Mn$_3$O$_4$ microspheres as an oxidase mimic for rapid detection of glutathione

Juqun Xi, Chunhua Zhu, Yanqiu Wang, Qiannan Zhang and Lei Fan

Exploiting a rapid and sensitive method for biomarker detection has important implications in the early diagnosis of diseases. Here, we synthesized Mn$_3$O$_4$ microspheres which worked as a nanozyme to exhibit outstanding oxidase-like activity for rapid colorimetric determination of glutathione (GSH). The Mn$_3$O$_4$ microspheres of about 800 nm in size could be prepared through a hydrothermal method, and we found that the as-prepared Mn$_3$O$_4$ microspheres could quickly oxidize 3,3',5,5'-tetramethylbenzidine (TMB) to its oxidized form (TMBox) in the absence of H$_2$O$_2$. After adding glutathione (GSH), TMBox was able to be changed into its original form and resulted in the corresponding decrease in absorbance value at 652 nm. The Mn$_3$O$_4$-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$_3$O$_4$ would enlarge and exploit the application fields of nanozymes in bio-analysis.

Introduction

Developing a simple and effective sensing assay to detect biomarkers rapidly has attracted widespread interest in the field of bio-analysis.\textsuperscript{1,2} 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.\textsuperscript{3,4} Among these strategies, nanozymes, as catalytic materials with enzyme mimicking activities, show great potential in bio-analysis owing to their tunable catalytic activities.\textsuperscript{5} Especially, peroxidase-mimicking nanozymes have been widely investigated to design versatile sensing platform biosensors.\textsuperscript{6,7} For instance, a selective glucose biosensor was constructed by combining peroxidase-like Fe$_3$O$_4$ nanozymes with glucose oxidase.\textsuperscript{8} This strategy now is generalized to detect other analytes, but this assay is only suitable to the H$_2$O$_2$-producing analytes.\textsuperscript{9} 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$_2$O$_2$ 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.\textsuperscript{10,11} 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$_2$O$_2$ as a co-substrate, to construct a colorimetric assay based on one-pot reaction will simply 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,\textsuperscript{12,13} and CeO$_2$, V$_2$O$_5$, MnO$_2$ can also oxidize a diverse range of substrates directly.\textsuperscript{14,15} Oxidases are important since they do not need H$_2$O$_2$ as a co-substrate. Mn$_3$O$_4$ is a versatile nanozyme and reported to have various enzyme-like activities, including catalase,\textsuperscript{16} superoxide dismutase\textsuperscript{17} and glutathione peroxidase (GPx).\textsuperscript{18} It was usually applied for wound healing, \textit{in vivo} anti-inflammation and cytoprotection. However, the oxidase-like activity of Mn$_3$O$_4$ has not been reported. The catalytic activities of Mn$_3$O$_4$ are attributed to the mixed oxidation states of Mn$^{3+}$ and Mn$^{2+}$, and related oxygen vacancies. As CeO$_2$ and V$_2$O$_5$, we speculated that Mn$_3$O$_4$ maybe possessed oxidase-like activity as a promising candidate for developing colorimetric assays.

Herein, we reported a hydrothermal synthesis method to prepare Mn$_3$O$_4$ microspheres, which exhibited outstanding oxidase-like activity to convert colorless TMB rapidly into TMBox (a naked-eye visible chromogenic substrate) in the...
absence of H$_2$O$_2$. It was found that glutathione (GSH) could selectively suppress the oxidation of TMB, converting blue TMBBox to colorless TMB. Based on this phenomenon, the GSH level could be measured by monitoring the decrease of TMBBox 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. 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. 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, Ag$^{2+}$ and MnO$_2$, with TMB for colorimetric detection of GSH. We here utilized a one-pot reaction, the oxidase-like activity of Mn$_3$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 Mn(HCO$_2$)$_2$·2H$_2$O, sodium acetate (NaAc), ethanol (C$_2$H$_5$OH), methanol (CH$_3$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). ZnCl$_2$, CaCl$_2$, and MgCl$_2$ were bought from Sangon Biotech (Shanghai, China). TMB (0.1 M), containing 5.0 µg mL$^{-1}$ Mn$_3$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.

**Synthesis of Mn$_3$O$_4$ microspheres**

Mn(HCO$_2$)$_2$·2H$_2$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, Mn$_3$O$_4$ microspheres were obtained through centrifugation, washing and drying.

**Characterization of Mn$_3$O$_4$ microspheres**

Scanning electron microscopy (SEM, S-4800II, Hitachi, Tokyo, Japan) was applied to characterize the morphology and structure of Mn$_3$O$_4$ microspheres. The FT-IR spectrum was monitored on a Bruker Tensor 27 FT-IR spectrometer (Germany). The composition and surface chemistry of Mn$_3$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 Mn$_3$O$_4$ microspheres and kinetic studies**

The major absorbance peaks of TMBBox in 0.1 M NaAc-HAc buffer (pH 4.5) appeared at 652 and 370 nm. For the evaluation of oxidase-like activity of Mn$_3$O$_4$ microspheres, chemicals were added into a 1.0 mL NaAc-HAc buffer solution in the order of Mn$_3$O$_4$ (varying amounts) and TMB (final concentration: 0.416 mM). The color change of TMB in the presence of Mn$_3$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 µg mL$^{-1}$ Mn$_3$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 Mn$_3$O$_4$ microspheres and TMB were 10.0 µg mL$^{-1}$ and 0.416 mM, respectively. After adding different concentrations of GSH in Mn$_3$O$_4$-TMB buffer system, the mixture was incubated for 5 min and then the change of absorbance was monitored in 652 nm was monitored. The calibration curve of GSH was obtained by plotting A at 652 nm as a function of the GSH concentration. Where $\Delta A = A_0 - A$, and A$_0$ correspond to the absorbance at 652 nm with and without GSH addition, respectively.

**Statistical analysis**

Quantitative data were expressed as the means ± standard deviations.

**Results and discussion**

Mn$_3$O$_4$ microspheres were synthesized according to a previously report.28 The morphology, crystalline phase and chemical composition of the obtained Mn$_3$O$_4$ microspheres were analyzed by SEM, XRD and XPS. The SEM image shown in Fig. 1A indicated that the prepared Mn$_3$O$_4$ microspheres were about 800 nm in diameter, and each microsphere was composed of small-sized Mn$_3$O$_4$ nanoparticles (~10 nm) (Fig. 1B). Elemental mapping of the Mn$_3$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 Mn$_3$O$_4$ [JCPDS card no. 24-0734], suggesting their crystalline nature. Furthermore, the surface of Mn$_3$O$_4$ nanocrystals was grafted with TMB and the oxidase-like activity of the Mn$_3$O$_4$ microspheres was investigated. The oxidase-like activity of Mn$_3$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 µg mL$^{-1}$ Mn$_3$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.
Mn$_3$O$_4$ microspheres was analyzed by XPS. As shown in Fig. 2B and C, Mn $2p_{3/2}$ and $2p_{1/2}$ peaks were located at 641.67 eV and 653.35 eV, respectively. The atom ratio of Mn$^{2+}$ and Mn$^{3+}$ was about 1/2, which was consistent with the theoretical value.25 Moreover, the FT-IR spectrum (Fig. 2D) of Mn$_3$O$_4$ microspheres showed three main bands centered at (407, 502 and 620 cm$^{-1}$), which were attributed to the Mn–O vibrations of the Mn$_3$O$_4$ framework.27 Thus, the Mn$_3$O$_4$ microspheres could be obtained successfully through a hydrothermal synthesis.

Recently, Mn$_3$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.26 However, the oxidase-like activity of Mn$_3$O$_4$ has not been reported. We here concentrated on the investigation of the oxidase-like activity possessed by Mn$_3$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 Mn$_3$O$_4$ microspheres were able to cause the oxidation of TMB directly. This reaction occurred without the assistance of H$_2$O$_2$. Increasing the concentration of Mn$_3$O$_4$ microspheres was beneficial for the occurrence of this oxidation reaction. The time-dependent absorbance change in Mn$_3$O$_4$-TMB was shown in Fig. 3B, and the oxidation of TMB reached a plateau at ~2 min at 25 °C, which was much faster than that reported oxidase-like reactions triggered by other nanomaterials for detection of GSH, such as V$_2$O$_5$ (13 min$^{-1}$) and Co, N-doped porous carbon hybrids (20 min).27 So, this quick response between TMB and Mn$_3$O$_4$ laid the foundation of rapid analysis. Similarity to natural enzymes, the catalytic activity of Mn$_3$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 °C, respectively. We also measured the maximum initial velocity ($V_{max}$) and Michaelis-Menten constant ($K_m$) of Mn$_3$O$_4$ microspheres (Fig. 3E), the obtained $K_m$ value was 0.02715 mM with TMB as the substrate, and the corresponding $V_{max}$ value was 126.7 nM s$^{-1}$. These results demonstrated that Mn$_3$O$_4$ microspheres exhibited the excellent oxidase-like activity.

As a most abundant intracellular nonprotein thiolated tripeptide,28,29 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.30 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 Mn$_3$O$_4$ microspheres to detect GSH directly. As displayed in Fig. 4A and B, an obvious blue color was
observed in the TMB-Mn3O4 system. However, when TMB, Mn3O4 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 Mn3O4 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 result revealed that the inhibition of the TMB color reaction was resulted in the reduction of the blue TMBox to colorless TMB again by GSH.

To find the possible mechanism of the oxidase-like activity of Mn3O4 microspheres, we used BMPO-trapped EPR spectra to detect ROS generated in the catalytic reaction. We speculated that oxidase-like activity of Mn3O4 microspheres might be resulted in the catalytic capacity for activation of O2 to generate ROS.

As shown in Fig. 5A, the BMPO/·OH signal with intensity of 1 : 2 : 2 : 1 were observed, demonstrating the generation of ·OH in Mn3O4 system. We also detected the ESR signal of TMBox in Mn3O4 + TMB + BMPO dispersion, which proved that TMB did happen the oxidation reaction (Fig. 5B). While the GSH was added, the signal of TMBox disappeared, corresponding to the results of colorimetric reaction. Thus, we speculated that the electrons provided by Mn3O4 microspheres were captured by the peroxyl radicals, which was converted to ·OH, and this process also led to the decrease of blue TMBox.

Table 1  Comparison of colorimetric detection of glutathione based on TMB

<table>
<thead>
<tr>
<th>Materials</th>
<th>H2O2 (+/−)</th>
<th>Linear range (µM)</th>
<th>LOD (nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnO2 nanosheets</td>
<td>−</td>
<td>1−25</td>
<td>300</td>
<td>35</td>
</tr>
<tr>
<td>BSA-MnO2 NPs</td>
<td>−</td>
<td>0.26−26</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>Co3 N-HPC</td>
<td>−</td>
<td>0.05−30</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>Gold nanoclusters</td>
<td>+</td>
<td>2−25</td>
<td>420</td>
<td>36</td>
</tr>
<tr>
<td>Fe3O4 magnetic nanoparticles</td>
<td>+</td>
<td>3−30</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>V2O5 nanoxide</td>
<td>−</td>
<td>0.01−0.5</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>Carbon quantum dots</td>
<td>+</td>
<td>0.05−20</td>
<td>0.016</td>
<td>38</td>
</tr>
<tr>
<td>Ag+</td>
<td>−</td>
<td>0.05−8.0</td>
<td>0.1</td>
<td>39</td>
</tr>
<tr>
<td>Cu1.8S nanoparticles</td>
<td>+</td>
<td>0.5−10</td>
<td>0.06</td>
<td>40</td>
</tr>
<tr>
<td>Mn3O4 microspheres</td>
<td>−</td>
<td>5−60</td>
<td>0.889</td>
<td>This work</td>
</tr>
</tbody>
</table>
by O₂ in the air to generate H₂O₂. In the Fenton-like reaction, the metal ion provides an electron to H₂O₂ and produces HO• to further react with the organic compounds. As reported in the previous work,²² the Mn²⁺ and/or Mn³⁺ species on the surfaces of Mn₃O₄ could be converted into Mn⁴⁺ species, and the released electrons were trapped by the dissolved O₂ in solution to generate H₂O₂. The H₂O₂ then created HO• 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 HO• radicals³³ and H₂O₂, respectively. Fig. 5C indicated that the oxidase-like activity of Mn₃O₄ microspheres was retarded in the presence of CAT and hypotaurine, verifying that production of intermediates (H₂O₂ and HO•). Moreover, the absorbance value at 652 nm of TMB oxidation slight changed in the N₂ atmosphere (bubbled with N₂ for 30 min), while increased after saturation with O₂ (Fig. 5D). This phenomenon also indicated that the dissolved O₂ in the reaction system played a key role in TMB oxidation reaction. Thus, GSH, known as antioxidant,³⁴ could exhaust HO• produced from Mn₃O₄-O₂ system and suppressed the TMB oxidation reaction. Moreover, GSH is also able to reduce the TMBox 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 Mn₃O₄ 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 μM, 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 ΔA = 0.1099 + 0.0191[GSH] (R² = 0.9908), and the detection limit of GSH was 0.889 μM (S/N = 3). Compared with other reported colorimetric GSH sensors based on TMB (Table 1),³⁵-⁴⁰ our assay was able to offer comparable performance in terms of limit of detection (LOD) and presented a wider liner range (5–60 μM).

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 (Na⁺, Mg²⁺, Zn²⁺, Ca²⁺ and 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 Mn₃O₄ 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 Mn₃O₄ microspheres for highly sensitive and selective detection of GSH. This GSH biosensor required neither natural peroxidase nor H₂O₂ 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.

Acknowledgements

This project was funded by the National Natural Science Foundation of China (No. 21703198), the University Natural Science Foundation of Jiangsu Province (16KJD150004). The authors also gratefully acknowledge financial support from the Priority Academic Program Development of Jiangsu Higher Education Institutions and Top-notch Academic Programs Project of Jiangsu Higher Education Institutions.

Notes and references