Oxidase-like manganese oxides nanoparticles: A mechanism of organic acids/aldehydes as electron acceptors and potential application in cancer therapy

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ABSTRACT: Identifying the underlying catalytic mechanisms of synthetic nanocatalysts or nanozymes is important in directing their design and applications. Herein, we revisited the oxidation process of 4,4'-diamino-3,3',5,5'-tetramethylbiphenyl (TMB) by Mn$_3$O$_4$ nanoparticles and revealed that it adopted an organic acids/aldehydes-triggered catalytic mechanism at a weakly acidic or neutral pH, which is O$_2$-independent and inhibited by pre-addition of H$_2$O$_2$. Importantly, similar organic acids/aldehydes-mediated oxidation was applied to other substrates of peroxidase in the condition of nanoparticulate or commercially available MnO$_2$ and Mn$_2$O$_3$ but not MnO. The selective oxidation of TMB by Mn$_3$O$_4$ over MnO was further supported by density functional theory calculation. Moreover, Mn$_3$O$_4$ nanoparticles enabled oxidation of indole 3-acetic acid, a substrate that can generate cytotoxic singlet oxygen upon a single-electron transfer oxidation, displaying a potential in nanocatalytic tumor therapy. Overall, we revealed a general catalytic mechanism of manganese oxides towards oxidation of peroxidase substrates, which could boost the design and various applications of these manganese-based nanoparticles.

KEYWORDS: Manganese oxides; Non-radical catalysis; Oxidase-like; Organic acids; Organic aldehydes
Natural oxidases and peroxidases mediate highly specific and efficient oxidation reactions at a mild biological condition using molecular oxygen or hydrogen peroxide as electron acceptor/oxidant, respectively.\textsuperscript{1-3} In addition to the biological functions, these enzymes also play an important role in biological and environmental sensing but they are challenged by the harsh chemical conditions. Fortunately, synthetic nanocatalysts (or nanozymes) that harbor the enzyme-like activity but higher stability at harsh conditions were developed and had been widely explored for cancer treatment, anti-bacteria and biosensing.\textsuperscript{4-9}

Manganese-based nanozymes are attractive owing to the multivalency nature-endowed redox behavior and multi-enzyme activity,\textsuperscript{10,11} allowing for their extensive applications across a range of biomedical fields.\textsuperscript{12-16} The excellent catalytic properties of manganese oxides-based nanoparticles, mainly Mn\textsubscript{3}O\textsubscript{4} and Mn\textsubscript{2}O\textsubscript{3}, have been largely attributed to the manganese-catalyzed generation of reactive oxygen species (ROS) from molecular oxygen. The highly active ROS can oxidize substrates like TMB. In most studies, catalytic studies are performed in a typical acetate buffer solution. However, this ROS-mechanism does not fully explain the exceptional catalytic performances observed in solutions with a low concentration of molecular oxygen (around 1.4 × 10\textsuperscript{-3} mol/L), nor does it account for similar catalytic effects observed in an oxygen-free buffer solution in our study. Therefore, we hypothesized the existence of an alternative catalytic mechanism for manganese oxides nanoparticles that does not rely on oxygen. Investigating this mechanism has the potential to revolutionize our comprehension of manganese-based nanozymes and provide valuable insights for their design and diverse applications.

Herein, we revisited the oxidation behavior of Mn\textsubscript{3}O\textsubscript{4} and revealed that acetate in acetate buffer solution, as electron acceptor, involves with the catalysis reaction and is reduced to CH\textsubscript{3}CHO and/or CH\textsubscript{3}CH\textsubscript{2}OH. Importantly, we further established acetic acid could be replaced with other organic acids and/or aldehydes. This organic acids/aldehydes-mediated oxidation of substrates could be a general mechanism and applied to other manganese oxides (MnO\textsubscript{2} and Mn\textsubscript{2}O\textsubscript{3}) at weakly acidic or even neutral pH. Furthermore, we explored the potential anticancer application of the oxidase-like nanoparticles using indole 3-acetic acid as a substrate.

Mn\textsubscript{3}O\textsubscript{4} nanoparticles were prepared and the structure confirmation was discussed in supporting information (Figure S1-S4). We employed a polymeric stabilizer poly(L-histidine) (PHis, Figure S5) to improve the dispersion of Mn\textsubscript{3}O\textsubscript{4} (Figure 1A). The obtained PHis-Mn NPs displayed enhanced dispersibility
and a slightly larger size relative to Mn₃O₄ (Figure S4). Other transition metal oxide-based NPs (PHis-M, M= Fe, Cu and Zn) were prepared similarly as control groups. PHis-M prepared at a feeding PHis/M molar ratio of 1:1 was selected for the size of 150-250 nm (Figure S6) and the presence of transition metals was verified by inductively coupled plasma optical emission spectrometry (Table S1). Next, we tested the TMB oxidation behaviors of these PHis-M NPs in the presence and absence of H₂O₂ (Figure 1C-F), and the sequential addition of H₂O₂ and TMB was also presented in Figure 1B. TMB is a classical substrate of peroxidase and undergoes a single electron transfer process to obtain blue oxidized TMB, which could further lose one electron to give a yellow product (Figure S8E).¹⁷ PHis-Zn as a control cannot oxidize TMB. In contrast, PHis-Fe and PHis-Cu caused strong absorption at 650 nm only in the presence of H₂O₂, which was consistent with previously reported H₂O₂-dependent oxidation of TMB by Fe/Cu oxides through Fenton and Fenton-like reactions.¹⁸,¹⁹ In stark contrast, PHis-Mn led to TMB oxidation in the absence of H₂O₂ but not in the condition of H₂O₂ addition before TMB (Figure 1C). For PHis-Mn/Cu/Fe, higher absorbances of ox-TMB was observed at an acidic pH than those at normal pH (5.5 vs 7.4 at ABS buffer). The above results were summarized in Figure 1G. Meanwhile, we screened and confirmed that the oxidation behavior of PHis-Mn was inherited from Mn₃O₄ (Figure S7).
Figure 1. (A) Synthesis of PHis-coated metal oxide NPs (PHis-M, M= Fe, Cu, Mn, and Zn). (B) A schematic of the sequence of adding H$_2$O$_2$ and TMB to figures C-F. (C-F) The impacts of H$_2$O$_2$ and pH on the TMB oxidation of PHis-M in ABS. (G) Table of oxidation degree of TMB in Figure C-F.

Besides of TMB, PHis-Mn was capable of oxidizing other substrates (2,2’-azinobis-(3-ethylenzthiazoline-6-sulphonate) (ABTS) and o-phenylenediamine (OPD)) of horseradish peroxidase (HRP) in ABS or HCl solution without H$_2$O$_2$ (Figure 2B-D, S8), but it cannot oxidize free-radical scavengers sodium L-ascorbate (NaA)$^{20}$ or methylene blue (MB)$^{21}$ (Figure S9), suggesting that PHis-Mn-mediated oxidation was probably involved with a single electron transfer process but not a reactive oxygen species (ROS)-generating one.$^{22}$ According to Figure S10, we utilized electron paramagnetic resonance (EPR) to observe the production of ROS. However, ROS signal was hardly detected, providing additional evidence that the oxidation of TMB did not occur through a ROS generation mechanism. Notably, the oxidation of TMB is more intense in ABS than in HCl solution at the same pH of 5.5. In addition, the addition sequences...
of \( \text{H}_2\text{O}_2 \) and substrates of HRP (TMB, ABTS and OPD) strongly affected the oxidation process (Figure 2A-D, curve a vs d), where \( \text{H}_2\text{O}_2 \) addition first would greatly hamper the oxidation process (curve d), indicating the competitive interactions with PHis-Mn between substrates and \( \text{H}_2\text{O}_2 \), which is consistent with the previous result (Figure 1C). Overall, we confirmed that PHis-Mn could oxidize the typical substrates of peroxidase but without the assistance of \( \text{H}_2\text{O}_2 \).

**Figure 2.** (A) Diagram and (B-D) the absorbance of TMB oxidation systems with different addition sequences of \( \text{H}_2\text{O}_2 \) and substrates (TMB, ABTS, and OPD) at pH 5.5 ABS. PHis-Mn: 70 \( \mu \text{g/mL} \); TMB: 0.8 mM; ABTS: 3 mg/mL; OPD: 0.16 mM. The (E) Mn 2p and (F) Mn 3s spectra of \( \text{Mn}_3\text{O}_4 \) upon treatment with TMB or \( \text{H}_2\text{O}_2 \) at pH 5.5 ABS. (G) Lineweaver-Burk plot of \( \text{Mn}_3\text{O}_4 \) and PHis-Mn (70 \( \mu \text{g/mL} \)) at 25 °C.

Inspired by the \( \text{H}_2\text{O}_2 \)-free oxidation of broad substrates of peroxidase, we speculated that PHis-Mn may act as an oxidase-mimicking nanozyme. To verify our conjecture, we performed a XPS spectroscopy comparison of PHis-Mn before and after the TMB oxidation assay (Figure 2E, 2F and S11). Obviously, the
average oxidation states (AOS) of Mn and the Mn(II)/Mn(III) composition in Mn$_3$O$_4$ did not significantly change after TMB oxidation (2.75 vs. 2.70). In contrast, H$_2$O$_2$ treatment led to a sharp AOS decrease to 2.11. These results manifested that Mn$_3$O$_4$ triggered a catalytic oxidation of TMB and reduction of Mn$_3$O$_4$ by H$_2$O$_2$ inhibited this oxidation, well supporting the oxidation failure when H$_2$O$_2$ was added before substrates (Figure 2A-D, curve d) and implying that Mn(III) in Mn$_3$O$_4$ was responsible for the catalysis.

Then, we moved our attention to the catalysis kinetics. Noteworthily, the TMB oxidation by PHis-Mn in ABS was rapid and the system turned blue in seconds. The kinetic parameters of Mn$_3$O$_4$ and PHis-Mn (Figure 2G, S12) at pH 5.5 ABS were measured at various concentrations of TMB within 5 s. PHis-Mn showed a higher $V_m$ but lower Michaelis constant ($K_m$) than Mn$_3$O$_4$, which might be due to the better dispersity of PHis-Mn that improved the catalytic efficiency.

Next, we tried to explore the underlying catalytic oxidation mechanism of TMB by PHis-Mn. First, we evaluated the role of O$_2$ in this oxidation. Comparing ABS and HCl solution filled with air and N$_2$ (Figure 3A), O$_2$ did not greatly contribute to the TMB oxidation. Instead, ABS buffer is a key factor for the strong oxidation of TMB, suggesting that H$^+$ or acetic acid played an important role in catalysis. We then use inorganic acid (HCl) to assess the role of H$^+$ in this catalytic process (Figure S13B). A very high concentration of H$^+$ (pH ≤ 3) is needed for obvious TMB oxidation; the possible mechanism would be discussed later. Noteworthily, all the above catalytic effects in ABS solution were stronger than those in HCl at the same pH ranges (pH 5-7). Intriguingly, a neutral pH ABS buffer can even enable PHis-Mn catalyze TMB (Figure S13A). These observations suggested that H$^+$ contributed to catalysis but carboxylate group played a dominant role in the context of ABS. To further explore the role of acetate in the catalysis, gas chromatography (GC) was used to examine the changes of acetic acid in the catalytic reactions (Figure 3B). A new peak of acetaldehyde appeared at 3.34 min after adding TMB into ABS. Meanwhile, another peak of ethanol at 5.33 min was observed in the condition of excessive TMB. These results demonstrated that acetic acid, as an oxidant, was reduced to acetaldehyde and/or ethanol by TMB as electron donors. If this explanation was valid, we believe that other organic acids/aldehydes should be applicable to this Mn$_3$O$_4$-catalyzed TMB oxidation. We next performed the catalysis of TMB by PHis-Mn or Mn$_3$O$_4$ in aqueous solutions of organic acids including polycarboxylic acids (like propanoic acid, citric acid, malic acid, etc.) or
aldehydes (acetaldehyde, chloral hydrate, and butylaldehyde), and the results clearly confirmed this speculation (Figure 3C and S14). In the context of aldehydes, the absorbance of ox-TMB changed a little in acidic pH that was tuned by HCl addition, confirming that H\(^+\) was not the main factor in a weakly acidic condition. Furthermore, alcohols (methanol and ethanol) cannot trigger the oxidation of TMB.

We further tested the catalytic performance of chemically pure manganese oxides (MnO-c, MnO\(_2\)-c, Mn\(_2\)O\(_3\)-c, and Mn\(_3\)O\(_4\)-c). Interestingly, all except MnO-c enabled similar TMB oxidation behaviors (Figure 3D), indicating the high valence of Mn (> 2) is required. Together, these results clearly corroborated the organic carboxylic acid/aldehyde-facilitated the catalysis of manganese oxides (except MnO) in a neutral or weakly acidic condition. In this reaction, carboxylic acid/aldehyde and TMB separately exerted an electron acceptor and donor while manganese oxides a nano-catalyst (Figure 3E). Interestingly, this catalytic performance of ABTS or OPD by commercial manganese oxides was similar to that of TMB (Figure S15), except that OPD was not oxidized by aldehyde because that o-phenylenediamine easily condensates with aldehydes to generate benzimidazole.\(^{23}\)
Figure 3. (A) N$_2$, O$_2$, and air purged solution showed very close TMB oxidation. (B) GC analysis of the reaction products after incubating PHis-Mn with TMB under ABS (pH 5.5). The effects of different kind of aldehydes, organic acids and alcohols on the catalytic performance of (C) PHis-Mn and (D) commercial manganese oxides (PHis-Mn or commercial manganese oxides: 70 $\mu$g/mL; TMB: 0.8 mM; H$_2$O at neutral pH as control). (E) Schematic illustration of organic acids/aldehydes-enabled catalysis of TMB by manganese oxides except MnO.
To elucidate the catalytic activity of Mn$_3$O$_4$ in the condition of organic acids or aldehydes, we conducted density functional theory (DFT) calculations to study the reduction of acetic acid on the Mn$_3$O$_4$ surface using TMB as the reducing agent. The change in Gibbs free energy ($\Delta G$) along the proposed electron transfer pathway and the optimized calculation model was shown in Figure 4A and Table S3. Mn$_3$O$_4$ (211) was represented as an octahedral lattice occupied by Mn (III). Mn$_3$O$_4$ (211) showed a lower adsorption energy of CH$_3$COOH ($\Delta G_{CH3COOH^*}$, -0.10 eV) and shorter length of Mn-O bond (2.039 Å) compared to MnO (111) (0.22 eV and 2.169 Å, Figure 4B and Table S3), suggesting Mn$_3$O$_4$ had a good adsorption configuration stability and was easier to adsorb and activate CH$_3$COOH. What’s more, the rate-limiting step was hydrogenation of CH$_3$CO* to CH$_3$CHO*, where Mn$_3$O$_4$ showed a much lower $\Delta G$ than MnO (0.55 vs. 0.83 eV) and lead to higher hydrogenation activity. To sum up, compared with the MnO, Mn(III) enhances Mn$_3$O$_4$’s ability to attract and transfer electrons significantly. This improvement in electron dynamics boosts the oxo group's basicity, thereby facilitating the efficient acquisition and oxidation of protons and electrons from the substrate TMB. DFT calculations confirmed that the spatial structure of hypervalent manganese dictated the superior catalytic activity, which was consistent with our observations in the previous experiments (Figures 3D and S15).

**Figure 4.** Theoretical investigation of catalytic activity over Mn$_3$O$_4$ (211) and MnO (111). (A) Proposed reaction pathways of CH$_3$COOH reduction to CH$_3$CH$_2$OH with optimized adsorption configurations on
material surface. The red, purple, gray and white balls represent the O, Mn, C and H atoms, respectively. (B) Free energy diagram for acetic acid reduction reaction on enzyme mimics with TMB as reductant.

Finally, we explored the possible mechanism of TMB oxidation by Mn₃O₄ at inorganic HCl solution (pH 3.0). Firstly, we ruled out the involvement of O₂ (Figure S16). Then, we detected the valence change of Mn after reaction with TMB under HCl. XPS results showed that the AOS of Mn₃O₄ at pH 3.0 (2.81) hardly changed (Figure 5A, B), but it significantly declined to 2.29 after addition of TMB. It suggested that Mn₃O₄ acted as an electron acceptor to directly oxidize TMB at a low pH, where the oxidation ability of manganese is greatly elevated like the well-known KMnO₄. The schematic diagram of the oxidative mechanism of Mn₃O₄ in inorganic strong acid was depicted in Figure 5C.

**Figure 5.** The mechanism of Mn₃O₄ catalyzed activation of TMB under inorganic acid. The (A) Mn 2p and (B) Mn 3s spectra of Mn₃O₄ upon treatment with TMB at HCl (pH 3.0). (C) Schematic illustration of oxidation of TMB by Mn₃O₄ under inorganic strong acid.

Taking together, we proposed the oxidation mechanisms of peroxidase substrates by manganese oxide nanoparticles (MnO₂, Mn₃O₄, or Mn₂O₃) in the absence of H₂O₂ (Scheme 1). At a neutral or weakly acidic condition, these NPs underwent a nanocatalyst mechanism using organic acids/aldehydes as electron acceptors (oxidants); in a strong acid solution, however, Mn₃O₄ per se acted as an oxidant.
Scheme 1. Proposed mechanisms of Mn₃O₄-mediated TMB/ABTS/OPD oxidation: as a nanocatalyst in the presence of organic acids/aldehydes at low H⁺ concentrations and as a nano-oxidant at a high H⁺ concentration.

Inspired by that PHis-Mn can catalyze single electron transfer from carboxylic acid, we further explored its potential in catalytic cancer therapy by catalyzing IAA into cytotoxic free radicals. As an acid, IAA similarly promoted the oxidation of TMB (Figure S17A), confirming the proposed organic acids-mediated TMB oxidation. Similarly, we expected that IAA as an acid could trigger the one-electron self-oxidation by PHis-Mn. Since the one-electron oxidation of IAA could produce singlet oxygen, we used 1,3-diphenylisobenzofuran (DPBF) as a singlet oxygen probe to verify this conjecture. As expected, DPBF decreased significantly only when both IAA and PHis-Mn (Figure 6A) were added and positively correlated with the concentration of PHis-Mn (Figure 6B) and IAA (Figure 6C). Compared with IAA/HRP system, IAA/PHis-Mn exhibited similar catalytic effect (Figure S17B). These results clearly demonstrated the IAA-mediated self-oxidation by PHis-Mn and subsequent ¹⁰₂ generation (Figure S18A). Notably, SOSG possessing carboxylic groups cannot be used as a ¹⁰₂ probe here because it can be reduced by IAA and cannot emit fluorescence (Figure S19). Inspired by the ¹⁰₂ generation capacity, we further performed a preliminary
application exploration in cancer therapy. We treated 4T1 breast cancer cells with IAA and PHis-Mn and the
cell viability was tested by a typical MTT assay. The PHis-Mn+IAA treatment led to an IAA dose-dependent
toxicity and 3.6 ± 2.1% cells survived at a concentration of 4 mM IAA, showing a much higher cytotoxicity
than IAA or PHis-Mn treatment alone (Figure 6D, Figure S18B). The high cytotoxicity could be attributed
to the efficient generation of \(^1\text{O}_2\) in cancer cells, as demonstrated by confocal laser scanning microscopy
(CLSM) using DPBF as a probe (Figure S20). Meanwhile, the cytotoxicity and increased intracellular \(^1\text{O}_2\)
level caused by IAA could be mitigated by the introduction of ROS scavenger sodium D-isoascorbate (D-
VC). This is evident from the significance increase in cell viability to approximately 60% (Figure S21), which
is comparable to the 65% viability observed when only pHis-Mn was added. Furthermore, the intracellular
fluorescence intensity was restored upon the introduction of D-VC (Figure S20). These observations clearly
demonstrated the anticancer effects were primary attributed to \(^1\text{O}_2\) generation but not by the reductive
byproducts of IAA. These results indicated that the IAA/PHis-Mn could produce cytotoxic free radicals,
holding a potential in tumor catalytic therapy (Figure S18C).
Figure 6. In vitro PHis-Mn-triggered activation of IAA to produce abundant cytotoxic free radicals to induce the apoptosis of 4T1 breast cancer cells. (A) The depletion of DPBF under different condition (PHis-Mn: 14 μg/mL; IAA: 500 μM; DPBF: 50 μM). UV–vis absorption of different concentrations of (B) PHis-Mn and (C) IAA. (D) Cell viability analysis of 4T1 cells after incubation with IAA and PHis-Mn for 48 h (n = 5, bars represent means ± SD; *** p < 0.001).

In summary, we revealed the different mechanisms of peroxidase substrates by manganese oxide nanoparticles in acidic and neutral pH conditions. At a neutral or weakly acidic condition, these NPs acted as a nanocatalyst using organic acids/aldehydes as electron acceptors (oxidants). In a strong acid solution, manganese oxides per se acted as an oxidant. In addition, we explored the potential application of manganese oxide NPs in cancer therapy in combination with IAA. Our study could provide insights into the understanding and design of Mn-based catalysis toward different applications.
Conflicts of interest

The authors declare no competing financial interest.

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References