

# Metallothionein-Inspired Asymmetric Heteroatom Doping of Single-Atom Nanozymes for Multi-Enzyme Biocatalysis

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Single-atom catalysts (SACs) exhibit enzyme-mimicking activity but are often limited by single-enzyme-like functions and modest catalytic efficiency. Here, a metallothionein-inspired heteroatom doping strategy is reported to construct asymmetric Fe single-atom catalysts ( $\text{FeN}_3\text{S}$ ).  $\text{Fe}^{3+}$  is coordinated with cysteine via strong mercaptide bond formation, followed by zeolitic imidazolate framework-8 (ZIF-8) biomineralization and pyrolysis. The  $\text{FeN}_3\text{S}$  catalyst displays markedly enhanced multi-enzyme activities—including NADH oxidase-, oxidase-, peroxidase-, and catalase-like activities—with 1.35–4.60-fold improvements compared to sulfur (S)-free analogues. This high multi-enzyme efficiency arises from i) atomically dispersed Fe from biomineralization; ii) the large surface area and pore volume retained from the original metal–organic framework, and iii) the S-doping achieved through the strong mercaptide coordination between Fe and S. The S doping not only tunes electronic structure of Fe single atom to reduce activation barriers and enhances substrate interaction, but also facilitates charge transfer. As a result,  $\text{FeN}_3\text{S}$  induces  $\approx 90\%$  tumor cell suppression within one day through reactive oxygen species generation and disruption of the NADH/ $\text{NAD}^+$  balance, highlighting its strong potential for cancer therapy. This work provides a bioinspired strategy for advancing SACs toward multifunctional biocatalysis and biomedical applications.

pH, low tolerance to organic solvents, and high production costs.<sup>[4,5]</sup> To address these limitations, nanozymes—nanomaterials with enzyme-like activity—have emerged.<sup>[6,7]</sup> Compared with natural enzymes, nanozymes offer advantages such as high stability, facile design, and low manufacturing cost.<sup>[8–11]</sup> However, their catalytic activity and selectivity are generally lower than those of natural enzymes, and their potential cytotoxicity remains a concern.<sup>[12–15]</sup> As one of the emerging nanozymes, single-atom catalysts (SACs) have recently attracted significant attention, demonstrating excellent structural stability, high catalytic activity, and selectivity.<sup>[16]</sup> For example, the developed SACs have been demonstrated to mimic the activities of peroxidase (POD),<sup>[17]</sup> oxidase (OXD),<sup>[18]</sup> catalase (CAT),<sup>[19]</sup> laccase,<sup>[20]</sup> and superoxide dismutase.<sup>[21]</sup> These advantages arise from atomically dispersed active sites, tunable coordination environments, and maximal metal utilization.<sup>[22]</sup> Consequently, SACs show broad application prospects in biosensing, disease therapy, environmental remediation, and energy conversion.<sup>[23–26]</sup>

## 1. Introduction

Natural enzymes are widely used in biocatalysis, medicine, and industry due to their remarkable catalytic efficiency and substrate specificity.<sup>[1–3]</sup> However, their practical applications are often limited by poor stability, sensitivity to temperature and

establishing them as a rapidly growing research hotspot in catalysis.

However, there remains significant room for improving the performance of SACs. Various strategies have been explored, including constructing porous structures to enhance substrate mass transfer,<sup>[27,28]</sup> introducing heteroatoms (such as N,<sup>[29]</sup>

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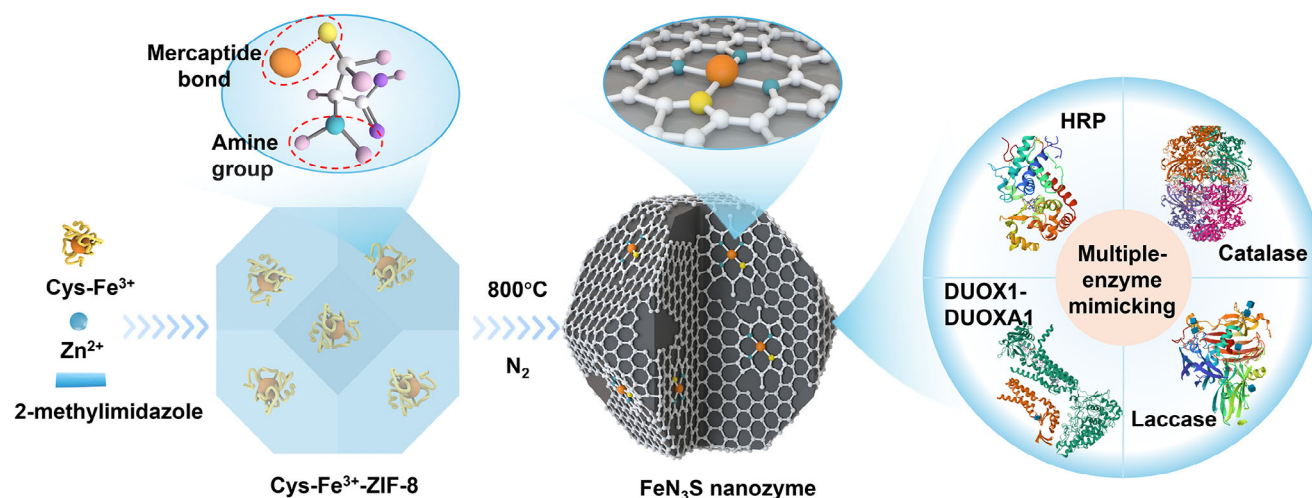
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**Scheme 1.** Illustration of the preparation process of FeN<sub>3</sub>S nanozyme for multiple-enzyme mimicking. Cys was coordinated with Fe<sup>3+</sup> through strong mercaptide bonds, while the amine group in Cys promoted the biomineralization of MOFs. After pyrolysis, the obtained FeN<sub>3</sub>S nanozyme exhibits multi-enzyme mimetic activities, including NOX, OXD-, POD-, and CAT-like activities.

S,<sup>[30,31]</sup> P,<sup>[7]</sup> B<sup>[32]</sup>) to modulate the electronic structure of metal centers, and improving the stability of active sites through optimized carriers and metal-carrier interactions.<sup>[23,33,34]</sup> While these approaches have achieved notable improvements in catalytic activity, they also present challenges, such as reduced structural stability, disruption of active site homogeneity, agglomeration at high metal loadings,<sup>[35–37]</sup> and restriction to single enzyme-like activity.<sup>[29]</sup> In particular, previous heteroatom-doping strategies have mainly relied on heteroatom-containing polymers<sup>[7,38,39]</sup> or small molecules<sup>[32]</sup> that lack specific anchoring sites for metal ions, and depending on host-guest self-adjustment during pyrolysis.<sup>[40]</sup> This often leads to poor coordination between metal and nonmetal atoms or excessive heteroatom loading without deliberate design. Moreover, although recent comprehensive reviews have highlighted both the remarkable potential and ongoing challenges in developing selective single-atom nanozymes,<sup>[41–45]</sup> the single enzyme-like activity of most SACs limits their effectiveness in complex environmental and biological processes that often require multiple catalytic functions.<sup>[46]</sup> Recently, Ir–N<sub>5</sub> SAC was developed, demonstrating multi-enzyme activities, including CAT-, OXD-, POD-, and NADH oxidase (NOX)-like activities.<sup>[47]</sup> Nevertheless, the catalytic efficiency of Ir–N<sub>5</sub> SAC remains limited, and the use of cerulenin was necessary to achieve effective cancer therapy.<sup>[47]</sup> Therefore, the development of SACs with precisely designed heteroatom doping capable of mimicking multiple enzyme activities while maintaining high catalytic efficiency remains a critical challenge in this field.

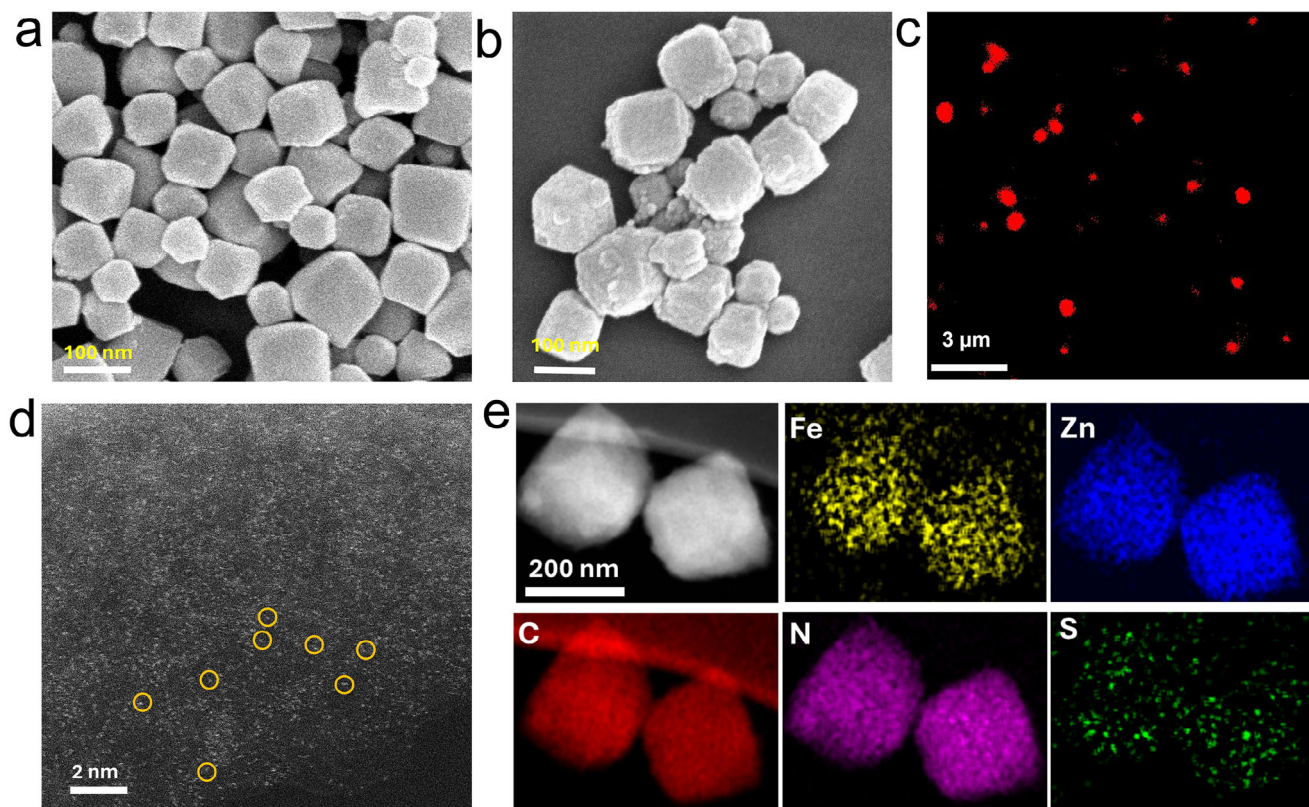
To develop highly efficient Fe-SACs with multi-enzyme activity for effective biocatalysis, a metallothionein-inspired heteroatom doping strategy was employed to engineer asymmetric Fe SACs (Scheme 1). This approach was inspired by the metal cation accumulation mechanism of metallothionein.<sup>[48]</sup> Compared with other S-containing ligands (e.g., 1,2-ethanedithiol, 1-butanethiol, and thioglycolic acid), L-cysteine methyl ester hydrochloride (Cys) not only coordinated with Fe<sup>3+</sup> ions through strong mercaptide bonds, but its amine group also promoted

the assembly of prenucleation clusters and facilitated the subsequent formation of MOFs around the Cys molecules.<sup>[49]</sup> This strategy enables highly dispersed and effective immobilization of Cys-Fe<sup>3+</sup> within the MOF framework. Following pyrolysis, asymmetric FeN<sub>3</sub>S SACs were obtained, exhibiting remarkable multi-enzyme mimetic activities, including NOX, OXD-, POD-, and CAT-like activities (Scheme 1), with 1.35–4.60-fold higher activity compared to FeN<sub>3</sub> SACs without S doping. The highly efficient multi-enzyme activity of FeN<sub>3</sub>S can be attributed to i) the highly dispersed single atoms originating from the biomineralization; ii) the large surface area and pore volume retained from the original MOF; and iii) the S-doping achieved through the strong mercaptide coordination between Fe and S. The S doping not only tunes electronic structure of Fe single atom to reduce activation barriers and enhances substrate interaction, but also facilitates charge transfer. Finally, FeN<sub>3</sub>S with multi-enzyme-like activity was applied to suppress tumor cell growth, achieving an ≈90% cell death rate within one day. This study provides a promising strategy for developing highly efficient, multi-enzyme-like SACs for complex environmental and biomedical applications.

## 2. Results and Discussion

### 2.1. Synthesis and Structural Characterization of the Fe-SACs

Zeolitic imidazolate framework-8 (ZIF-8), rich in nitrogen and carbon and featuring abundant micropores, has been widely employed for the construction of Fe-SACs, as its structure effectively hosts Fe–Nx active sites.<sup>[7,29]</sup> Cys, an amino acid, contains a thiolate group that serves as the primary binding site for metal ions, while the histidyl and aspartyl side chains contribute to the stability of the resulting complexes.<sup>[50]</sup> Compared to N, S has a lower electronegativity, making it a highly effective nonmetal dopant.<sup>[40]</sup> To introduce S atoms into the Fe SAC structure, Cys was first mixed with Fe<sup>3+</sup> ions. A distinct blue color developed upon introducing Fe<sup>3+</sup> solution into Cys solution (Figure S1, Supporting Information), attributable to the



**Figure 1.** Structural characterization of Fe-SAC. SEM images of a) Cys-Fe<sup>3+</sup>-ZIF and b) FeN<sub>3</sub>S, c) CLSM image of Cys-Fe<sup>3+</sup>-ZIF, NHS 633 ester was used to labeled Cys. d) Aberration-corrected HAADF-STEM image of FeN<sub>3</sub>S, showing atomically dispersed metal single-atom sites as bright dots (yellow circles indicate single atoms). e) HAADF-STEM image and corresponding EDS mapping of FeN<sub>3</sub>S, showing uniform distributions of Fe, Zn, C, N, and S.

[Fe-Cys]<sup>+</sup> complex, which shows an absorption maximum at 614 nm ( $\epsilon = 1030 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ).<sup>[51]</sup> The subsequent addition of 2-methylimidazole (HmIm) and Zn<sup>2+</sup> did not immediately alter the [Fe-Cys]<sup>+</sup> complex (Figure S1, Supporting Information). Although this [Fe-Cys]<sup>+</sup> complex is transient, our previous work and other groups revealed that MOFs can rapidly lock such short-lived biomolecular conformations through rapid nucleation around biomolecules,<sup>[52,53]</sup> with the amine group of Cys facilitating MOF biomineralization.<sup>[49,54,55]</sup> The interaction between Fe<sup>3+</sup> and Cys was confirmed by Fourier transform infrared (FTIR) spectra (Figure S2, Supporting Information), where the peak at  $2550 \text{ cm}^{-1}$ , corresponding to the S-H stretching vibration of Cys, disappeared after self-assembly with Fe<sup>3+</sup>, indicating coordination via strong mercaptide bonds.<sup>[56]</sup> The resulting biomineralized MOF was then pyrolyzed at  $800^\circ\text{C}$  under a nitrogen atmosphere to yield SAC, denoted FeN<sub>3</sub>S. Controlled samples lacking Fe<sup>3+</sup>, Cys, or both were also prepared and denoted as SNC, FeN<sub>3</sub>, and NC, respectively. For comparison, the higher pyrolysis temperature of  $900^\circ\text{C}$  was also examined, which are designated FeN<sub>3</sub>S-900, SNC-900, FeN<sub>3</sub>-900, and NC-900, while the corresponding unpyrolyzed MOF samples were denoted Cys-ZIF-8, Fe<sup>3+</sup>-ZIF-8, and Fe<sup>3+</sup>-Cys-ZIF-8.

Scanning electron microscopy (SEM) images revealed that all unpyrolyzed MOF samples displayed a uniform rhombic dodecahedral morphology<sup>[57]</sup> with an average particle diameter ranging from 75 to 95 nm (Figure S3, Supporting Information). In

contrast to Fe<sup>3+</sup>-Cys-ZIF-8, which has a smooth surface, FeN<sub>3</sub>S displays a rough surface morphology due to high-temperature pyrolysis (Figure 1a,b). Cys was labeled with Atto 633 NHS ester. Confocal laser scanning microscopy (CLSM) images show that Cys (red) is homogeneously distributed within the ZIF-8 nanocrystals (Figure 1c), indicating that the Fe-Cys complex is encapsulated inside the ZIF-8 framework rather than attached to its external surface. The powder X-ray diffraction (PXRD) patterns of Cys-ZIF-8, Fe<sup>3+</sup>-ZIF-8, and Fe<sup>3+</sup>-Cys-ZIF-8 exhibited the same characteristic peaks as those of pure ZIF-8, confirming the successful synthesis and preservation of the crystalline structure after encapsulation of Fe<sup>3+</sup> and Cys (Figure S4, Supporting Information). Moreover, FeN<sub>3</sub>S, SNC, FeN<sub>3</sub>, and NC, all samples exhibited consistent morphology and particle size (Figure S5, Supporting Information), indicating the well preservation of structure after pyrolysis treatment. However, the average particle size of SEM images of FeN<sub>3</sub>-900 and FeN<sub>3</sub>S-900 decreased slightly (Figure S6, Supporting Information), which may be attributed to structural shrinkage resulting from the decomposition of organic ligands and Zn volatilization during higher temperature pyrolysis.<sup>[58]</sup> These results are also in agreement with TEM images (Figure S7, Supporting Information). After pyrolysis at  $800^\circ\text{C}$  and  $900^\circ\text{C}$ , the characteristic peaks of ZIF-8 in PXRD patterns disappeared, and two broad reflections corresponding to (002) and (101) planes emerged at  $2\theta = 24^\circ$  and  $43^\circ$ , indicating the formation of amorphous graphitic carbon structures (Figure S8,

Supporting Information). The Raman spectra of pyrolyzed samples further confirmed the formation of graphitic carbon, as only two characteristic peaks corresponding to the D and G bands of carbon materials were observed (Figure S9, Supporting Information). The  $I_D/I_G$  values of NC, SNC,  $\text{FeN}_3$ , and  $\text{FeN}_3\text{S}$  were estimated as 1.057, 1.035, 1.028, and 1.040, with similar values observed for the samples pyrolyzed at 900 °C. These results imply abundant structural imperfections and a significant degree of graphitization,<sup>[29]</sup> consistent with the PXRD patterns (Figure S8, Supporting Information). Moreover, it is observed that the  $I_D/I_G$  values all decrease after the introduction of S, Fe, or both of them, reflecting that they facilitate MOF graphitization. This enhanced graphitic structure might be beneficial for accelerating interfacial electron transfer between the catalyst and reactants, thereby improving the potential catalytic activity of the SACs.<sup>[34]</sup>

The X-ray photoelectron spectroscopy (XPS) measurements revealed that all samples contained C, O, N, and Zn elements (Table S1, Supporting Information). In the N 1s XPS spectra of ZIF-8, Cys-ZIF-8,  $\text{Fe}^{3+}$ -ZIF-8, and  $\text{Fe}^{3+}$ -Cys-ZIF-8, a major peak at 398.5 eV is clearly observed, corresponding to the C=N bond in HmIm (Figure S10, Supporting Information). After pyrolysis, three main peaks at 398.4, 400.5, and 401.2 eV, assigned to pyridinic N, pyrrolic N, and graphitic N appeared, respectively<sup>[29]</sup> (Figures S11 and S12, Supporting Information). Pyridinic N serves as a primary anchoring site for single metal atoms, while pyrrolic N and graphitic N modulate the electronic structure of the carbon materials, thereby enhancing the catalytic activity of the SACs.<sup>[59–61]</sup> The presence of single metal (Zn and Fe) atoms was confirmed by high-angle aberration-corrected annular dark-field scanning transmission electron microscopy (HAADF-STEM), presented as the bright spots marked with yellow circles in the images (Figure 1d and Figure S13, Supporting Information), indicating highly dispersed single atoms derived from MOF biomineralization<sup>[55]</sup> followed by pyrolysis. Meanwhile, the energy-dispersive spectrometry (EDS) exhibited uniform distribution of Fe, Zn, C, N, and S elements in  $\text{FeN}_3\text{S}$  and  $\text{FeN}_3\text{S-900}$  (Figure 1e; Figures S14 and S15, Supporting Information), confirming the incorporation of Fe atoms and S doping. This is also consistent with the electron energy-loss spectrum (EELS) mappings (Figure S16a, Supporting Information), Fe shows a pronounced Fe  $L_2$  edge at  $\approx 708$  eV (Figure S16b,c, Supporting Information), providing further evidence of the Fe in  $\text{FeN}_3\text{S}$ . However, inductively coupled plasma (ICP) results show that the Zn content is over ten times higher than that of Fe (Table S2, Supporting Information), making it challenging to clearly distinguish Fe single atoms from Zn in Figure 1d. Importantly, no aggregation of either Zn or Fe is observed. EDS and EELS mapping (Figures S14 and S16, Supporting Information) confirm that Fe exists as isolated single atoms rather than aggregated clusters.

## 2.2. Atomic Structure Analysis of Fe-SACs by X-ray Absorption Fine Structure Spectroscopy

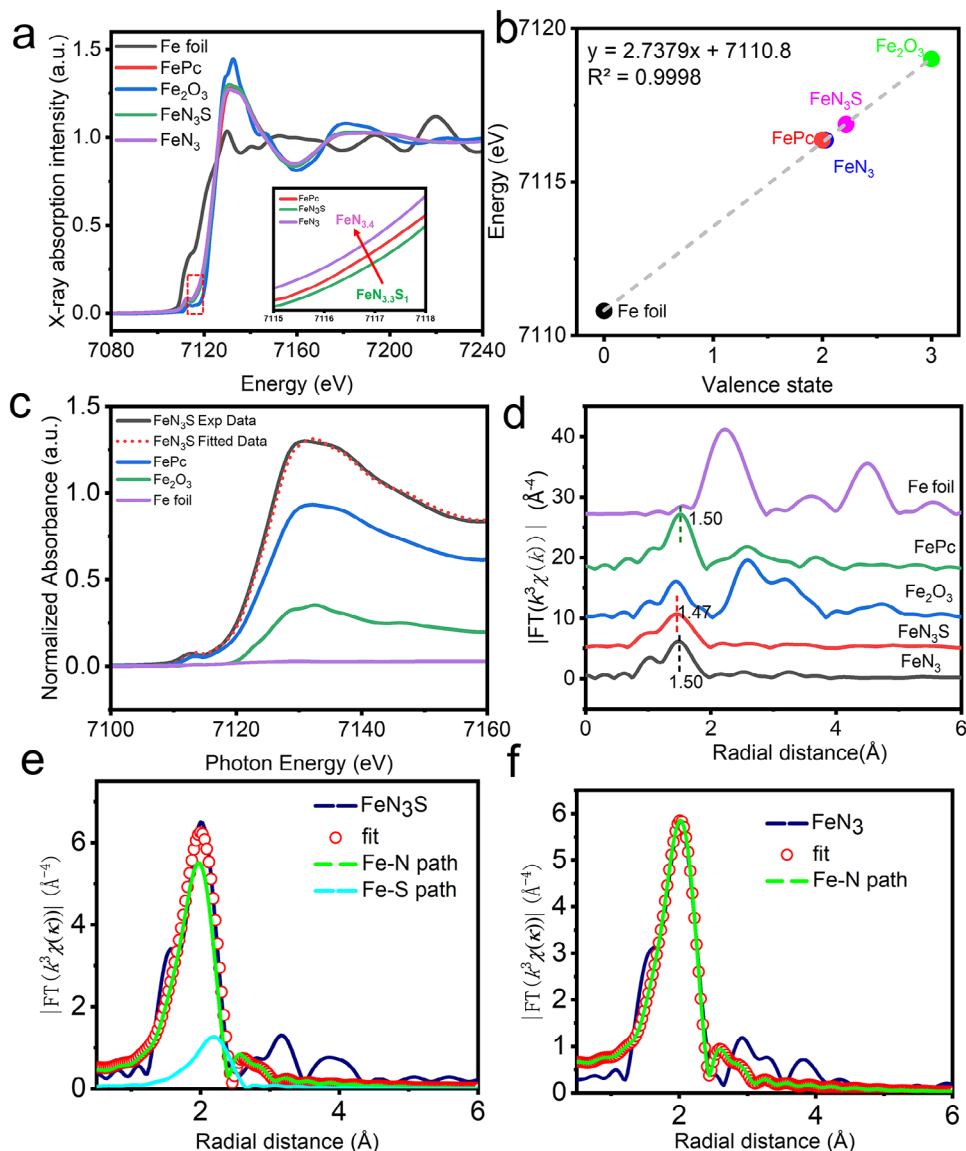
The chemical state and coordination environment of the Fe-SACs were examined using Fe K-edge X-ray absorption spectroscopy (XAS). In the Fe K-edge XANES spectra, the near-edge adsorption energy of the Fe K-edge in the  $\text{FeN}_3\text{S}$  is located between to that of FePc and  $\text{Fe}_2\text{O}_3$ , which manifests the dominant valence state of

between +2 and +3. Specifically, the near-edge adsorption energy of Fe decreases from  $\text{FeN}_3\text{S}$  to  $\text{FeN}_3$ , whose definite oxidation states could be estimated by comparison with Fe foil, FePc, and  $\text{Fe}_2\text{O}_3$  references (Figure 2a). After enlarging the energy range from 7116 to 7118 eV, a slight shift toward lower energy from  $\text{FeN}_3\text{S}$  to FePc and to  $\text{FeN}_3$  was observed (Figure 2a, inset), suggesting a slightly decreased oxidation state of Fe.<sup>[62,63]</sup> The oxidation states are +2.22 in  $\text{FeN}_3\text{S}$  and +2.03 in  $\text{FeN}_3$  (Figure 2b). The linear combination fitting of the Fe K-edge XANES spectra demonstrates the calculated percentages of the redox states suggest the predominance of Fe(II) ( $\approx 72.6\%$ ), with smaller contributions from Fe (0) ( $\approx 2.9\%$ ) and Fe(III) ( $\approx 24.5\%$ ) for  $\text{FeN}_3\text{S}$  (Figure 2c; Figure S17, Supporting Information). The reduced chi-square value (0.0004348) indicates a robust fit, further validating the results (Table S3, Supporting Information).

The formation of asymmetric coordination configurations is confirmed by the Fourier-transform (FT) of Fe K-edge EXAFS spectra (Figure 2d). The position of the main peaks corresponding to Fe–N scattering paths shifts slightly and negatively from  $\text{FeN}_3$  to  $\text{FeN}_3\text{S}$ , which is in accordance with both the average Fe–N bond length by the quantitative EXAFS curve-fittings.  $\text{FeN}_3\text{S}$  exhibits a shorter average Fe–N distance (1.997 vs 2.008) (Table S4, Supporting Information). These results demonstrate the presence of local asymmetric configurations upon doping of heteroatoms.<sup>[64]</sup> The quantitative EXAFS curve-fitting analysis was conducted using least-squares FT-EXAFS fitting to investigate the coordination structure (Figure 2e,f; Figures S18 and S19, Supporting Information). The best fitting results for  $\text{FeN}_3\text{S}$  and  $\text{FeN}_3$  indicate Fe–N with coordination numbers of 3.3 and 3.4 at 2.008 and 1.997 Å, respectively, along with Fe–S coordination number of 1.1 at 2.185 Å for  $\text{FeN}_3\text{S}$  (Figure 2e,f and Table S4, Supporting Information), consisting with the previous report.<sup>[65]</sup> Hence, the most likely coordination configuration of Fe single atoms is  $\text{FeN}_3\text{S}$  with S doping and  $\text{FeN}_3$  without S doping (Figure 2e,f). Additionally, experimental 2D wavelet transform extended X-ray absorption fine structure (2D WT EXAFS) plots also further convince the existence and length of Fe–N bonds (Figures S20 and S21, Supporting Information). In contrast to the Fe foil standard, the Fe–Fe scattering path at  $\approx 2.15$  Å disappeared in both curves of  $\text{FeN}_3\text{S}$  and  $\text{FeN}_3$ , suggesting negligible iron aggregates on  $\text{FeN}_3\text{S}$  and  $\text{FeN}_3$  (Figure 2d; Figures S20 and S22, Supporting Information). Furthermore, hematite  $\text{Fe}_2\text{O}_3$  was also used as a control to exclude the formation of aggregated nanoparticles after pyrolysis (Figure 2d and Figure S21, Supporting Information). Thereby, these results confirm that Fe species exist exclusively as single atoms.<sup>[66]</sup>

## 2.3. Multi-Enzyme Activity Performance of Fe-SACs

NOX-like activity was first evaluated. In this reaction, NADH is catalytically oxidized to  $\text{NAD}^+$  by Fe SAC, and the progress of the reaction was monitored at 340 nm using a plate reader (Figure S23, Supporting Information). All NADH oxidation reactions followed typical Michaelis–Menten kinetics. The catalytic efficiency ( $K_{\text{cat}}/K_m$ ) of  $\text{FeN}_3\text{S}$  was determined to be  $267.42 \mu\text{M}^{-1}\text{h}^{-1}$ , whereas that of  $\text{FeN}_3$  was  $65.99 \mu\text{M}^{-1}\text{h}^{-1}$ , corresponding to a 4.1-fold enhancement for  $\text{FeN}_3\text{S}$  (Figure 3a,b). However, increasing the pyrolysis temperature to 900 °C led to a significant de-

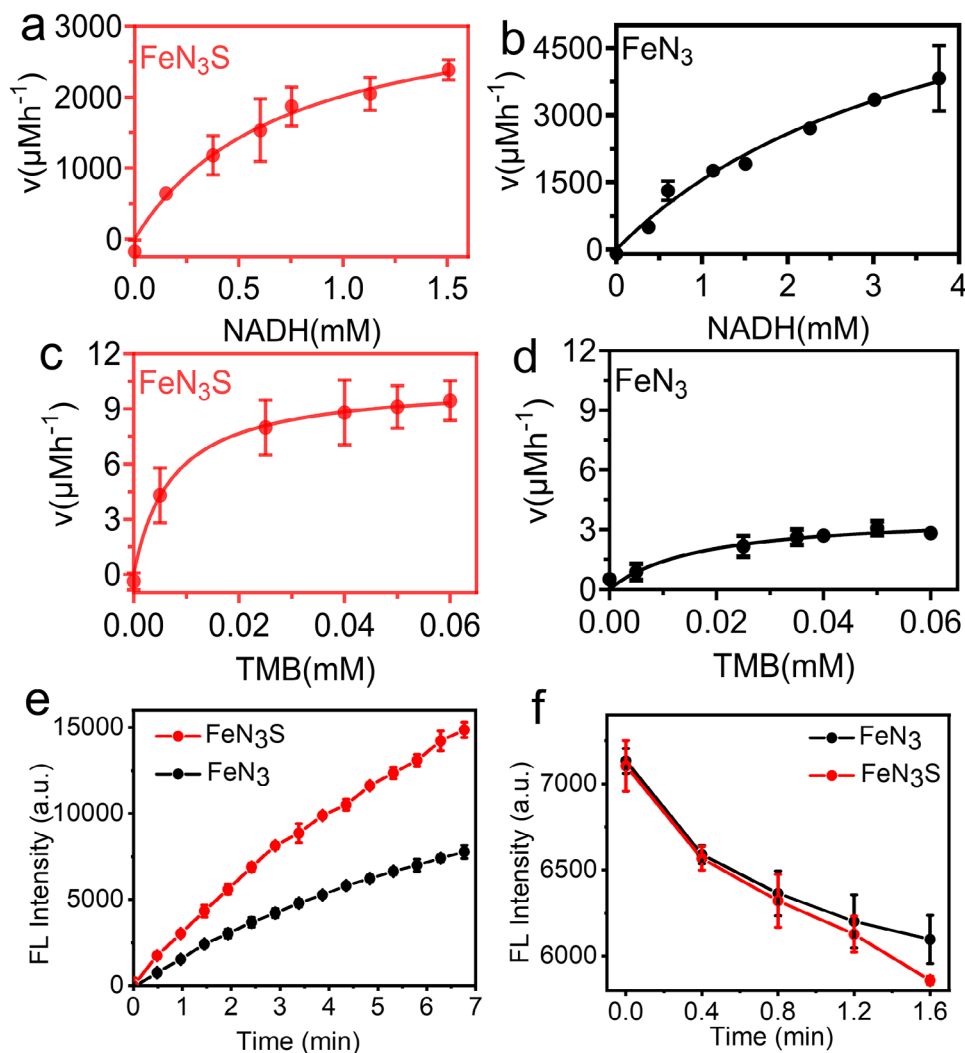


**Figure 2.** X-ray absorption spectra of Fe-SACs. a) Fe K-edge XANES spectra of Fe foil,  $\text{Fe}_2\text{O}_3$ , FePc,  $\text{FeN}_3\text{S}$ , and  $\text{FeN}_3$ . The inset shows the enlarged energy of  $\text{FeN}_3\text{S}$  and  $\text{FeN}_3$  range from 7116 to 7118 eV. b) The position of the near-edge as a function of the valence state of Fe in different samples. c) Linear combination fitting of the XANES data of  $\text{FeN}_3\text{S}$  collected at the Fe K-edge. d) Fe K-edge FT-EXAFS spectra of Fe foil,  $\text{Fe}_2\text{O}_3$ , FePc,  $\text{FeN}_3\text{S}$ , and  $\text{FeN}_3$ . e, f) Phase-corrected Fe K-edge EXAFS fitting results in R-space for e)  $\text{FeN}_3\text{S}$  and f)  $\text{FeN}_3$ .

crease in the catalytic efficiency of both  $\text{FeN}_3\text{S}$ -900 and  $\text{FeN}_3$ -900, with  $\text{FeN}_3\text{S}$ -900 even exhibiting lower activity than  $\text{FeN}_3$ -900, highlighting the critical influence of pyrolysis temperature on Fe SAC performance (Figure S24, Supporting Information). In contrast, the control samples NC, NC-900, SNC, and SNC-900, which lack Fe single atoms, displayed very low catalytic efficiencies of only  $\approx 4\text{--}6\ \mu\text{M}^{-1}\text{h}^{-1}$  (Figure S24, Supporting Information), without considering the possible coupling effect between Zn and Fe, indicating the indispensable role of Fe single atoms in NOX-like activity. Next, OXD-like activity was assessed using a 3,3',5,5'-tetramethylbenzidine (TMB) colorimetric assay. In this process, the Fe-SAC catalyzes the oxidation of TMB to a blue-colored product in the presence of  $\text{O}_2$ , which exhibits a characteristic absorbance peak at  $\approx 652\ \text{nm}$ .  $\text{FeN}_3$ ,

$\text{FeN}_3$ -900,  $\text{FeN}_3\text{S}$ , and  $\text{FeN}_3\text{S}$ -900 all displayed typical Michaelis–Menton behavior in their TMB oxidation, and the catalytic efficiency of  $\text{FeN}_3\text{S}$  reached  $65.71\ \mu\text{M}^{-1}\text{h}^{-1}$ , corresponding 4.6-fold improvement over  $\text{FeN}_3$ . However, after pyrolysis at  $900\ ^\circ\text{C}$ , the OXD-like activity of  $\text{FeN}_3\text{S}$ -900 and  $\text{FeN}_3$ -900 decreased, with  $\text{FeN}_3\text{S}$ -900 showing only a 1.35-fold higher than that of  $\text{FeN}_3$ -900 (Figure 3c,d; Figure S25a,b, Supporting Information). In contrast, NC, NC-900, SNC, and SNC-900 showed negligible catalytic activity, further confirming the indispensable role of Fe single atoms in OXD-like activity (Figure S25c–f, Supporting Information).

Furthermore, POD-like activity of Fe SAC was evaluated. Terephthalic acid (TA) was employed to semi-quantify  $\cdot\text{OH}$  in the catalytic system, as it reacts with  $\cdot\text{OH}$  to form the fluores-



**Figure 3.** a,b) Michaelis–Menten kinetic analysis of NOX-like activity of FeN<sub>3</sub>S and FeN<sub>3</sub>. Data are shown as mean  $\pm$  standard deviation (SD) ( $n = 3$ ). c,d) Michaelis–Menten kinetic analysis of OXD-like activity of FeN<sub>3</sub>S and FeN<sub>3</sub>. Data are shown as mean  $\pm$  SD ( $n = 3$ ). e)  $\cdot\text{OH}$  generation detected by TA (excitation at 320 nm, emission at 425 nm) with each sample containing equal Fe single-atom loading. Data are shown as mean  $\pm$  SD ( $n = 3$ ). f) O<sub>2</sub> generation detected by [Ru(bpy)<sub>3</sub>]<sup>2+</sup> (excitation at 450 nm, emission at 625 nm) with each sample containing equal Fe single-atom loading. All experiments were conducted in NaAc/HAc buffer pH 4.5. Data are shown as mean  $\pm$  SD ( $n = 3$ ).

cent compound 2-hydroxyterephthalic acid.<sup>[67,68]</sup> The POD-like activity of FeN<sub>3</sub>S was approximately twofold higher than that of FeN<sub>3</sub> with each sample containing equal Fe single-atom loading (Figure 3e). However, further increasing the pyrolysis temperature to 900 °C reduced the POD-like activity (Figure S26a,b, Supporting Information). A gradual increase in fluorescence intensity at 420–460 nm was observed for FeN<sub>3</sub>S with increasing H<sub>2</sub>O<sub>2</sub> concentration (Figure S26c, Supporting Information). In contrast, no such fluorescence signal was detected for NC, NC-900, SNC, and SNC-900 (Figure S26d, Supporting Information), confirming that the POD-like activity was induced by Fe single atoms. The enzymatic reaction mechanism of Fe-SACs was further investigated by electron spin resonance (ESR) spectroscopy.  $\cdot\text{OH}$  generation was detected during the POD-like catalytic process in the presence of FeN<sub>3</sub>S, FeN<sub>3</sub>, FeN<sub>3</sub>S-900, or FeN<sub>3</sub>-900 (Figures S27 and S28, Supporting Information). More importantly, the additional H<sub>2</sub>O<sub>2</sub> generated by the NOX-like activity

also induced the POD-like activity of FeN<sub>3</sub>S (Figure S27, Supporting Information), indicating a cascade reaction between the NOX- and POD-like activities of FeN<sub>3</sub>S. Finally, [Ru(bpy)<sub>3</sub>]<sup>2+</sup>, a strongly luminescent complex that emits intense orange luminescence under UV excitation ( $\lambda_{\text{exc}} = 450$  nm), was employed to probe CAT-like activity. Since the luminescence of [Ru(bpy)<sub>3</sub>]<sup>2+</sup> can be quenched by O<sub>2</sub>, it serves as an effective probe for monitoring O<sub>2</sub> generation.<sup>[69]</sup> FeN<sub>3</sub>S exhibited a 1.35-fold higher O<sub>2</sub> generation capacity than FeN<sub>3</sub>, with each sample containing equal Fe single-atom loading (Figure 3f). A slight increase in O<sub>2</sub> generation was observed for FeN<sub>3</sub>S-900 (Figure S29a,b, Supporting Information). Moreover, a decrease in fluorescence intensity at 550–700 nm was observed for FeN<sub>3</sub>S with increasing H<sub>2</sub>O<sub>2</sub> concentration (Figure S29c, Supporting Information), indicating enhanced O<sub>2</sub> generation. In contrast, no fluorescence signal was detected for NC, NC-900, SNC, and SNC-900 (Figure S29d, Supporting Information), confirming that the CAT-like activity originated

from Fe single atoms. Although a significant Zn content (290–1800 mm kg<sup>-1</sup>) in the catalyst was found (Table S2, Supporting Information), Figures S25, S26 and S29 (Supporting Information) show that NC, SNC, SNC-900, and NC-900 exhibit negligible OXD-, POD-, and CAT-like activities, while Figure S24 (Supporting Information) indicates that Zn SAC display some NOX-like activity. To eliminate the potential contribution of Zn to NADH catalysis, the catalytic efficiency of Zn was subtracted from that of Fe-SACs based on ICP results (Figure S24, Supporting Information). Our previous work demonstrated that there is no synergistic effect between Zn and Fe single atoms, as confirmed by pyrolyzing pristine ZIF-8 at 1000 °C to completely remove Zn, followed by re-doping with Zn and Fe.<sup>[29,63,70]</sup> This observation is consistent with earlier reports, which also found no synergistic effect between Zn and Fe in Zn/Fe dual-atom systems.<sup>[71]</sup> Therefore, the possibility that the enhanced activity originates from Fe–Zn dual-atom sites was ruled out. Since the same amount of Fe was added during MOF synthesis, both FeN<sub>3</sub> and FeN<sub>3</sub>S exhibited similar Fe contents after pyrolysis (161.25 vs 165.73 mm kg<sup>-1</sup>). Although FeN<sub>3</sub>S-900 showed the highest Fe content (176.11 mm kg<sup>-1</sup>) (Table S2, Supporting Information), this was likely due to Zn evaporation at 900 °C. However, as the multi-enzyme activity is determined by the activity of a single Fe atom, the influence of overall Fe loading on multi-enzyme performance can be considered negligible. In addition, other S-containing ligands (1,2-ethanedithiol, 1-butanethiol, and thioglycolic acid) serve as alternatives; however, no NOX-, OXD-, POD-, or CAT-like activities were observed despite Fe loading (Figure S30; Table S2, Supporting Information). This indicates that the choice of S-ligand is critical for enhancing multi-enzyme-like activity, otherwise, it may hinder or completely inactivate the catalytic functions. In summary, the multi-enzyme activity of FeN<sub>3</sub>S primarily arises from the synergistic effects between the MOF-derived carbon structures and Fe single atoms, while the S from Cys induced asymmetric coordination of Fe further amplifies these activities.

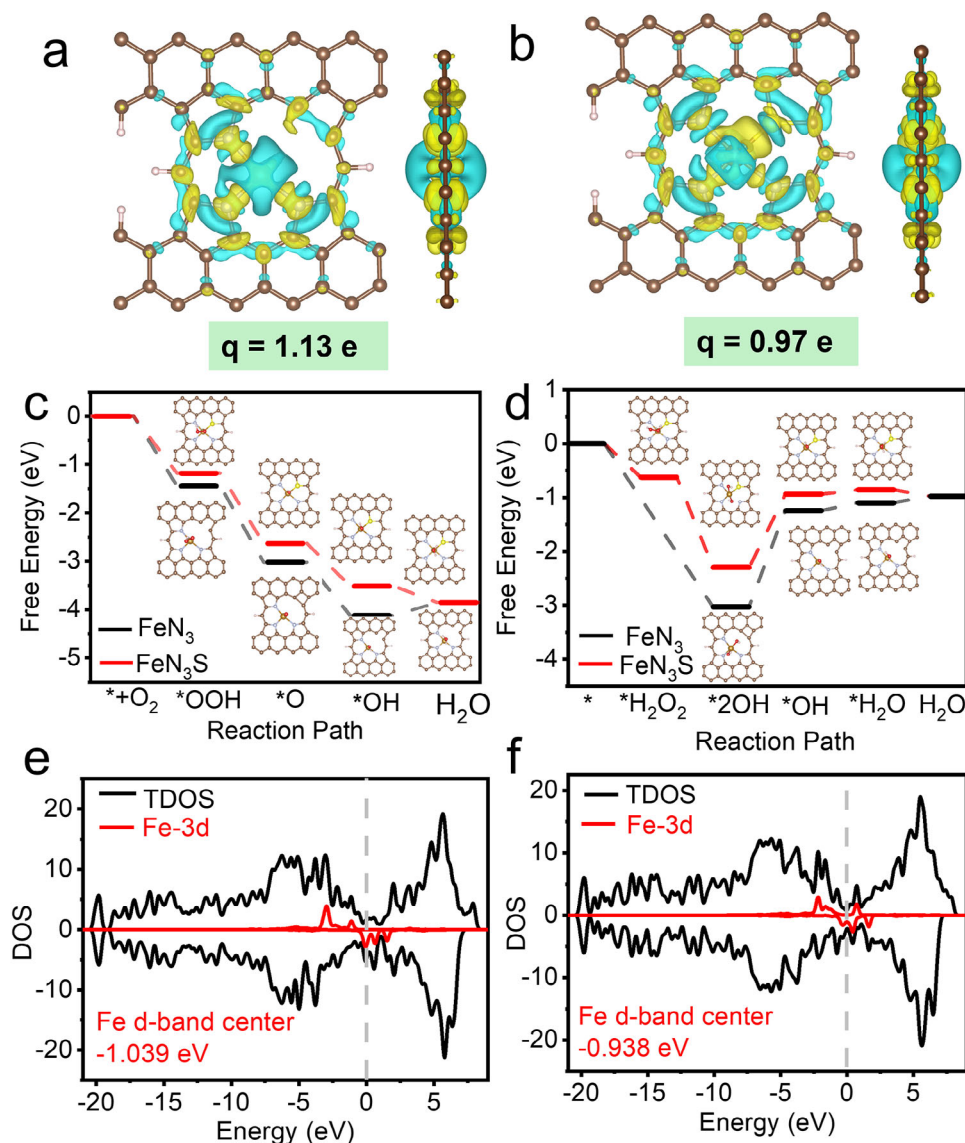
The temperature-dependent and pH-dependent multi-enzyme-like catalytic activities of FeN<sub>3</sub>S were also investigated (Figure S31a,b). The NOX-like and POD-like activities remained relatively stable and retained considerable activity within the temperature range of 20 to 50 °C, indicating good thermal tolerance (Figure S31a). In contrast, both OXD-like and CAT-like activities exhibited significantly reduced performance at lower temperatures but reached their optimal catalytic activity at 40 °C (Figure S31a). These results suggest that the temperature sensitivity of the nanozyme varies depending on the type of enzymatic function it mimics, with certain activities (e.g., OXD-like and CAT-like) being more reliant on thermal activation to achieve effective catalysis. Overall, 40 °C is most suitable for all enzymatic functions. In terms of pH dependence, POD-like activity increased and then decreased with pH, showing a maximum at pH 4.5. OXD-like activity remained nearly the same at pH 3 and pH 4.5 but decreased at higher pH values. NOX-like activity decreased with increasing pH, whereas CAT-like activity increased with pH (Figure S31b). Finally, the stability of FeN<sub>3</sub>S has also been investigated. The FeN<sub>3</sub>S demonstrates significant stability at various high temperature (20–100 °C) and pH (2–9) environments (Figure S31c,d), implying their potential for industrial applications.

## 2.4. Density Functional Theory (DFT) Study on Multi-enzyme-like Activity of Fe-SACs

To further elucidate the origin of the enhanced multi-enzyme catalytic activities of FeN<sub>3</sub>S, the potential catalytic mechanism of FeN<sub>3</sub> and FeN<sub>3</sub>S was investigated using DFT calculations. Although pyridinic N is generally very stable, Raman spectra indicate that numerous defects are generated during pyrolysis (Figure S9, Supporting Information). XPS analysis further shows that, in FeN<sub>3</sub>, 20.13% of the N species are pyridinic, 14.67% are graphitic, and 61.55% are pyridine-N (Figure S11, Supporting Information). Previous studies have also reported that structural rearrangements of carbon precursors during pyrolysis (800–1000 °C) can generate carbon vacancies (C<sub>v</sub>).<sup>[72,73]</sup> Such defect formation likely accounts for the absence of a pyrrole ring in FeN<sub>3</sub>. Consistent with this, similar FeN<sub>3</sub> vacancies have been described in the literature,<sup>[74,75]</sup> which exhibit well-balanced free energy barriers for reaction intermediates. Bader charge analysis demonstrates electron accumulation between the central Fe and S atoms.<sup>[76,77]</sup> Fe atom carries charges of 1.13 |e| and 0.97 |e| in FeN<sub>3</sub> and FeN<sub>3</sub>S, respectively (Figure 4a,b), indicating that the electron localization around the Fe–S and Fe–N bonds (green regions between Fe and S, and Fe and N) in FeN<sub>3</sub>S is less pronounced than that around the Fe–N bonds in FeN<sub>3</sub> (Figure 4a,b). This can be attributed to the weaker electron-withdrawing nature of S compared to N.<sup>[76]</sup> In principle, a higher Bader charge corresponds to a higher oxidation state. However, it has been reported that Bader charges are not always reliable indicators of oxidation states.<sup>[78,79]</sup> In this case, the Bader charge trend appears opposite to the formal oxidation state of FeN<sub>3</sub>S, which is more positively charged (Figure 2b). This discrepancy may arise from charge delocalization within the framework, resulting in an uneven redistribution of electron density,<sup>[80]</sup> which in turn significantly alters the catalytic activity.

For the case of the OXD-like process, O<sub>2</sub> first adsorbs onto the Fe single-atom site to form an adsorbed intermediate (Figure 4c).<sup>[81]</sup> Although FeN<sub>3</sub> shows stronger O<sub>2</sub> adsorption capacity, its higher \*O free energy leads to a large energy barrier in the final step, which requires a substantial barrier of 0.27 eV in the final step, whereas this barrier vanishes in the FeN<sub>3</sub>S pathway. This suggests a more favorable thermodynamic and kinetic profile of FeN<sub>3</sub>S for OXD-like catalysis (Figure 4c). In the POD-like catalytic process, adsorption of H<sub>2</sub>O<sub>2</sub> molecules on the Fe single atom site occurs first (Figure 4d). FeN<sub>3</sub> exhibits stronger H<sub>2</sub>O<sub>2</sub> adsorption, after which the adsorbed H<sub>2</sub>O<sub>2</sub> undergoes homolytic dissociation to form two \*OH groups. The subsequent desorption of \*OH radicals from the Fe single atom site is the rate-limiting step<sup>[82]</sup> (Figure 4d). Here, FeN<sub>3</sub>S demonstrates a lower energy barrier (1.78 eV) compared with FeN<sub>3</sub> (2.21 eV), making \*OH formation more favorable (Figure 4d).<sup>[83]</sup> Meanwhile, the synergistic coordination of Fe with N and S effectively optimizes the transition-state free energy. Thus, the central Fe atom and the asymmetric spatial configuration of FeN<sub>3</sub>S are the key factors underlying its superior enzyme-like activity.

Furthermore, density of states (DOS) analysis offered additional insights into the catalytic mechanism underlying the enhanced NOX-, OXD-, POD-, and CAT-like activities. The DFT-calculated DOS results provide clear evidence that S coordination profoundly tunes the electronic structure of the Fe center.



**Figure 4.** DFT calculation for FeN<sub>3</sub> and FeN<sub>3</sub>S. a,b) The charge density difference and Bader charges of a) FeN<sub>3</sub> and b) FeN<sub>3</sub>S. c) Free energy diagram for OXD-like catalytic reaction on FeN<sub>3</sub> and FeN<sub>3</sub>S, and their schematic illustration of the mechanism. d) Free energy diagram for POD-like catalytic reaction of FeN<sub>3</sub> and FeN<sub>3</sub>S, and their schematic illustration of the mechanism. e,f) DOS and Fe d-band center of FeN<sub>3</sub> and FeN<sub>3</sub>S. Color code: Fe, dark yellow; C, brown; N, blue; O, red; H, light pink; S, bright yellow.

The introduction of S in FeN<sub>3</sub>S leads to a noticeable increase in the DOS near the Fermi level ( $E_F$ ), accompanied by the formation of new hybrid orbitals (Figure 4e,f). Furthermore, the activation of Fe by S doping is confirmed by the upward shift of the Fe d-band center ( $\epsilon_d$ ) in FeN<sub>3</sub>S (−0.938 eV) compared with that in FeN<sub>3</sub> (−1.309 eV) (Figure 4e,f), implying that the Fe 3d orbitals in FeN<sub>3</sub>S approach the Fermi level and become more chemically active. Such an upshift indicates stronger electronic interactions and more efficient charge transfer between FeN<sub>3</sub>S and the reaction substrates than in FeN<sub>3</sub>,<sup>[47,84]</sup> consistent with the frontier orbital theory, thereby enhancing its catalytic activity.

EPR spectroscopy was employed to probe the spin states of the Fe active site. As shown in Figure S32 (Supporting Information), a notable difference is observed between the EPR spectra

of FeN<sub>3</sub> and FeN<sub>3</sub>S. The FeN<sub>3</sub>S sample displays a broader peak, whereas FeN<sub>3</sub> exhibits a sharp resonance signal, reflecting the increased electron density around Fe due to the formation of an asymmetric, low-coordinated FeN<sub>3</sub> structure.<sup>[85]</sup> The reduced signal intensity in FeN<sub>3</sub>S supports a transition from a high-spin ( $\uparrow\uparrow\uparrow$ ) to a low-spin ( $\uparrow$ ) state of the Fe atoms.<sup>[86]</sup> These changes in signal intensity, along with the g-value shift from 2.002 (FeN<sub>3</sub>) to 2.088 (FeN<sub>3</sub>S), indicate a spin-state transition upon S doping, suggesting the pivotal role of S incorporation in modulating the electronic structure and magnetic properties of the Fe centers. Unlike previous reports that aim to develop high-spin Fe sites, which possess more unpaired electrons in the  $e_g$  orbitals and facilitate substrate penetration to enhance adsorption,<sup>[87]</sup> our results show that excessively strong substrate adsorption on FeN<sub>3</sub>

is detrimental to catalytic activity, as it slows the desorption of intermediates, the rate-limiting step (Figure 4c,d). Consequently, FeN<sub>3</sub>S achieves overall improved multi-enzyme activity.

## 2.5. Pore Size Analysis

The N<sub>2</sub> adsorption–desorption isotherms, the corresponding Brunauer–Emmett–Teller (BET) surface area, and pore size distribution were further analyzed to support the improved multi-enzyme activity (Figures S33 and S34; Table S5, Supporting Information). For adsorption characterization at low P/P<sub>0</sub>, FeN<sub>3</sub>S and FeN<sub>3</sub> exhibit features typical of microporous carbon materials. Nitrogen uptake begins at very low relative pressures ( $\approx 5 \times 10^{-6}$  P/P<sub>0</sub>) (Figure S33a,b, Supporting Information), indicating the presence of slit-shaped pores with intra-wall ultramicropores (< 1.2 nm) accessible to N<sub>2</sub> molecules.<sup>[88]</sup> In the case of FeN<sub>3</sub>S-900 and FeN<sub>3</sub>-900 (Figure S33c,d, Supporting Information), a small hysteresis loop appears in the adsorption–desorption isotherm, characteristic of adsorption in interconnected mesopores.<sup>[89]</sup> The open-ended isotherms observed for samples pyrolyzed at 800 and 900 °C at low relative pressures can be attributed to the formation of abundant ultramicropores at 800 °C and structural defects-induced mesopores at 900 °C during high-temperature pyrolysis. These features enhance adsorbate–adsorbent interactions and hinder complete desorption,<sup>[90]</sup> resulting in nonclosure of the isotherm at low P/P<sub>0</sub>. The results are consistent with the pore size analysis (Figure S34; Table S5, Supporting Information). It was found that all samples pyrolyzed at 800 °C retained the original MOF pore size ( $\approx 1$  nm), whereas those pyrolyzed at 900 °C exhibited enlarged pores of 3–10 nm. Moreover, the 900 °C samples showed a dramatic reduction in BET surface area (139–764 m<sup>2</sup> g<sup>-1</sup>) compared with the 800 °C samples (831–1627 m<sup>2</sup> g<sup>-1</sup>), along with a decrease in total pore volume from 0.5–0.7 to 0.2–0.4 cm<sup>3</sup> g<sup>-1</sup>. This can be ascribed to the Zn evaporation, structural collapse, and enhanced graphitization induced by the elevated pyrolysis temperature, as indicated by the reduction of I<sub>D</sub>/I<sub>G</sub> values in Raman spectra (Figure S9, Supporting Information). This graphitization process promotes the formation of a denser carbon framework, resulting in the partial elimination of micropores and consequently diminishing the specific surface area.<sup>[91]</sup> It is found that the high pore volumes and surface areas of support remarkably promoted the exposure of active sites and mass transport of intermediates, thus significantly improving catalytic performance.<sup>[92,93]</sup> Therefore, FeN<sub>3</sub>S demonstrates higher NOX, OXD, CAT and POD-like activity than FeN<sub>3</sub>S-900.

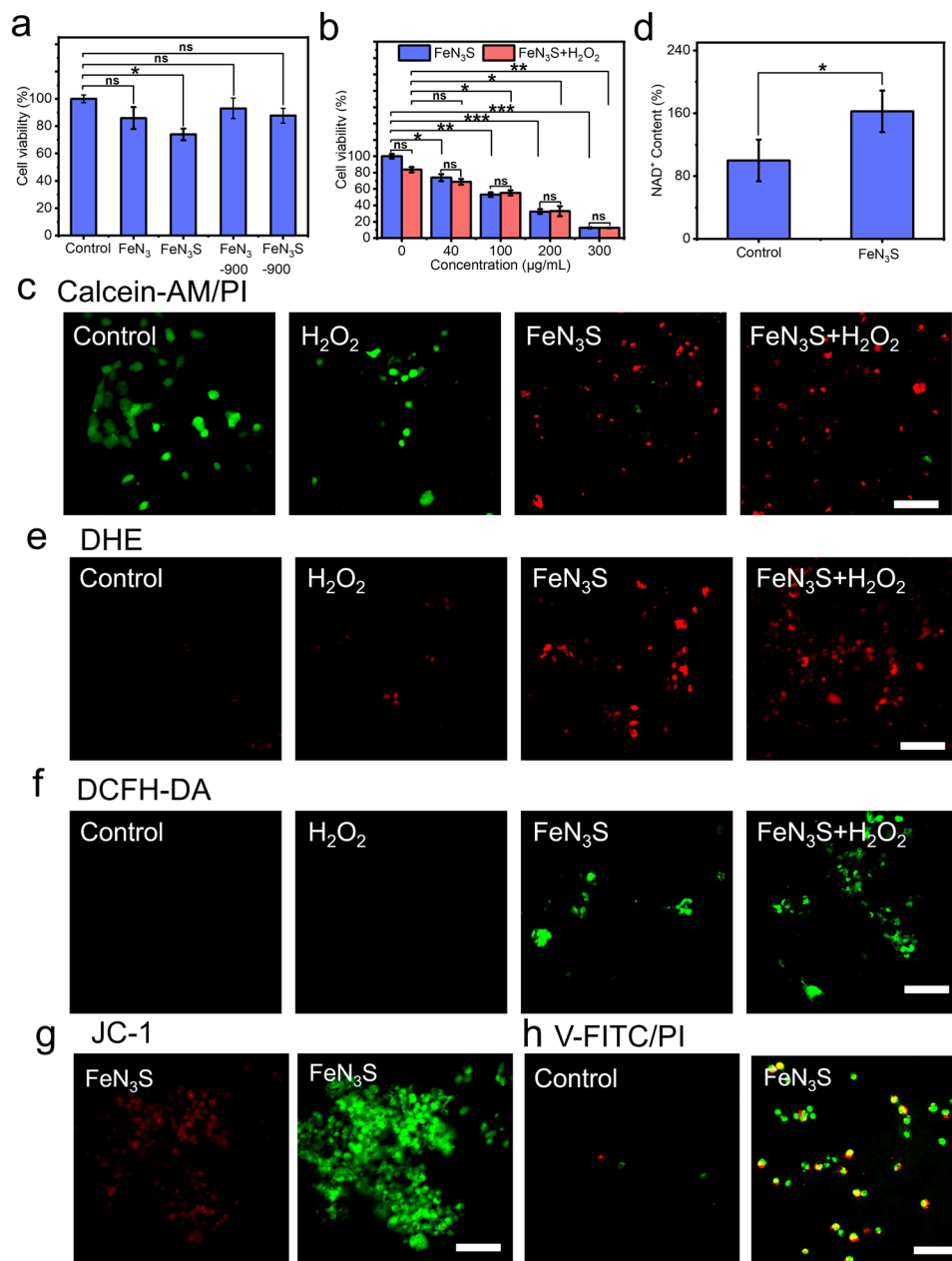
Compared with previous reports (Table S6, Supporting Information), asymmetric SACs were typically developed by mixing supports with heteroatom-containing chemicals and metal precursors, without precise control over heteroatom doping. For example, glucosamine and dicyandiamide were employed as carbon sources to anchor single atoms,<sup>[76]</sup> thiourea,<sup>[76]</sup> trithiocyanuric acid<sup>[77]</sup> and bis(4-hydroxyphenyl) sulfone,<sup>[94]</sup> were used to introduce S; while poly-(cyclotriphosphazene-co-4,4'-diaminodiphenylether)<sup>[7]</sup> and phosphonitric chloride trimer<sup>[94]</sup> were used to introduce P. However, these approaches typically yielded catalysts with only single or bi-enzyme activity. In some cases, additional natural enzymes had to be loaded to achieve

better performance,<sup>[95]</sup> which limits their broader applications. In contrast, our work employs a precisely designed heteroatom-doping strategy to develop asymmetric FeN<sub>3</sub>S SACs with enhanced catalytic functionality: i) the highly dispersed Fe single atoms originating from biomineralization. The thiolate group of Cys serves as the primary binding site for Fe, while the amine group of Cys promotes MOF biomineralization, enabling the formation of highly dispersed Fe single atoms; ii) the large surface area and pore volume retained from the original MOF. After pyrolysis at 800 °C, the MOF retains its original pore size, surface area, and pore volume; iii) S-doping tunes the electronic structure of the Fe single atom, reducing activation barriers, enhancing substrate interactions, and facilitating charge transfer. Collectively, these effects contribute to the improved multi-enzyme activity of Fe SAC. In addition, this strategy could potentially be applied to other metal single atoms owing to the metal cation accumulation ability of Cys.<sup>[50]</sup>

## 2.6. The Role of Fe-SACs in Suppressing Tumor Cell Growth

Given the outstanding multi-enzyme activity of FeN<sub>3</sub>S, its potential in suppressing tumor cell growth was further investigated through a series of in vitro cytotoxicity assays on 4T1 cells. As shown in Figure 5a, FeN<sub>3</sub>S exhibited the most effective suppression of tumor cell growth among FeN<sub>3</sub>, FeN<sub>3</sub>S-900, and FeN<sub>3</sub>-900 at 40 µg mL<sup>-1</sup>. Furthermore, the cytotoxicity of FeN<sub>3</sub>S was dose-dependent, with cell viability decreasing as its concentration increased. At 300 µg mL<sup>-1</sup>, FeN<sub>3</sub>S induced  $\approx 90\%$  cell death within 24 h. However, no significant difference was observed between FeN<sub>3</sub>S with or without H<sub>2</sub>O<sub>2</sub> addition (Figure 5b), suggesting that intracellular H<sub>2</sub>O<sub>2</sub> generated from cell metabolism was sufficient to drive FeN<sub>3</sub>S-activated catalytic reactions.<sup>[7]</sup> A control experiment using normal 3T3 fibroblasts was also conducted. The results showed that over 80% of cells remained viable at concentrations below 100 µg mL<sup>-1</sup>, while  $\approx 60\%$  remained viable at 200–300 µg mL<sup>-1</sup> (Figure S35, Supporting Information), indicating a certain degree of selectivity of FeN<sub>3</sub>S toward tumor cells. Fluorescence imaging confirmed extensive cell death (Figure 5c). The long-term stability and degradation behavior of FeN<sub>3</sub>S in biological environments were systematically evaluated. FeN<sub>3</sub>S was incubated with cells in an intracellular-mimicking medium containing 10% serum and 90% culture medium for 24 and 72 h. After 24 h, 12.2% of Fe and 19.7% of Zn were released, whereas after 72 h, 12.7% of Fe and 36% of Zn were released (Table S7, Supporting Information). In addition, 4000 ng/mL of S was detected after 72 h. The potential cytotoxicity of the released Fe, S, and Zn species toward healthy 3T3 fibroblasts was subsequently assessed. The results indicated that Fe had no appreciable effect on cell viability, while S induced a slight decrease. Notably, co-doping Fe and Zn with S did not cause any additional reduction in cell viability (Figure S36, Supporting Information).

It is found that FeN<sub>3</sub>S can effectively increase NAD<sup>+</sup> content (60%) in cancer cells by mimicking NOX-like activity (Figure 5d), indicating the disruption of the NADH/NAD<sup>+</sup> cycle among cancer cells. Then, the  $\cdot\text{O}_2^-$  at the cellular level was detected by specific fluorescent probes dihydroethidium (DHE). Enhanced red fluorescence was measured in 4T1 cells after being treated with FeN<sub>3</sub>S (Figure 5e), indicating that FeN<sub>3</sub>S can generate  $\cdot\text{O}_2^-$  in



**Figure 5.** Cytotoxicity assessment of 4T1 cells after being treated with a), FeN<sub>3</sub>, FeN<sub>3</sub>S-900, and FeN<sub>3</sub>S-900; Quantitative data are shown as mean  $\pm$  SD ( $n = 3$ ). One-way ANOVA and Sidak's multiple comparisons test were performed. b) different concentrations of FeN<sub>3</sub>S with or without 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> addition. Quantitative data are shown as mean  $\pm$  SD ( $n = 3$ ). Two-way ANOVA (mixed model) and Sidak's multiple comparisons test were performed. c) Live/dead cell fluorescence microscopy images of 4T1 cells stained with Calcein-AM and propidium iodide (PI) after treatment with H<sub>2</sub>O<sub>2</sub>, FeN<sub>3</sub>S, and FeN<sub>3</sub>S+H<sub>2</sub>O<sub>2</sub>. Scale bar, 100  $\mu$ m. d) The NAD<sup>+</sup> content of cancer cells with or without FeN<sub>3</sub>S treatment using the NAD<sup>+</sup> /NADH Assay Kit. Quantitative data are shown as mean  $\pm$  SD ( $n = 3$ ). Two-tailed unpaired *t*-test was performed for the comparison between the two groups. e) Intracellular  $\cdot$ O<sub>2</sub><sup>-</sup> generation probed by DHE after treatment with H<sub>2</sub>O<sub>2</sub>, FeN<sub>3</sub>S, and FeN<sub>3</sub>S+H<sub>2</sub>O<sub>2</sub>. f) Intracellular total ROS generation probed by DCFH-DA after treatment with H<sub>2</sub>O<sub>2</sub>, FeN<sub>3</sub>S, and FeN<sub>3</sub>S+H<sub>2</sub>O<sub>2</sub>. The scale bar is 50  $\mu$ m in c, e-f. g) Fluorescence microscopy images showing mitochondrial depolarization in 4T1 cells cultured with FeN<sub>3</sub>S (200  $\mu$ g mL<sup>-1</sup>) for 24 h. Scale bar, 100  $\mu$ m. Representative fluorescence images from  $n = 3$  independent experiments in c, e-g (each experiment: 3 wells per condition,  $\geq 5$  random fields per well). Acquisition settings were identical across groups. h) Fluorescence microscopy images of 4T1 cells cultured without and with FeN<sub>3</sub>S (200  $\mu$ g mL<sup>-1</sup>) for 24 h and stained with Annexin V-FITC/PI. Scale bar, 20  $\mu$ m. Two-tailed unpaired *t*-test was performed for the comparison between the two groups. Statistical significance was denoted as ns ( $p \geq 0.05$ ), \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), and \*\*\*\* ( $p < 0.0001$ ).

cells through OXD-like activity. However, there is no noticeable signal in the presence of the  $\text{H}_2\text{O}_2$  (Figure 5e). Furthermore,  $\cdot\text{O}_2^-$  production of  $\text{FeN}_3\text{S}$  could be further enhanced after adding  $\text{H}_2\text{O}_2$  due to its intrinsic CAT-like activity. Meantime, a significantly increased green fluorescence was observed after treatment with  $\text{FeN}_3\text{S}$ , indicating total intracellular ROS generation (including  $\cdot\text{OH}$  and  $\cdot\text{O}_2^-$ ) (Figure 5f). Similar to the  $\cdot\text{O}_2^-$ , there is no signal detected in the presence of the  $\text{H}_2\text{O}_2$  alone, indicating the ROS was produced by  $\text{FeN}_3\text{S}$  rather than by intracellular  $\text{H}_2\text{O}_2$ .

To further investigate mitochondrial effects and apoptotic/necrotic pathways, additional experiments were performed. The mitochondrial membrane potential (MMP) is crucial for mitochondrial homeostasis and serves as a key indicator of cell apoptosis. A sustained decrease in MMP from normal levels signals cell death and associated pathologies.<sup>[84]</sup> Therefore, JC-1 staining (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) was employed to assess MMP. Compared with the control (Figure S37, Supporting Information),  $\text{FeN}_3\text{S}$  treatment decreased red fluorescence and increased green fluorescence (Figure 4g), indicating MMP reduction and cell apoptosis, consistent with elevated ROS levels (Figure 5f). Additionally, ATP production in  $\text{FeN}_3\text{S}$ -treated cells was significantly inhibited, with ATP levels reduced by  $\approx 24.7\%$  relative to controls (Figure S38, Supporting Information). Thus, the inhibition of energy production in cancer cells may result from disruption of the NADH/NAD<sup>+</sup> cycle, as endogenous NADH is essential for transmembrane proton translocation and for maintaining the proton motive force required for ATP synthesis.<sup>[47]</sup> Apoptosis analysis using annexin V-FITC/PI staining revealed that 41.5% of 4T1 cells were positive for both V-FITC and PI, indicating late apoptosis or necrosis, while 25% were V-FITC positive but PI negative, reflecting early apoptosis with intact membranes. Only 1% of cells were PI positive but V-FITC negative, suggesting minimal direct necrotic death (Figure 5h; Table S8, Supporting Information). These results demonstrate that  $\text{FeN}_3\text{S}$  predominantly induces apoptosis in 4T1 cells.

In comparison, the single-enzyme catalysts  $\text{Fe-N}_4$ ,<sup>[96]</sup> noble-metal (e.g., Pt/Pd)- $\text{N}_4$ ,<sup>[97]</sup> and bi-enzyme  $\text{Cu-N}_4$ <sup>[98]</sup> achieved only limited therapeutic effects, inducing 56% mortality of 4T1 cells within 10 h, 70% mortality of CNE1<sup>LMPI</sup> cells after 24 h, and 63% mortality of 4T1 cells after 24 h, respectively (Table S9, Supporting Information). Although other reported systems such as BSA-Cu,  $\text{Fe-N}_5$ , and  $\text{Cu-N}_4$  exhibited multi-enzyme activities (POD, OXD, glutathione oxidase (GSHOx) for BSA-Cu,<sup>[99]</sup> OXD, POD, GSHOx for  $\text{Fe-N}_5$ ,<sup>[100]</sup> and POD, CAT, OXD, GSHOx for  $\text{Cu-N}_4$ <sup>[101]</sup>), their therapeutic efficacy remained moderate, with only 66.2% mortality of HCT116 cells after 24 h, 59% mortality of 4T1 cells after 24 h, and  $\approx 78\%$  mortality of 4T1 cells after 12 h, respectively. Moreover, the multi-enzyme Ir- $\text{N}_5$  SAC induced merely  $\approx 60\%$  4T1 cell death under comparable conditions.<sup>[47]</sup> Therefore,  $\text{FeN}_3\text{S}$  catalyst exhibited stronger tumor cell suppression than other reported SACs (Table S9, Supporting Information), highlighting the superior therapeutic efficiency of  $\text{FeN}_3\text{S}$ . In summary, the developed  $\text{FeN}_3\text{S}$  integrates the advantages of multi-enzyme properties (NOX, OXD, POD, and CAT) induce cell apoptosis (Figure S39, Supporting Information): i) The NOX-like property of  $\text{FeN}_3\text{S}$  perturbs intracellular redox equilibrium and disrupts metabolic stability in cancer cells, thereby sensitizing them to oxidative stress. ii) The OXD- and POD-like activities fur-

ther accelerate ROS generation, leading to oxidative damage that inhibits cell growth and promotes apoptotic signaling pathways. iii) The CAT-like activity of  $\text{FeN}_3\text{S}$  unexpectedly enhances  $\cdot\text{O}_2^-$  production, amplifying oxidative stress. Collectively, these synergistic redox activities shift the intracellular balance toward excessive ROS accumulation. This oxidative overload compromises mitochondrial integrity, decreases ATP production, and triggers apoptosis as the dominant mode of cell death. These characteristics highlight its strong potential as a therapeutic agent in cancer treatment. However, the biological evaluation in this study is limited to 4T1 cells and 3T3 fibroblasts in vitro. To further advance the translation of  $\text{FeN}_3\text{S}$  for cancer therapy, in vivo studies will be required in the future. To adapt  $\text{FeN}_3\text{S}$  for in vivo applications, surface modification strategies such as PEGylation<sup>[102]</sup> or coating with zwitterionic polymers<sup>[103]</sup> can be employed to form hydrophilic barriers that minimize protein adsorption and reduce immune recognition. To enhance targeted delivery,  $\text{FeN}_3\text{S}$  can be further functionalized with macrophage membranes<sup>[104]</sup> or site-specific antibodies<sup>[105]</sup>, enabling selective accumulation at inflammatory sites.

### 3. Conclusion

A metallothionein-inspired heteroatom doping strategy was employed to engineer an asymmetric  $\text{FeN}_3\text{S}$  nanozyme.  $\text{FeN}_3\text{S}$  exhibited 1.35-4.60-fold higher catalytic efficiency in NOX-, OXD-, POD-, and CAT-like activities compared with  $\text{FeN}_3$  without S doping. The highly efficient multi-enzyme activity of  $\text{FeN}_3\text{S}$  can be attributed to the highly dispersed single atoms originating from the biomineralization, the large surface area and pore volume retained from the original MOF, and the S-doping achieved through the strong mercaptide coordination between Fe and S. The S doping not only tunes electronic structure of Fe single atom to reduce activation barriers and enhances substrate interaction, but also facilitates charge transfer. Owing to its highly efficient multi-enzyme activity,  $\text{FeN}_3\text{S}$  achieved  $\approx 90\%$  cancer cell death within 24 h. This work highlights a promising strategy for developing advanced SACs with multi-enzyme activity for complex environmental and biomedical applications.

### 4. Experimental Section

**Reagent and Materials:** Zinc nitrate hexahydrate (98.0%), 2-methylimidazole (HmIm, 99.0%), L-cysteine methyl ester hydrochloride (Cys), 1,2-ethanedithiol, 1-butanethiol and thioglycolic acid, iron(III) chloride ( $\text{FeCl}_3$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), 3,3',5,5'-tetramethylbenzidine (TMB), nicotinamide adenine dinucleotide (NADH), terephthalic acid (TA), tris(2,2'-bipyridine)ruthenium(II) hexafluorophosphate (Tris II), NAD<sup>+</sup>/NADH Assay Kit (MAK460), Annexin V-FITC Apoptosis Detection Kit, fluorescent probes dihydroethidium (DHE), acetoxymethyl ester form (Calcein-AM), propidium iodide (PI), 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), NAD<sup>+</sup>/NADH Assay Kit, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide and phosphate-Buffered Saline (PBS) were purchased from Sigma Aldrich (Australia). DyLight<sup>TM</sup> 633 NHS Ester, Zeba<sup>TM</sup> Dye and biotin removal columns (0.5 mL), ATP Determination Kit (A22066), JC-1 Dye (Mitochondrial Membrane Potential Probe) were purchased from Thermo Scientific (Australia). All other reagents were purchased from Sigma-Aldrich (Australia) and used without further purification.

**Catalyst Preparation:** For the synthesis of Fe SACs, 40 mL of an aqueous solution containing 9.020 g of HmIm was first mixed with 0.704 mL

of  $\text{FeCl}_3$  (0.023 M), 0.704 mL of Cys (0.023 M), or a combination of both (rapidly mix). To investigate the impact of different S precursors to the synthesis of Fe SACs, 1,2-Ethanedithiol, 1-Butanethiol, and Thioglycolic acid were used to replace cysteine in equivalent molar amounts. After rapid mixing, immediately add 4 mL of an aqueous solution containing 327.4 mg of  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  was added, and the mixture was stirred at room temperature for 30 min. The resulting products were then centrifuged at 12500 rpm, washed three times with DI water, and dried at 80 °C overnight. The dried samples were placed in a quartz boat and pyrolyzed at 800 or 900 °C for 2 h in a tube furnace under a flowing nitrogen atmosphere ( $10\text{--}20\text{ mL min}^{-1}$ ) with a heating rate of  $5\text{ °C min}^{-1}$ . After cooling to room temperature, the pyrolyzed samples were collected and ground in a mortar for further use.

**Characterizations:** Scanning electron microscopy (SEM) images were taken on a FEI Nova NanoSEM 230 under 10 kV acceleration voltage with a secondary electron detector. The samples were coated with Pt using Emitech K575x evaporative premium coater before imaging. The crystallinity of enzyme-ZIF-8 and SAC was also measured by an Empyrean XRD Xpert Materials Research diffractometer (MRD) system with a  $\text{Cu K}\alpha$  anode ( $\lambda = 0.15406\text{ nm}$ ) at 40 kV and 40 mA. Nitrogen adsorption-desorption measurements were performed using a MicroActive for TriStar II Plus 2.02, samples were degassed by heating at 120 °C for 12 h under vacuum. An X-ray photoelectron spectrometer (Thermo ESCALAB250Xi) was deployed to record the X-ray photoelectron spectra (XPS). Raman spectra were obtained by a Renishaw Raman system at a 514 nm excitation. The metal ion content was determined by ICP-OES (Optima7000 and Avio from PerkinElmer, USA). JEOL JEM-F200 with electron acceleration energy of 200 kV was used to record bright field TEM and HRTEM images, and XEDS elemental mappings. Samples were prepared by dispersing in ethanol using a sonication bath and drop-casting 10  $\mu\text{L}$  of solution onto a holey carbon Cu film. A JEOL JEM-GrandARM300F2 double aberration corrected scanning transmission electron microscope (STEM) operated at 300 kV was used to obtain annular dark field scanning (ADF) STEM images and electron energy loss spectroscopy (EELS) spectra of the pyrolyzed samples. ADF-STEM images were acquired with a camera length of 200 mm with corresponding inner and outer acceptance angles of 32 and 175 mrad, respectively. Under these conditions, it was not possible to distinguish between the Fe and Zn species directly from the images due to the similar Z numbers of the two species and the varying background intensity due to thickness. To confirm the presence of Fe and Zn, EELS spectra were collected using a Gatan Image Filter (GIF) Continuum spectrometer equipped with a single electron detector, K3. The EELS data presented have been background-subtracted. All nanozyme activity measurements were recorded by a CLARIOstar microplate reader (BMG LABTECH Germany) at room temperature.

**XAS Measurements and Data Processing:** The X-ray absorption fine structure spectra (Fe K-edge) were collected at XAS Beamline (Australian Synchrotron, VIC, Australia), the data collection was carried out in transmission mode using an ionization chamber for Fe foil, and in fluorescence excitation mode using a Lytle detector for  $\text{FeN}_3$ ,  $\text{FeN}_3$ ,  $\text{FeN}_3$ -900,  $\text{FeN}_3$ -S-900. All spectra were collected in ambient conditions. The XAS data were processed according to the standard procedures using the Athena module implemented in the IFEFFIT software packages. The EXAFS spectra were obtained by subtracting the post-edge background from the overall absorption and then normalizing with respect to the edge-jump step. Subsequently, the  $\chi(k)$  data were Fourier transformed into real (R) space using a Hanning windows ( $dk = 1.0\text{ Å}^{-1}$ ) to separate the EXAFS contributions from different coordination shells. To obtain the quantitative structural parameters around central atoms, least-squares curve parameter fitting was performed using the ARTEMIS module of IFEFFIT software packages.<sup>[106,107]</sup> Fitting of the magnitude of the Fourier transform of the  $k^3$ -weighted EXAFS (data-blue and fit-red) for the sample and standards, and the corresponding EXAFS fitting curves of Sample and Standards at  $k$  space. For Wavelet Transform analysis, the  $\chi(k)$  exported from Athena was imported into the Hama Fortran code.<sup>[108]</sup> The parameters were listed as follows:  $R$ -range, 0.0–6.0 Å,  $k$ -range, 0–14.0  $\text{Å}^{-1}$  for sample and standards;  $k$  weight, 2; and Morlet function with  $\kappa = 8$ ,  $\sigma = 1$  was used as the mother wavelet to provide the overall distribution.

**Preparation of Dye-Labeled Cys:** Cys were tagged with NHS-633 ester. The mixtures of Cys and NHS-633 ester were allowed to stand for 20 min to ensure thorough interaction. Excess dyes were then removed using Zeba™ Dye and Biotin Removal Spin Columns and Filter Plates, which cleared unreacted dyes and other residues via centrifugation at 1000 rcf for 2 min. The obtained dye-labeled Cys followed the same synthesis of  $\text{Fe}^{3+}$ -Cys-ZIF-8.

**Detection of thNOX-like and OXD-like Activity:** Michaelis–Menten kinetic analysis of NOX-like and OXD-like activities was performed using HAc–NaAc buffer (0.2 M, pH 4.5) with 2 mg  $\text{mL}^{-1}$  samples. For NOX-like activity, 50  $\mu\text{L}$  of varying concentrations of NADH and 5  $\mu\text{L}$  of samples were added, and the absorbance at 340 nm was measured over time using a CLARIOstar microplate reader (BMG LABTECH, Germany). A plot of absorbance at 340 nm versus time was generated to create a reaction-time curve. For OXD-like activity, the absorbance was monitored at 652 nm ( $\epsilon = 39000\text{ M}^{-1}\text{ cm}^{-1}$ ). The initial rate of absorbance change was determined from the slope of the linear portion at the beginning of the curve. A Michaelis plot was then constructed by plotting the initial rate against substrate concentration, and the Michaelis–Menten equation was fitted using nonlinear least-squares regression in GraphPad Prism (version 9) to calculate  $K_m$  and  $V_{\text{max}}$ . The turnover number ( $k_{\text{cat}}$ ) was obtained by dividing  $V_{\text{max}}$  by the molar concentration of the catalyst. The Fe content in the samples was determined by inductively coupled plasma optical emission spectrometry (Optima 7000 and Avio, PerkinElmer, USA).

**Detection of the POD-like and CAT-like Activity:** Michaelis–Menten kinetic analysis of POD-like and CAT-like activities was performed using HAc–NaAc buffer (0.2 M, pH 4.5) with 2 mg  $\text{mL}^{-1}$  SACs. To evaluate the catalytic production of hydroxyl radicals from  $\text{H}_2\text{O}_2$  using terephthalic acid (TA), 50  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (0.0979 M) and SACs containing the same amount of Fe single atoms were added, and the change in fluorescence intensity over time was measured (excitation at 320 nm, emission at 425 nm) using a CLARIOstar microplate reader (BMG LABTECH, Germany). For assessing the catalytic production of oxygen from  $\text{H}_2\text{O}_2$  using Tris(2,2'-bipyridine)ruthenium(II) hexafluorophosphate ( $[\text{Ru}(\text{bpy})_3]^{2+}$ ), the excitation and emission wavelengths were 450 and 625 nm, respectively.

**MTT Assay to Evaluate Cytotoxicity:** To evaluate the ability of the prepared Fe-SACs to suppress cancer cell growth and simulate the in vivo environment, 4T1 cells were selected for live/dead staining after treatment. The cells were cultured in 24-well plates for 24 h, followed by the addition of different samples at varying concentrations, with or without 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , for another 24 h. Cytotoxicity was assessed using the MTT assay. Specifically, 100  $\mu\text{L}$  of thiazolyl blue tetrazolium bromide (0.5 mg  $\text{mL}^{-1}$ ) was added to each well. After incubation for 2 h at 37 °C, the supernatants were removed, 1 mL of DMSO was added, and the absorbance at 550 nm was measured using a Benchmark Plus microplate spectrophotometer (BMG LABTECH, Germany). Data are presented as the mean of three independent experiments. In parallel, a non-tumorigenic control was conducted using 3T3 fibroblast cells following the identical protocols to assess off-target cytotoxicity and comparative biocompatibility relative to 4T1 cells.

**Detection of Live and Dead Cells:** Live and dead cells were stained with Calcein-AM and PI, respectively. 4T1 cells were seeded into 24-well plates and cultured for 24 h. The cells were then treated with different groups: i) Control, ii) 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , iii)  $\text{FeN}_3$ S, and iv)  $\text{FeN}_3$ S + 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . After 24 h of treatment, the cells were incubated with Calcein-AM (2  $\mu\text{M}$ ) and PI (5  $\mu\text{g mL}^{-1}$ ) for 20 min, and the fluorescence images of live and dead cells were captured using a fluorescence microscope.

**Detection of Cell Death States:** Early apoptotic and late apoptotic cells were stained with V-FITC, and necrotic cells were stained with PI. 4T1 cells were seeded into 24-well plates and cultured for 24 h. The cells were then treated with or without 200  $\mu\text{g mL}^{-1}$   $\text{FeN}_3$ S for 24 h. Then, cells were incubated with V-FITC (5  $\mu\text{L}$ ) and PI (5  $\mu\text{g mL}^{-1}$ ) for 10 min in the dark. The fluorescence images of apoptotic and necrotic cells were captured using a fluorescence microscope. The proportions of cells in different death states were quantified based on analysis of more than 10 random fields.

**Intracellular ROS Groups and  $-\text{O}_2\text{O}_2^-$  Production:** Intracellular ROS and  $-\text{O}_2\text{O}_2^-$  generation were probed using DCFH-DA and DHE, respectively. 4T1 cells were seeded into 18-well plates and cultured for 24 h, followed by treatment with different groups: i) Control, ii) 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , iii)  $\text{FeN}_3$ S,

and iv)  $\text{FeN}_3\text{S} + 100 \mu\text{M H}_2\text{O}_2$ . After 24 h, the treated cells were incubated with DCFH-DA ( $10 \mu\text{M}$ ) or DHE ( $30 \mu\text{M}$ ) for 20 min, and the ROS and  $-\text{O}_2^-$  production were visualized using a fluorescence microscope.

**Detection of Intracellular ATP Content:** Intracellular ATP levels were determined using an ATP Determination Kit (A22066, Thermo Scientific). 4T1 cells were seeded in 24-well plates and cultured for 24 h, followed by treatment with or without  $200 \mu\text{g mL}^{-1} \text{FeN}_3\text{S}$  for an additional 24 h. After treatment,  $200 \mu\text{L}$  of CellLytic™ M reagent was added to each well to lyse the cells. The resulting lysates were centrifuged, and the supernatants were collected for ATP quantification using the ATP assay kit.

**Detection of Mitochondrial Membrane Potential (MMP):** The mitochondrial membrane potential was assessed using JC-1 dye (Thermo Scientific). 4T1 cells were seeded in 24-well plates and cultured for 24 h, followed by treatment with or without  $200 \mu\text{g mL}^{-1} \text{FeN}_3\text{S}$  for another 24 h. After treatment, the cells were incubated with JC-1 dye ( $2 \mu\text{M}$ ) for 20 min in the dark. Fluorescence images of green JC-1 monomers (indicating low MMP) and red JC-1 aggregates (indicating high MMP) were captured using a fluorescence microscope.

**Detection of Intracellular NADH Content:** Intracellular NADH levels were measured using a  $\text{NAD}^+/\text{NADH}$  Assay Kit (Sigma). 4T1 cells were seeded into culture bottles and cultured for 24 h, then treated with  $\text{FeN}_3\text{S}$  for 24 h. After treatment, the cells were rinsed with PBS and collected by centrifugation at 3000 rpm. The cell pellets were suspended in 1 mL of  $\text{NAD}^+/\text{NADH}$  extraction buffer, and the supernatants were collected for NADH quantification using the assay kit.

**Computational Details:** All the DFT calculations were carried out in the Vienna ab initio simulation (VASP5.4.4) code.<sup>[109]</sup> The exchange-correlation was simulated with the PBE functional, and the ion-electron interactions were described by the PAW method.<sup>[110,111]</sup> The vdWs interaction was included by using the empirical DFT-D3 method.<sup>[112]</sup> The Monkhorst-Pack-grid-mesh-based Brillouin zone k-points were set as  $2 \times 2 \times 1$  for all periodic structures with the cutoff energy of 450 eV. The convergence criteria were set as  $-0.03 \text{ eV Å}^{-1}$  and  $10^{-5} \text{ eV}$  in force and energy, respectively.

**Statistical Analysis:** All experiments were conducted in at least three independent biological replicates. Quantitative data were presented as mean  $\pm$  SD. The exact sample size (n) for each analysis refers to the number of independent biological replicates and is provided in the figure legends. For two-group comparisons, a two-tailed unpaired Welch's *t*-test (equal variances not assumed) was used. For multiple-group (one-factor) analyses, one-way ANOVA followed by Šidák's multiple comparisons test was applied. For two-factor datasets, two-way ANOVA (mixed-effects model with REML when appropriate) followed by Šidák's multiple comparisons test was used; interaction *P*-values were reported where relevant. All tests were two-sided with  $\alpha = 0.05$ . Statistical significance was denoted as ns ( $p \geq 0.05$ ), \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), and \*\*\*\* ( $p < 0.0001$ ). For imaging-based assays, each replicate used three wells per condition and  $\geq 5$  randomly selected fields per well. Field-level measurements were background-subtracted using identical thresholds across groups and averaged within wells; well means were then averaged to yield one value per biological replicate (statistical unit). Analyses were performed in GraphPad Prism 10 and Origin 2024. Data failing predefined quality-control criteria (e.g., out-of-focus or saturated images) were excluded a priori before analysis. Where indicated, viability or ratio-type readouts were normalized to the control condition (set to 100%).

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

biocatalysis, biomineralization, metal-organic frameworks, nanozymes, single atom catalysts

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