2018 protocol. The important difference relates to the equation used to determine the SA of the enzyme. Here, the SA is related to the number of active sites (nmol) of nanozyme where in the previous version, the divisor was the weight of the nanozyme sample.

11. Dissolve six to ten different amounts of the nanozyme samples (from 0 to 20 μg) in 2 mL of NaAc–HAc buffer (0.2 M, pH 3.6) in tubes.
    ▲ **CRITICAL STEP** As the reaction activity and the amount of nanozyme have a linear relationship, an appropriate nanozyme concentration range should be chosen to obtain an accurate nanozyme activity curve. The nanozyme concentration should be much lower (at least tenfold) than the sum of the substrate concentration and the K_m to ensure the validity of the Michaelis–Menten equation[41,42].

▲ **CRITICAL STEP** The peroxidase-like activity of the nanozymes is highly dependent on pH. In this assay, the NaAc–HAc buffer (0.2 M, pH 3.6) is optimal for determining the peroxidase-like activity of nanozymes ('Reagent setup').

12. Add 100 µL of TMB solution to each nanozyme solution and mix by vortexing.
▲ **CRITICAL STEP** The TMB colorimetric solution is light sensitive and thus should be handled in the dark and used only on the day of preparation.
▲ **CRITICAL STEP** The TMB concentration used for measuring the activity of nanozymes should greatly exceed that of the nanozyme to ensure that the nanozymes added in the reaction solution are saturated with TMB substrate. Generally, the TMB concentration should be five times greater than the Michaelis constant (K_m) of the measured nanozyme.
▲ **CRITICAL STEP** ABTS (λ_{max} = 420 nm) and OPD (λ_{max} = 417 nm) colorimetric substrates can be used when the tested peroxidase nanozymes severely interfere with the TMB absorption spectrum.
13. Incubate the reaction mixture at 37 °C in a thermostatic water bath in the dark for 1 min. The water bath is connected to the UV/visible spectrometer.
14. Add H_2O_2 to a final concentration of 1 M in the reaction solution.
▲ **CRITICAL STEP** H_2O_2 should be used fresh to minimize H_2O_2 decomposition. In particular, the H_2O_2 stock solution bottle (30% (wt/vol) aqueous solution) should not be open for more than 1 month.
▲ **CRITICAL STEP** The H_2O_2 concentration used for measuring the activity of nanozymes should be excessive to ensure that the nanozymes added in the reaction solution are saturated with H_2O_2 substrate.
15. In addition to the solutions prepared in Step 14, prepare a solution without addition of H_2O_2. This solution will serve as the background measurement for Step 17.
16. Mix and incubate the reaction solution at 37 °C in the dark and measure the colorimetric response by absorbance at 652 nm every 10 s for up to 800 s after H_2O_2 addition.
▲ **CRITICAL STEP** Users can end the reaction early if the reaction plateau has been reached (see Fig. 2b for examples).
▲ **CRITICAL STEP** Peroxidase nanozymes show catalytic activity under a wide range of temperatures (between 20 and 50 °C), whereas natural peroxidases show optimal catalytic activity at 37 °C (ref. 19). To make a valid comparison between nanozymes and natural peroxidases, we recommend performing all catalytic activity measurements at 37 °C.
▲ **CRITICAL STEP** We recommend performing the experiment in triplicate to ensure reproducibility and consistency of the results.
◆ **TROUBLESHOOTING**
17. Plot the absorbance intensities at 652 nm against the reaction time to obtain the reaction–time curve (see Fig. 2b for examples).
▲ **CRITICAL STEP** The resulting absorbance should be calculated by subtraction of the background absorbance at 652 nm caused by the nanomaterials themselves.
18. Calculate the catalytic activity (units) of the measured nanozymes using the following equation:

$$b_{\text{nanozyme}} = V/(\varepsilon \times l) \times (\Delta A/\Delta t)$$

where b_{nanozyme} is the catalytic activity of the measured nanozymes and expressed in unit (U). One U is defined as the amount of nanozyme that catalytically produces one µmol of product per minute measured at 37 °C under the assay conditions employed. V is the total volume of reaction solution (µL); ε is the molar absorption coefficient of the used colorimetric substrate, which is typically maximized at 39,000 M^{-1} cm^{-1} at 652 nm for TMB (the ε value of the other two commonly used substrates: OPD: $\varepsilon_{417\,nm}$ = 16,700 M^{-1} cm^{-1}, ABTS: $\varepsilon_{420\,nm}$ = 36,000 M^{-1} cm^{-1})[19]; l is the path length of light travel in the cuvette (cm); A is the absorbance after subtracting the blank value; $\Delta A/\Delta t$ is the initial rate of change in absorbance at 652 nm per min.
▲ **CRITICAL STEP** For a short period after the start of the reaction, generally within the period of 5% change of the initial substrate concentration, the product of the nanozyme

is generated at an initial rate that is approximately linear to the reaction time (see Fig. 2c for examples). Therefore, the $\Delta A/\Delta t$ value (i.e., the slope value in the initial rate period of the reaction) is constant, and the calculated $b_{nanozyme}$ value from $\Delta A/\Delta t$ is thus a constant. The length of the initial rate period is generally determined when the R^2 coefficients are close to 1 after a linear regression analysis.

◆ **TROUBLESHOOTING**

19. Calculate the SA of the tested nanozyme sample (U/nmol) by using the following formula:

$$a_{nanozyme} = b_{nanozyme}/E_t$$

where $a_{nanozyme}$ is the SA defined as the activity units (U) per active site of nanozyme (U/nmol) and E_t is the number of active sites (nmol) of nanozyme.

▲ **CRITICAL STEP** As the peroxidase-like activity of nanozymes ($b_{nanozyme}$) is linearly proportional to the number of active sites, the $a_{nanozyme}$ value will be calculated by plotting the catalytic activity of $b_{nanozyme}$ against E_t and measuring the slope of the resultant straight line (see Fig. 2d for examples).

Part 3: characterization of the catalytic kinetics for peroxidase nanozymes

● **TIMING ~2 h**

20. Dissolve 10 μg of nanozyme in 2 mL Hac–NaAc buffer (0.2 M, pH 3.6).
21. Add various volumes (from 0 to 100 μL) of the TMB stock solution (10 mg/mL in DMSO) to the reaction mixture.

    ▲ **CRITICAL STEP** Generally, the reaction velocities are recorded by fixing the concentration of one substrate ([H_2O_2] or [TMB]) while varying that of the other one. The kinetic constants thus can be obtained by the fitted Michaelis–Menten equation for the reaction velocity and the initial substrate concentration. In the examples provided in this protocol, we record the reaction velocities by fixing the concentration of H_2O_2 while varying TMB.

    ▲ **CRITICAL STEP** To obtain a well-fitted Michaelis–Menten curve, this assay should be performed over a suitable TMB substrate concentration range. Usually, concentrations of TMB substrate range straddle the K_m value and range from $0.5 \times K_m$ to $5 \times K_m$.
22. Add H_2O_2 to the reaction mixture to a final concentration of 1 M.

    ▲ **CRITICAL STEP** The H_2O_2 concentration used for the kinetic measurement of peroxidase nanozymes should be excessive to ensure that the added nanozymes in the reaction solution are saturated with H_2O_2 substrate.
23. Mix the samples, incubate the reaction mixture in a 37 °C water bath, and record the change of absorbance at 652 nm every 10 s for up to 100 s (see Part 2, Steps 16–17).
24. Calculate the initial rate from the $\Delta A/\Delta t$ value (see Part 2, Step 18).
25. Plot the calculated initial rate against the TMB substrate concentrations to produce the Michaelis–Menten curve (see Fig. 3 for examples).

    ▲ **CRITICAL STEP** To obtain a well-fitted Michaelis–Menten curve, choose an appropriate TMB substrate concentration range.

    ◆ **TROUBLESHOOTING**
26. Calculate the kinetic constants of v_{max} and K_m by fitting the reaction velocity values and the substrate concentrations to the Michaelis–Menten equation as follows:

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

where v is the initial reaction velocity, v_{max} is the maximal reaction rate that is observed at saturating TMB substrate concentrations, $[S]$ is the concentration of TMB substrate and K_m is the Michaelis constant. K_m reflects the affinity of the nanozyme for TMB substrate.

▲ **CRITICAL STEP** Sometimes the accurate v_{max} cannot be obtained due to insufficient substrate being added. A double-fitting method (see ref. 43 for examples) is suggested to confirm the accuracy of the obtained v_{max} of the tested peroxidase nanozyme.
27. Calculate the catalytic constant (k_{cat}) using the following equation:

$$k_{cat} = v_{max}/[E_t]$$

where, k_{cat} is the rate constant defining the maximum number of TMB substrates converted to product per second and $[E_t]$ is the molar concentration of the active sites on a nanozyme particle (M), which is determined in Step 8.

▲ **CRITICAL STEP** $[E_t]$ is determined by the unique physicochemical properties of the tested nanozyme. The physicochemical characterization described in Part 1 should be performed carefully.

28. Repeat Steps 20–27 and obtain the catalytic kinetic constants of the nanozyme for another substrate of H_2O_2 by fixing the concentration of TMB while varying that of H_2O_2.

▲ **CRITICAL STEP** The concentration of TMB used should be excessive to ensure that the added nanozymes in the reaction solution are saturated with TMB.

▲ **CRITICAL STEP** The used range of H_2O_2 concentration should straddle the K_m value and range from $0.5 \times K_m$ to $5 \times K_m$ to obtain a well-fitted Michaelis–Menten curve.

Part 4: computational kinetics for a single site of peroxidase nanozymes

● **TIMING ~36 h**

29. Browse and download crystal structures (in cif format) of nanozymes from the materials project (https://next-gen.materialsproject.org/).
30. Prepare input files in text format (INCAR, POSCAR, POTCAR and KPOINTS) used by the VASP program to optimize the geometry of the crystal structures. Examples of these files can be found at https://github.com/xingfagao/PyPOD.
    - The INCAR and KPOINTS (KPOINTS_For_Bulk) files can be used directly (remember to copy KPOINTS_For_Bulk to KPOINTS).
    - The POSCAR file can be exported from the crystal structure using the VESTA software.
    - POTCAR files are provided with the VASP program and are license protected (therefore we have not included an example of this file). The POTCAR file used in the calculations can be prepared by merging the POTCAR files for different atoms in the order they appear in the POSCAR file.
    - Upload these files to a workstation or supercomputer and submit a job to execute the VASP program.
31. Create nanosurfaces (slab models) using the optimized crystal structure at all positions for surface cut and select the most appropriated one. The slab models can be created using the 'Cleavage Surface' module in the Materials Studio software and are exported as '.cif' files.

    ▲ **CRITICAL STEP** There are many possible exposed surfaces of nanozymes with different miller indices. It is recommended to refer to the experimental results or literature to select the nanozyme with the most exposed surfaces. If such information is unknown, the most stable exposed surface is recommended.
32. Prepare input files (INCAR, POSCAR, POTCAR, and KPOINTS) for VASP calculations of adsorbates and nanosurfaces, then perform VASP calculations.
    - The INCAR and POTCAR files can be the same as those used in Step 30.
    - The KPOINT file should be changed to the $3 \times 3 \times 1$ Monkhorst-Pack k-point mesh (the KPOINTS_For_Slab file was provided at at https://github.com/xingfagao/PyPOD for reference).
    - The POSCAR files for slabs can be exported from the crystal structures (exported cif files in Step 31) using the VESTA software. Upload these files to a workstation or supercomputer and submit a job to execute the VASP program.

    ◆ **TROUBLESHOOTING**
33. Generate the adsorption structures of adsorbates (H_2O_2 and OH) on nanosurfaces (slab models) and prepare input files for VASP calculations.
34. Execute the VASP program for calculations of adsorbates–adsorbed structures in a workstation or supercomputer.

    ▲ **CRITICAL STEP** There are usually many available adsorption sites on the surfaces of nanozymes. All these sites should be considered in the calculations, which are time consuming. The use of a supercomputer is highly recommended.

35. Extract the energy values for the surface with molecular adsorbate ($E_{mol@surface}$), separated surface ($E_{surface}$) and molecular adsorbate (E_{mol}) to calculate the adsorption energy ($E_{ads,mol}$) using the following equation:

$$E_{ads,mol} = E_{mol@surface} - E_{surface} - E_{mol}$$

where $E_{mol@surface}$, $E_{surface}$ and E_{mol} are energies of the surface with molecular adsorbate, separated surface and molecular adsorbate, respectively. A workflow to perform such calculations is available at https://github.com/xingfagao/PyPOD.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
16	Background interference at the 652 nm absorption peak	The absorption spectrum of the tested nanozyme solution interferes at 652 nm	Subtract the background absorbance or use other colorimetric substrates such as DAB or OPD with no overlapping absorption
18	The slope value in the initial rate period of the reaction is not constant	Choose an inappropriate initial rate period for determining the initial rate	Determine the length of the initial rate when the R^2 coefficients are close to 1 after a linear regression analysis
	Fail to calculate the initial rate	The substrate concentration used should greatly exceed that of the nanozyme to ensure that the added nanozymes in the reaction solution are saturated with substrate	The substrate concentration (TMB or H_2O_2) should be 5 times greater than the Michaelis constant (K_m) of the measured nanozyme
25	Fail to fit the Michaelis–Menten curve from the measured data	Choose an inappropriate range of TMB substrate concentrations	A suitable TMB substrate concentration range should straddle the K_m value and range roughly from $0.5 \times K_m$ to $5 \times K_m$
32	Surface structures are severely changed	The selected surface models are unreasonable	Change the position of the surface cut in constructing surface models or discard the unreasonable structures
	Calculations fail to convergence	The electronic structures are too complex	Change the initial assignments of magnetic centers (antiferromagnetic or ferromagnetic)

Timing

Part 1, Steps 1–10, characterization of active site, surface area, shape and size of nanozymes: 28 h
Steps 1–8, measurement of active sites of nanozyme: 2 h
Step 9, measurement of surface areas of nanozyme: 12 h
Step 10, measurement of shape, and size of nanozyme: 14 h

Part 2, Steps 11–19, characterization of the peroxidase-like activity of nanozymes: 2 h
Part 3, Steps 20–28, characterization of the catalytic kinetics for peroxidase nanozymes: ~2 h
Part 4, Steps 29–35, computational kinetics for a single site of peroxidase nanozymes: ~36 h
Steps 29–30, calculation of bulk crystal: 1.5 h
Steps 31–32, calculations of adsorbates and nanosurfaces: 10 h
Steps 33–34, calculation of adsorbate-adsorbed surfaces: 24 h
Step 35, data analysis: 30 min

Anticipated results

In this protocol, we employed three classic peroxidase nanozymes, including Fe_3O_4 NPs, Pt NPs and Pt-SAzyme, as representative examples to present how to determine the catalytic activity and kinetics of nanozymes. Figure 2 shows an example of how to determine the peroxidase-like activity of Fe_3O_4 NPs, Pt NPs and Pt-SAzyme using this updated protocol. As shown in Fig. 2a, Fe_3O_4 NPs, Pt NPs or Pt-SAzyme show the typical peroxidase-like activity by catalyzing the oxidation of peroxidase substrates of TMB, DAB or OPD to produce the unique color reactions. Figure 2b,c shows the typical reaction-time curves of TMB color reaction catalyzed by these three nanozymes, and Fig. 2d shows their typical SA (U per nmol of active sites). The number of active sites (nmol) of nanozyme (E_t) was measured using the carbon monoxide (CO) adsorption method (Supplementary Fig. 1). Generally, each CO molecule will occupy one catalytic site of nanozyme via chemisorption, and thus, the E_t value can be determined by measuring the amount of CO adsorption on the surface of nanozymes. Specifically, E_t is determined by the unique physicochemical properties of nanozymes, including their composition, defects, size, shape, surface modification and atomic configuration. Figure 2d shows that the catalytic activity unit is linearly proportional to the number of active sites of E_t, thus yielding a unity slope (i.e., SA) of 0.0261 units for Fe_3O_4 NPs, 1.14 units for Pt NPs and 24.7 units for Pt-SAzyme per nmol of metal active sites, clearly indicating the catalytic activity of a single site of the tested nanozyme that is dependently determined by the unique physicochemical properties of nanozymes. For example, the catalytic activity of Pt catalytic sites was increased from 1.14 to 24.7 units per nmol of Pt atoms after atomization of Pt atom from Pt metal crystals into Pt single atom chemically coordinated with N, P and S atoms. The results show that the SA (i.e., units per nmol of active sites) determined using this updated protocol thus can truly indicate the impact of the unique physicochemical properties on the catalytic activity of nanozymes.

Figure 3 shows a typical example of determining the kinetic constants of peroxidase Fe_3O_4 NPs, Pt NPs and Pt-SAzyme using the procedures described in this protocol. The reaction velocity values were recorded by fixing the concentration of H_2O_2 while varying that of TMB, and the kinetic constants of the tested nanozymes for TMB were obtained by fitting to the Michaelis–Menten equation using a nonlinear regression analysis (Fig. 2). Similarly, by fixing the concentration of TMB while varying that of H_2O_2, dependency of reaction rate on the H_2O_2 substrate in the form of Michaelis–Menten equation can also be obtained. The results indicate that even the same Pt active sites will show a considerably different catalytic kinetics along with the changes in shape, size, atomic configuration and electronic structure of the central Pt atoms, showing that this updated protocol can practically indicate the catalytic activity of nanozymes with completely different unique physicochemical properties.

We further demonstrate the close relationship between the catalytic kinetics of peroxidase nanozymes and their binding affinities to H_2O_2 and hydroxyl group (OH). To obtain $E_{ads,H2O2}$ and $E_{ads,OH}$ of Fe_3O_4 NPs, we performed DFT calculations according to Part 4 of the procedure. As shown in Fig. 4, the surface structures of Fe_3O_4 NPs were considered in our calculations. These structures have different exposed surfaces (001 and 111) and different numbers of iron vacancies. The corresponding adsorption energies of H_2O_2 and OH on these surfaces are given in Table 1. The different adsorption energies between these various nanozyme surfaces suggest their distinct catalytic kinetics. Since the exposed surfaces are dependent on the morphologies of nanozymes, the calculated results are consistent with the morphology-dependent catalytic activity of nanozymes observed in the above experiments. In addition, the vacancies also significantly influence the catalytic kinetics of the same surface. For example, on the Fe_3O_4(001) surface, the $E_{ads,H2O2}$ varies from –0.77 to –1.22 eV, indicating different K_m values. Equation 6 shows that the K_m for the H_2O_2 substrate decreases, indicating better catalytic kinetics, as the $E_{ads,H2O2}$ becomes more negative. Similarly, equation 5 demonstrates that $E_{ads,OH}$ affects the υ_{max} values, but its influence is not as straightforward as that of $E_{ads,H2O2}$ on K_m. This is because the $\Delta G^{\ddagger}$ of the rate-determining step does not increase or decrease linearly with the increase in $E_{ads,OH}$ (less negative). On the other hand, the $\Delta G^{\ddagger}$ initially decreases and then increases as $E_{ads,OH}$ becomes more negative, reaching a minimum at $E_{ads,OH}$ of –2.6 eV (ref. 31). Therefore, if the tested

nanozyme has an $E_{ads,OH}$ value close to −2.6 eV, a large v_{max} value is expected. The results suggest that the Fe_3O_4 nanozymes containing vacancies exhibit better peroxidase-like catalytic activity than those with high purity. This result also explains the observed differences in peroxidase-like activity of Fe_3O_4 NPs from various groups[1,44]. These computed adsorption energies thus can be combined with the experimentally characterized physicochemical properties of nanozymes to better describe the catalytic performance of nanozymes. More importantly, these energy values can further guide the rational design of nanozymes by engineering the electronic, geometric structure and atomic configuration of their catalytic sites. For example, the $Fe_3O_4(001)$-(Fe_v) has an $E_{ads,OH}$ value closer to −2.6 eV than that of $Fe_3O_4(111)_B$-$(Fe_v)_4$ (Table 2), suggesting improved peroxidase-like activity and kinetics. Thus, experimental efforts should focus on controlling the morphology of Fe_3O_4 with a more exposed 001 surface to achieve high peroxidase-like activity of Fe_3O_4 NPs. Such criteria have been successfully applied to understand the relative peroxidase-like activity of nanomaterials and design better peroxidase-like nanozymes[24,45,46].

Data availability

All data are available in the accompanying Supplementary Information.

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Author contributions

M.L. and X.G. conceived and designed the experiments. J.-J.Z., F. Z. and J.H. performed the experiments. M.L., X.G. and J.-J.Z. wrote the paper. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Jiuyang He, Xingfa Gao or Minmin Liang.

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