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# Mn<sub>3</sub>O<sub>4</sub> microspheres as an oxidase mimic for rapid detection of glutathione

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Exploiting a rapid and sensitive method for biomarker detection has important implications in the early diagnosis of diseases. Here, we synthesized Mn<sub>3</sub>O<sub>4</sub> microspheres which worked as a nanozyme to exhibit outstanding oxidase-like activity for rapid colorimetric determination of glutathione (GSH). The Mn<sub>3</sub>O<sub>4</sub> microspheres of about 800 nm in size could be prepared through a hydrothermal method, and we found that the as-prepared Mn<sub>3</sub>O<sub>4</sub> microspheres could quickly oxidize 3,3',5,5'-tetramethylbenzidine (TMB) to its oxidized form (TMB<sub>ox</sub>) in the absence of H<sub>2</sub>O<sub>2</sub>. After adding glutathione (GSH), TMB<sub>ox</sub> was able to be changed into its original form and resulted in the corresponding decrease in absorbance value at 652 nm. The Mn<sub>3</sub>O<sub>4</sub>-TMB system had good linearity with GSH concatenation in the range of 5–60 μM, and the limit of detection was 0.889 μM. Furthermore, this assay possessed high selectivity specificity, which made it possible to detect GSH in human serum samples. Thus, the obtained assay based on the oxidase mimic of Mn<sub>3</sub>O<sub>4</sub> would enlarge and exploit the application fields of nanozymes in bio-analysis.

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## Introduction

Developing a simple and effective sensing assay to detect biomarkers rapidly has attracted world-wide interest in the field of bio-analysis.<sup>1,2</sup> Till now, numerous analytical techniques based on colorimetric, fluorescence, electrochemistry and surface enhanced Raman scattering have made great efforts in the detection of different disease biomarkers, including promoting the sensitivity and accuracy, and shortening the time of measurement.<sup>3–5</sup> Among these strategies, nanozymes, as catalytic materials with enzyme mimicking activities, show great potential in bio-analysis owing to their tunable catalytic activities.<sup>6</sup> Especially, peroxidase-mimicking nanozymes have been widely investigated to design versatile sensing platform biosensors.<sup>7,8</sup> For instance, a selective glucose biosensor was constructed by combining peroxidase-like Fe<sub>3</sub>O<sub>4</sub> nanozymes with glucose oxidase.<sup>9</sup> This strategy now is generalized to detect other analytes, but this assay is only suitable to the H<sub>2</sub>O<sub>2</sub>-producing analytes.<sup>10</sup> Taking an example, the colorimetric assay of glucose detection includes two cascade reactions: (1) glucose oxidation in the presence of glucose oxidase, (2)

peroxidase-based H<sub>2</sub>O<sub>2</sub> detection. The different optimum reaction conditions for glucose oxidase and nanozymes give rise to two separate steps of reactions rather than one-pot reaction, leading to the complicated analytical processes.<sup>11</sup> Moreover, the two cascade reactions usually cause the error transfer, finally reducing the sensitivity and accuracy of detection. Thus, exploiting nanozymes possessing new enzymatic activities, such as oxidase-like activity that does not require unstable H<sub>2</sub>O<sub>2</sub> as a co-substrate, to construct a colorimetric assay based on one-pot reaction will simplify the measurement steps, thereby solving the above conundrum.

It is noteworthy that very few nanozymes have oxidase-like activity. The best known examples are Au nanoparticles for glucose oxidation,<sup>12,13</sup> and CeO<sub>2</sub>, V<sub>2</sub>O<sub>5</sub>, MnO<sub>2</sub> can also oxidize a diverse range of substrates directly.<sup>14,15</sup> Oxidases are important since they do not need H<sub>2</sub>O<sub>2</sub> as a co-substrate. Mn<sub>3</sub>O<sub>4</sub> is a versatile nanozyme and reported to have various enzyme-like activities, including catalase,<sup>16</sup> superoxide dismutase<sup>17</sup> and glutathione peroxidase (GPx).<sup>18</sup> It was usually applied for wound healing, *in vivo* anti-inflammation and cytoprotection. However, the oxidase-like activity of Mn<sub>3</sub>O<sub>4</sub> has not been reported. The catalytic activities of Mn<sub>3</sub>O<sub>4</sub> are attributed to the mixed oxidation states of Mn<sup>3+</sup> and Mn<sup>2+</sup>, and related oxygen vacancies. As CeO<sub>2</sub> and V<sub>2</sub>O<sub>5</sub>, we speculated that Mn<sub>3</sub>O<sub>4</sub> maybe possessed oxidase-like activity as a promising candidate for developing colorimetric assays.

Herein, we reported a hydrothermal synthesis method to prepare Mn<sub>3</sub>O<sub>4</sub> microspheres, which exhibited outstanding oxidase-like activity to convert colorless TMB rapidly into TMB<sub>ox</sub> (a naked-eye visible chromogenic substrate) in the

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absence of  $\text{H}_2\text{O}_2$ . It was found that glutathione (GSH) could selectively suppress the oxidation of TMB, converting blue TMB<sub>ox</sub> to colorless TMB. Based on this phenomenon, the GSH level could be measured by monitoring the decrease of TMB<sub>ox</sub> peak intensity at 652 nm. It is well known that GSH plays a critical role in the biological system as one of the most common intracellular non-protein biothiols.<sup>8</sup> Many diseases, such as cancer, liver damage, acquired immunodeficiency syndrome (AIDS), psoriasis, heart problems and leukocyte loss, are closely related to the abnormal level of cellular glutathione.<sup>19</sup> Therefore, the accurate detection of GSH in serum is a matter of cardinal significance for disease prevention and diagnosis. Several studies have combined materials, such as carbon quantum dots,<sup>20</sup>  $\text{Ag}^{+21}$  and  $\text{MnO}_2$ ,<sup>22</sup> with TMB for colorimetric detection of GSH. We here utilized a one-pot reaction, the oxidase-like activity of  $\text{Mn}_3\text{O}_4$ , to detect the GSH level in human serum samples directly and rapidly. Unlike previous peroxidase-based biosensors including two cascade reactions, the proposed strategy only needed a one-pot reaction, simplifying the steps of measurement and improving the sensitivity and accuracy.

## Experimental

### Materials

Manganese formate dehydrate  $\text{Mn}(\text{HCO}_2)_2 \cdot 2\text{H}_2\text{O}$ , sodium acetate (NaAc), ethanol ( $\text{C}_2\text{H}_5\text{OH}$ ), methanol ( $\text{CH}_3\text{OH}$ ), acetic acid (HAc), glucose (Glu), sodium chloride (NaCl), potassium chloride (KCl), ascorbic acid (AA), and dopamine (DA) were purchased from Sinopharm Chemical Reagent (Shanghai, China). TMB (3,3',5,5'-tetramethylbenzidine) was obtained from Sigma-Aldrich (USA).  $\text{ZnCl}_2$ ,  $\text{CaCl}_2$ , and  $\text{MgCl}_2$  were bought from Sangon Biotech (Shanghai, China). L-serine (Ser), glycine (Gly), L-histidine dihydrochloride (His), L-threonine (Thr), tryptophan (Try), L-arginine (Arg), cysteine (Cys) and bovine protein serum (BSA) were acquired from BBI Life Sciences (Shanghai, China). Glutathione (GSH) was purchased from Adamas Reagent (Shanghai, China). IgG was purchased from Solarbio (Beijing, China).

### Synthesis of $\text{Mn}_3\text{O}_4$ microspheres

$\text{Mn}(\text{HCO}_2)_2 \cdot 2\text{H}_2\text{O}$  (0.2040 g) was dissolved in methanol (40 mL) with continuous stirring for 30 min, and then the mixture was transferred to a Teflon autoclave and kept at 180 °C for 12 h. After finishing the reaction,  $\text{Mn}_3\text{O}_4$  microspheres were obtained through centrifugation, washing and drying.

### Characterization of $\text{Mn}_3\text{O}_4$ microspheres

Scanning electron microscopy (SEM, S-4800II, Hitachi, Tokyo, Japan) was applied to characterize the morphology and structure of  $\text{Mn}_3\text{O}_4$  microspheres. The FT-IR spectrum was monitored on a Bruker Tensor 27 FT-IR spectrometer (Germany). The composition and surface chemistry of  $\text{Mn}_3\text{O}_4$  microspheres were characterized by X-ray powder diffraction (XRD, D8 Advance, Bruker AXS, Germany) and X-ray photoelectron spectroscopy (XPS, Thermo ESCALAB 250 spectrometer, USA).

### Oxidase-like activity of $\text{Mn}_3\text{O}_4$ microspheres and kinetic studies

The major absorbance peaks of TMB<sub>ox</sub> in 0.1 M NaAc-HAc buffer (pH 4.5) appeared at 652 and 370 nm. For the evaluation of oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres, chemicals were added into a 1.0 mL NaAc-HAc buffer solution in the order of  $\text{Mn}_3\text{O}_4$  (varying amounts) and TMB (final concentration: 0.416 mM). The color change of TMB in the presence of  $\text{Mn}_3\text{O}_4$  microspheres was monitored by a UV-vis spectrometer (Hitachi UV2010, Japan). For kinetic parameters, the experiments were carried out in the NaAc-HAc buffer (0.1 M), containing 5.0  $\mu\text{g mL}^{-1}$   $\text{Mn}_3\text{O}_4$  microspheres and TMB with concentration ranging from 0 to 0.416 mM. The kinetic determination was monitored in a time-scan mode at 652 nm and the Michaelis-Menten constant was obtained through the Lineweaver-Burk plot analyzed by GraphPad.

### Colorimetric detection of GSH

The measurement of GSH was performed at room temperature. The final working concentrations of  $\text{Mn}_3\text{O}_4$  microspheres and TMB were 10.0  $\mu\text{g mL}^{-1}$  and 0.416 mM, respectively. After adding different concentrations of GSH in  $\text{Mn}_3\text{O}_4$ -TMB buffer system, the mixture was incubated for 5 min and then the change of peak intensity (652 nm) was monitored. The calibration curve of GSH was obtained by plotting  $\Delta A$  at 652 nm as a function of the GSH concentration. Where  $\Delta A = A_0 - A$ ,  $A$  and  $A_0$  corresponded to the absorbance at 652 nm with and without GSH addition, respectively.

Colorimetric assay of GSH in human serum samples was performed as follows. First, the human serum samples from our local hospital (Northern Jiangsu People's Hospital, Jiangsu Province) were centrifuged at 3000 rpm for 10 min. Afterwards, 5.0  $\mu\text{L}$  of serum supernatant,  $\text{Mn}_3\text{O}_4$  microspheres (final concentration: 10.0  $\mu\text{g mL}^{-1}$ ), and TMB (final concentration: 0.416 mM) were incubated together for 5 min, and then the absorbance at 652 nm was measured.

### Statistical analysis

Quantitative data were expressed as the means  $\pm$  standard deviations.

## Results and discussion

$\text{Mn}_3\text{O}_4$  microspheres were synthesized according to a previously report.<sup>20</sup> The morphology, crystalline phase and chemical composition of the obtained  $\text{Mn}_3\text{O}_4$  microspheres were analyzed by SEM, XRD and XPS. The SEM image shown in Fig. 1A indicated that the prepared  $\text{Mn}_3\text{O}_4$  microspheres were about 800 nm in diameter, and each microsphere was composed of small-sized  $\text{Mn}_3\text{O}_4$  nanoparticles ( $\sim 10$  nm) (Fig. 1B). Elemental mapping of the  $\text{Mn}_3\text{O}_4$  microsphere (Fig. 1C) showed that O and Mn elements were homogeneously distributed within a whole microsphere. The main diffraction peaks in XRD spectrum (Fig. 2A) matched to the standard pattern of hausmannite  $\text{Mn}_3\text{O}_4$  [JCPDS card no. 24-0734],<sup>23,24</sup> confirming their crystalline nature. Furthermore, the surface of



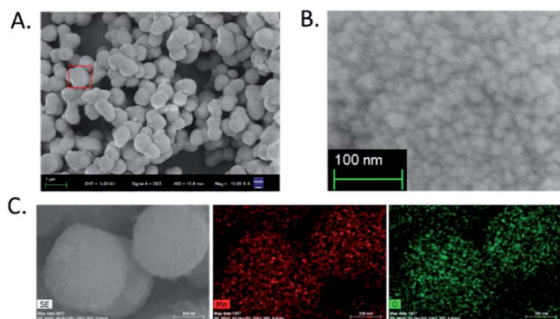


Fig. 1 (A) SEM image of  $\text{Mn}_3\text{O}_4$  microspheres. (B) High-magnification SEM image of an individual  $\text{Mn}_3\text{O}_4$  microsphere. (C) Elemental mapping images of Mn and O in  $\text{Mn}_3\text{O}_4$  microspheres.

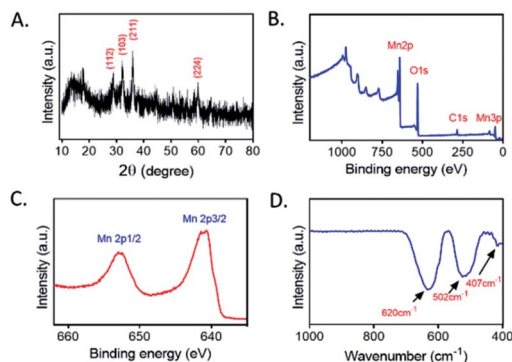


Fig. 2 (A) XRD patterns of  $\text{Mn}_3\text{O}_4$  microspheres. (B) XPS spectrum of  $\text{Mn}_3\text{O}_4$  microspheres. (C) Mn 2p peak of  $\text{Mn}_3\text{O}_4$  microspheres. (D) FT-IR spectrum of  $\text{Mn}_3\text{O}_4$  microspheres.

$\text{Mn}_3\text{O}_4$  microspheres was analyzed by XPS. As shown in Fig. 2B and C, Mn 2p<sub>3/2</sub> and 2p<sub>1/2</sub> peaks were located at 641.67 eV and 653.35 eV, respectively. The atom ratio of  $\text{Mn}^{2+}$  and  $\text{Mn}^{3+}$  was about 1/2, which was consistent with the theoretical value.<sup>25</sup> Moreover, the FT-IR spectrum (Fig. 2D) of  $\text{Mn}_3\text{O}_4$  microspheres showed three main bands centered at (407, 502 and 620  $\text{cm}^{-1}$ ), which were attributed to the Mn–O vibrations of the  $\text{Mn}_3\text{O}_4$  framework.<sup>17</sup> Thus, the  $\text{Mn}_3\text{O}_4$  microspheres could be obtained successfully through a hydrothermal synthesis.

Recently,  $\text{Mn}_3\text{O}_4$  materials were reported to have the catalase-like and superoxide dismutase-like activities under neutral conditions for elimination of reactive oxygen species both *in vitro* and *in vivo*.<sup>26</sup> However, the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  has not been reported. We here concentrated on the investigation of the oxidase-like activity possessed by  $\text{Mn}_3\text{O}_4$  microspheres, and TMB was used as the substrate of oxidase-like reaction. As presented in Fig. 3A, a deep blue color with maximum absorption peak at 652 nm indicated that  $\text{Mn}_3\text{O}_4$  microspheres were able to cause the oxidation of TMB directly. This reaction occurred without the assistance of  $\text{H}_2\text{O}_2$ . Increasing the concentration of  $\text{Mn}_3\text{O}_4$  microspheres was beneficial for the occurrence of this oxidation reaction. The time-dependent absorbance change in  $\text{Mn}_3\text{O}_4$ -TMB was shown in Fig. 3B, and the oxidation of TMB reached a plateau at  $\sim 2$  min at 25  $^\circ\text{C}$ , which was much faster than that reported oxidase-like reactions triggered by other nanomaterials for

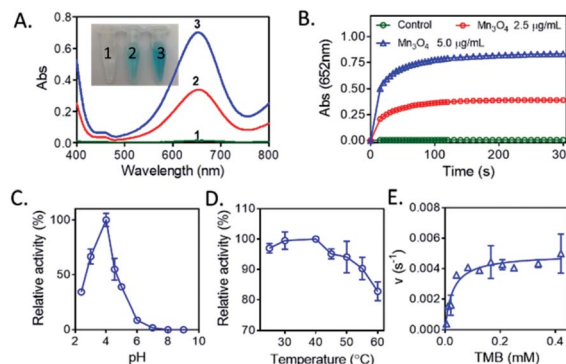


Fig. 3 (A) The visual color changes and absorbance spectra of TMB. (1) Control, (2)  $2.5 \mu\text{g mL}^{-1}$   $\text{Mn}_3\text{O}_4$  and (3)  $5.0 \mu\text{g mL}^{-1}$   $\text{Mn}_3\text{O}_4$ . (B) Time-dependent absorbance changes of TMB at 652 nm in the presence of  $\text{Mn}_3\text{O}_4$  microspheres. (C) The oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres was dependent on pH. TMB: 0.416 mM,  $\text{Mn}_3\text{O}_4$ :  $2.5 \mu\text{g mL}^{-1}$ . (D) The oxidase mimetic activity of  $\text{Mn}_3\text{O}_4$  microspheres was dependent on temperature. TMB: 0.416 mM,  $\text{Mn}_3\text{O}_4$ :  $5.0 \mu\text{g mL}^{-1}$ . (E) The steady-state kinetic assay of  $\text{Mn}_3\text{O}_4$  ( $5.0 \mu\text{g mL}^{-1}$ ).

detection of GSH, such as  $\text{V}_2\text{O}_5$  (13  $\text{min}^{-1}$ ) and Co, N-doped porous carbon hybrids (20  $\text{min}$ ).<sup>27</sup> So, this quick response between TMB and  $\text{Mn}_3\text{O}_4$  laid the foundation of rapid analysis. Similarity to natural enzymes, the catalytic activity of  $\text{Mn}_3\text{O}_4$  microspheres was also related to pH (Fig. 3C) and temperature (Fig. 3D). The results showed that the optimal pH and temperature were about 4.0 and 37  $^\circ\text{C}$ , respectively. We also measured the maximum initial velocity ( $V_{\text{max}}$ ) and Michaelis-Menten constant ( $K_{\text{m}}$ ) of  $\text{Mn}_3\text{O}_4$  microspheres (Fig. 3E), the obtained  $K_{\text{m}}$  value was 0.02715 mM with TMB as the substrate, and the corresponding  $V_{\text{max}}$  value was 126.7  $\text{nM s}^{-1}$ . These results demonstrated that  $\text{Mn}_3\text{O}_4$  microspheres exhibited the excellent oxidase-like activity.

As a most abundant intracellular nonprotein thiolated tripeptide,<sup>28,29</sup> GSH plays an important role in regulating oxidative stress for cell function and growth. An abnormal level of GSH related closely to many diseases, such as cancer, Alzheimer's disease and cardiovascular disease.<sup>30</sup> Therefore, the sensitive and rapid determination of GSH in biological samples is a matter of cardinal significance. So, we here utilized the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres to detect GSH directly. As displayed in Fig. 4A and B, an obvious blue color was

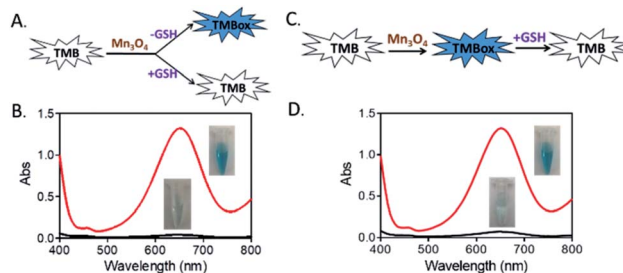


Fig. 4 (A) and (B) Absorption spectra and reaction process of the TMB- $\text{Mn}_3\text{O}_4$  system with/without GSH addition at the same time. (C) and (D) Absorption spectra and reaction process of TMB- $\text{Mn}_3\text{O}_4$  system with/without adding GSH into the solution after catalytic reaction. TMB:  $5.0 \mu\text{g mL}^{-1}$ , GSH: 60  $\mu\text{M}$ .



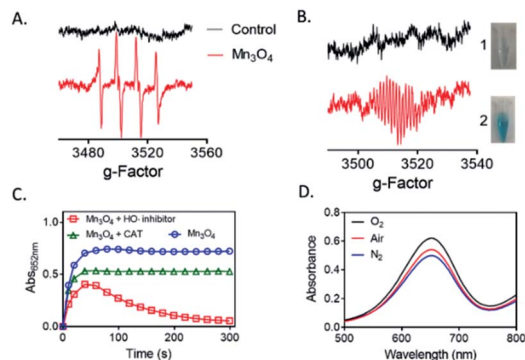


Fig. 5 (A) BMPO trapped EPR spectra over  $\text{Mn}_3\text{O}_4$  microspheres after 1 min of reaction. ( $V = 100 \mu\text{L}$ ,  $\text{pH} = 4.5$ ,  $\text{Mn}_3\text{O}_4 = 100 \mu\text{g mL}^{-1}$ ,  $\text{BMPO} = 10 \text{ mM}$ ). (B) BMPO trapped EPR spectra over GSH reducing the colored TMB to colorless TMB after 1 min of reaction. ( $V = 1 \text{ mL}$ ,  $\text{pH} = 4.5$ ,  $\text{Mn}_3\text{O}_4 = 10.0 \mu\text{g mL}^{-1}$ ,  $\text{TMB} = 0.416 \text{ mM}$ ,  $\text{GSH} = 60 \mu\text{M}$ ,  $\text{BMPO} = 10 \text{ mM}$ ). (C) Effect of  $\text{HO}^\bullet$  scavenger (hypotaurine, 10%) and  $\text{H}_2\text{O}_2$  scavenger (CAT,  $15 \mu\text{g mL}^{-1}$ ) on the oxidase-like activity of  $\text{Mn}_3\text{O}_4$ . (D) Effect of  $\text{O}_2$  concentration on the oxidase-like activity of  $\text{Mn}_3\text{O}_4$ .

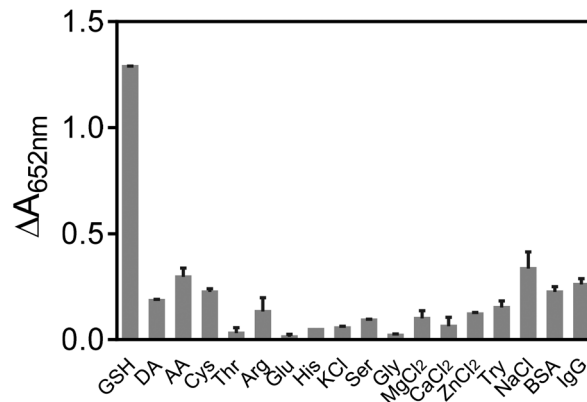


Fig. 7 Selectivity of  $\text{Mn}_3\text{O}_4$ -TMB system for GSH over other potential interferences. Reaction conditions:  $0.416 \text{ mM}$  TMB,  $10 \mu\text{g mL}^{-1}$   $\text{Mn}_3\text{O}_4$  microspheres,  $\text{pH} = 4.5$  and room temperature. Concentrations of the tested substances:  $600 \mu\text{M}$ , except for  $60 \mu\text{M}$  GSH,  $6 \mu\text{M}$  DA,  $6 \mu\text{M}$  Cys,  $20 \mu\text{M}$  AA,  $1 \text{ mg mL}^{-1}$  of BSA, and  $1 \text{ mg mL}^{-1}$  IgG.

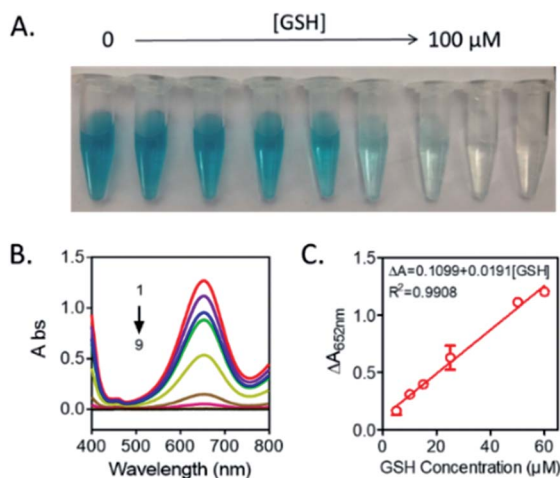


Fig. 6 (A) The photos of the TMB- $\text{Mn}_3\text{O}_4$  system in different concentrations of GSH (from left to right: 0, 5, 10, 15, 25, 50, 60, 80, 100  $\mu\text{M}$ ). (B) UV-vis absorption spectra of TMB- $\text{Mn}_3\text{O}_4$  system with various GSH amounts (1–9: 0, 5, 10, 15, 25, 50, 60, 80, 100  $\mu\text{M}$ ). (C) The calibration curve of GSH. Reaction condition:  $0.416 \text{ mM}$  TMB,  $10 \mu\text{g mL}^{-1}$   $\text{Mn}_3\text{O}_4$  microspheres,  $\text{pH} = 4.5$  and room temperature.

observed in the TMB- $\text{Mn}_3\text{O}_4$  system. However, when TMB,  $\text{Mn}_3\text{O}_4$  microspheres and GSH were mixed together at the same time, no color change was found. This result revealed that the presence of GSH inhibited the color reaction of TMB. Moreover, when TMB and  $\text{Mn}_3\text{O}_4$  microspheres were first mixed to trigger the color reaction of TMB, a deep blue color would then fade after the addition of GSH into the above system (Fig. 4C and D). This results revealed that the inhibition of the TMB color reaction was resulted in the reduction of the blue TMB to colorless TMB again by GSH.

To find the possible mechanism of the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres, we used BMPO-trapped EPR spectra to detect ROS generated in the catalytic reaction. We speculated that oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres might be resulted in the catalytic capacity for activation of  $\text{O}_2$  to generate ROS.<sup>31</sup> As shown in Fig. 5A, the BMPO/ $\cdot\text{OH}$  signal with intensity of 1 : 2 : 2 : 1 were observed, demonstrating the generation of  $\cdot\text{OH}$  in  $\text{Mn}_3\text{O}_4$  system. We also detected the ESR signal of TMB in  $\text{Mn}_3\text{O}_4 + \text{TMB} + \text{BMPO}$  dispersion, which proved that TMB did happen the oxidation reaction (Fig. 5B). While the GSH was added, the single of TMB disappeared, corresponding to the results of colorimetric reaction. Thus, we speculated that the electrons provided by  $\text{Mn}_3\text{O}_4$  microspheres were captured

Table 1 Comparison of colorimetric detection of glutathione based on TMB

Materials	$\text{H}_2\text{O}_2$ (+/–)	Linear range	LOD	Ref.
$\text{MnO}_2$ nanosheets	–	1–25 $\mu\text{M}$	300 nM	35
BSA- $\text{MnO}_2$ NPs	–	0.26–26 $\mu\text{M}$	100 nM	22
Co, N-HPC	–	0.05–30 $\mu\text{M}$	36 nM	27
Gold nanoclusters	+	2–25 $\mu\text{M}$	420 nM	36
$\text{Fe}_3\text{O}_4$ magnetic nanoparticles	+	3–30 $\mu\text{M}$	3 $\mu\text{M}$	37
$\text{V}_2\text{O}_5$ nanozyme	–	0.01–0.5 $\mu\text{M}$	2.4 nM	2
Carbon quantum dots	+	0.05–20 $\mu\text{M}$	0.016 $\mu\text{M}$	38
$\text{Ag}^+$	–	0.05–8.0 $\mu\text{M}$	0.1 $\mu\text{M}$	39
$\text{Cu}_{1.8}\text{S}$ nanoparticles	+	0.5–10 mM	0.06 mM	40
$\text{Mn}_3\text{O}_4$ microspheres	–	5–60 $\mu\text{M}$	0.889 $\mu\text{M}$	This work



Table 2 Recovery test results of GSH in human serum samples

Samples	Detected ( $\mu\text{M}$ )	Added ( $\mu\text{M}$ )	Total found ( $\mu\text{M}$ )	Recovery (%)	RSD (%)
Serum 1	1.13	1.0	$2.16 \pm 0.13$	103.07	2.24
		5.0	$5.99 \pm 0.25$	97.37	2.56
Serum 2	4.40	1.0	$5.41 \pm 0.18$	101.39	1.92
		5.0	$9.13 \pm 0.15$	94.69	1.19
Serum 2	1.63	1.0	$2.63 \pm 0.17$	99.72	2.76
		5.0	$6.90 \pm 0.20$	105.41	1.96

by  $\text{O}_2$  in the air to generate  $\text{H}_2\text{O}_2$ . In the Fenton-like reaction, the metal ion provides an electron to  $\text{H}_2\text{O}_2$  and produces  $\text{HO}^\bullet$  to further react with the organic compounds. As reported in the previous work,<sup>32</sup> the  $\text{Mn}^{2+}$  and/or  $\text{Mn}^{3+}$  species on the surfaces of  $\text{Mn}_3\text{O}_4$  could be converted into  $\text{Mn}^{4+}$  species, and the released electrons were trapped by the dissolved  $\text{O}_2$  in solution to generate  $\text{H}_2\text{O}_2$ . The  $\text{H}_2\text{O}_2$  then created  $\text{HO}^\bullet$  radicals to catalyze the oxidation of substrate. In order to further confirm this result, we investigated the impacts of hypotaurine and catalase (CAT), which specifically scavenges  $\text{HO}^\bullet$  radicals<sup>33</sup> and  $\text{H}_2\text{O}_2$ , respectively. Fig. 5C indicated that the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres was retarded in the presence of CAT and hypotaurine, verifying that production of intermediates ( $\text{H}_2\text{O}_2$  and  $\text{HO}^\bullet$ ). Moreover, the absorbance value at 652 nm of TMB oxidation slight changed in the  $\text{N}_2$  atmosphere (bubbled with  $\text{N}_2$  for 30 min), while increased after saturation with  $\text{O}_2$  (Fig. 5D). This phenomenon also indicated that the dissolved  $\text{O}_2$  in the reaction system played a key role in TMB oxidation reaction. Thus, GSH, known as antioxidant,<sup>34</sup> could exhaust  $\text{HO}^\bullet$  produced from  $\text{Mn}_3\text{O}_4\text{-O}_2$  system and suppressed the TMB oxidation reaction. Moreover, GSH is also able to reduce the TMB to TMB again, causing the color of the whole system fade.

According to the above results, a new bio-analysis assay sensor based on the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres could be proposed for the colorimetric detection of GSH. As displayed in Fig. 6, the absorption peak intensity was decreased with the GSH concentration from 0 to 100  $\mu\text{M}$ , observing the color fading of the solution (Fig. 6A). This phenomena verified the possibility of the colorimetric detection of GSH again. Moreover, it was found that the dependency of the absorbance on the GSH concentration could be well presented by the equation of  $\Delta A = 0.1099 + 0.0191[\text{GSH}]$  ( $R^2 = 0.9908$ ), and the detection limit of GSH was 0.889  $\mu\text{M}$  ( $\text{S/N} = 3$ ). Compared with other reported colorimetric GSH sensors based on TMB (Table 1),<sup>35–40</sup> our assay was able to offer comparable performance in terms of limit of detection (LOD) and presented a wider linear range (5–60  $\mu\text{M}$ ).

In order to test the specificity of the assay, several common species existed in blood were investigated. As presented in Fig. 7, various amino acids (Cys, Ser, Gly, His, Thr, Try and Arg), biologically relevant metal ions ( $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$ ), monosaccharides (glucose), ascorbic acid (AA), dopamine (DA), BSA and IgG were checked. The results shown in Fig. 7 indicated that only GSH could induce the obvious inhibition of TMB color reaction, showing the good selectivity of our assay for GSH

detection. Furthermore, the practical possibility of the GSH colorimetric method was investigated in human serum samples by the standard addition method, and the recovery studies were also performed, giving recoveries of 94.69–105.41% (Table 2). These results showed that the proposed bio-analysis assay based on the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres had great potential to detect GSH in biological samples accurately and rapidly.

## Conclusions

In this study, we constructed a one-pot reaction based on the oxidase-like activity of  $\text{Mn}_3\text{O}_4$  microspheres for highly sensitive and selective detection of GSH. This GSH biosensor required neither natural peroxidase nor  $\text{H}_2\text{O}_2$  with good repeatability, and could be finished in 5 min, showing the simple and rapid properties. Importantly, this assay also exhibited good reliability for GSH determination in clinical samples.

## Conflicts of interest

There are no conflicts to declare.

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