Feasibility Study on Facile and One-step Colorimetric Determination of Glutathione by Exploiting Oxidase-like Activity of Fe₃O₄-MnO₂ Nanocomposites

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A facile and one-step colorimetric assay is described for the determination of glutathione (GSH). It is based on the use of manganese dioxide-decorated magnetic (Fe₃O₄@MnO₂) nanocomposite that was prepared by an *in-situ* redox reaction. It exhibits oxidase-mimicking activity and can catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) without H₂O₂ to form a blue colored product (oxTMB) with an absorption maximum at 651 nm. Once GSH is introduced, the component of MnO₂ can be rapidly reduced to Mn²⁺ ions, which leads to inhibit the formation of oxTMB. Based on these findings, a one-step colorimetric assay was developed for the detection GSH in the range of 0.2 to 25 μM with a low detection limit of 0.2 μM without using any procedures of separation and washing. Importantly, the proposed approach is also used to accurately evaluate the intracellular GSH levels. In our perception, the assay is rapid, sensitive and specific.

Keywords Fe₃O₄-MnO₂ nanocomposite, oxidase-like, glutathione, cell lines, visual detection

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Introduction

Glutathione (GSH) is the most abundant intracellular biothiol, which consists of a tripeptide of glutamic acid, cysteine, and glycine.¹ It plays a crucial role in the defense of cells against free radicals and other electrophiles, and is involved in intracellular signal transduction, gene regulation and xenobiotic metabolism.^{2,3} An abnormal level of GSH is considered to be a reliable sign of some diseases, such as cancer, AIDS, liver damage, diabetes, and aging.^{4,5} Therefore, based on its biological and clinical importance, the rapid and sensitive detection of GSH in cells for early diagnosis has attracted continuing great interest.

Umezara *et al.* developed FRET-based ratiometric probes to quantify the GSH concentration in various cell types while enabling real-time live cell imaging of GSH dynamics in seconds, which pioneered the rapid detection of GSH.⁶ In addition, a variety of analytical methods, including high-performance liquid chromatography (HPLC), liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS), and spectrofluorimetry,⁷⁻⁹ have been established for the determination of GSH. However, these methods are time-consuming and require tedious sample pre-treatments, highly qualified professional technicians and sophisticated instruments,¹⁰ making them inconvenient for practical use in certain circumstances. Thus, colorimetric analytical strategies have aroused special attention due to their simple, rapid, cost-

effective detection without any advanced apparatus.¹¹⁻¹³ One of the most common types of colorimetric methods focuses on enzyme-chromogenic substrates. The limitation of the high-cost and low stability under harsh environmental conditions makes natural enzymes to be restricted to analytical applications. As alternatives, nanozymes are a class of synthetic nanomaterials based on carbon,¹⁴ metal oxides,¹⁵ or noble metals¹⁶ with enzyme-like characteristics, which have become increasingly important research tools in recent years.

Compared to noble metal oxides, ferroferric oxide nanoparticles (Fe₃O₄ NPs) as a typical kind of transition-metal oxides have received tremendous attention due to an outstanding peroxidase-like activity, low-cost, and non-toxicity.^{17,18} However, the Fe₃O₄ nanozyme-based catalytic Fenton reaction typically requires a high dose of H₂O₂ (approximately 10⁻³ to 10⁻⁴ M),^{19,20} due to its low affinity to the substrate H₂O₂. Because H₂O₂ as an oxidant is unstable to the surrounding environment and may damage target analytes,²¹ which makes a peroxidase-like activity of the Fe₃O₄ NPs-based nanozyme unviable for practical use. Fortunately, manganese dioxide (MnO₂) NPs with high intrinsic oxidase-like activity have been developed to address this problem,²² which can catalytically oxidize the substrate 3,3',5,5'-tetramethylbenzidine (TMB) without H₂O₂ to produce a blue color with a maximum absorption peak at 651 nm.^{23,24} However, when detecting GSH by MnO₂ NPs, there were still some drawbacks, such as the containing noble metal nanoparticles in the sensing system,²⁵ or requiring extremely acidic reaction conditions,²⁶ which is not conducive to its promotion and application in practical projects. To overcome these drawbacks, we combined the advantages of the Fe₃O₄ NPs and MnO₂ NPs, and designed a new class of

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Scheme 1 Schematic diagram of the proposed colorimetric assay for GSH detection in cell lines based on the inhibition effect of GSH on the oxidase-like activity of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite.

hybrid nanomaterials based on them. This has been extensively developed, owing to its easy magnetic separation capability and high catalytic activity.²⁷ $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs synthesized by this method are easy to prepare and have high stability. Therefore, the previous findings showed that $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs are highly recommended as a novel and facile tool for colorimetric detection.

With these insights, to develop a novel approach for GSH detection and identifying cancer cells in a simple mode for relevant biomedical diagnosis, we proposed a method for the rapid and accurate detection of GSH to overcome the shortcomings of the existing methods. Scheme 1 illustrates the mechanism of the proposed method for GSH detection. $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs possess strong oxidase-like activity, which can catalyze the oxidation of TMB to form a blue-colored product (oxTMB) that depends on dissolved molecular oxygen in the solution.²⁸ Upon the addition of GSH, the component of MnO_2 can be rapidly reduced to Mn^{2+} ions, which leads to a concentration-dependent inhibition of the formation of oxTMB.^{29,30} Based on these results, the intensity of the absorption maximum at 651 nm was proportionally decreased.^{23,29} Based on the colorimetric method, it has been proven to be sensitive and selective to GSH in aqueous solution and living cells. Importantly, our one-step approaches mean there is no need for any procedures of separation and washing, no needs for H_2O_2 , and has no special requirement for the pH and temperature. Taking advantage of the easy preparation and high stability of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs, the detection limit of this assay was as low as 0.2 μM and the total detection time was 25 min.

Experimental

The details of the materials, the protocol for synthesizing the $\text{Fe}_3\text{O}_4@\text{MnO}_2$ magnetic nanocomposites and the detection of GSH in living cells were described in Supporting Information.

Determination of GSH

A typical colorimetric procedure by using $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs for GSH determination was conducted as follows: 100 μL of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs (100 $\mu\text{g}/\text{mL}$) and 100 μL of different GSH concentrations (from 0.2 to 25 μM) were added into 300 μL of

a PBS solution (pH 7.2). The mixture was gently vortexed and incubated at room temperature for 20 min. Next, 7.5 μL of TMB (10 mg/mL) was added to the above mixture. DDW was used as a negative control. After coloration for 5 min, the absorbance of the supernatant was recorded from 420 to 800 nm by using a UV-Vis spectrophotometer. The total detection time of our assay was only 25 min.

For a selectivity study, 100 μL of other amino acid samples (Gly, Arg, Trp, Cys, Hcy) (25 μM) was used as interfering species. The test protocol was performed as described above, except that GSH was replaced by other amino acid samples.

Statistical analysis

All of the data, except for a special label, are expressed as the means $\pm$ standard deviations ($x \pm \text{SD}$) of three parallel samples. Paired t-tests were conducted for a statistical analysis using SPSS Statistics software (Ver. 22.0, IBM, USA). The statistical significance was set at 0.05.

Results and Discussion

Characterization of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs

$\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs were characterized by TEM, EDS, SEAD, FT-IR, XRD and VSM. TEM images (Figs. 1a and 1b) show that both Fe_3O_4 NPs and $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs were roughly spherical in shape and well dispersed. After being decorated with MnO_2 NPs, the average size of the NPs slightly increased from 112.0 ± 1.4 to 125.6 ± 1.8 nm. A particle composition analysis was performed by EDS (Fig. S1). Mn $K\alpha_1$ and Fe $K\alpha_1$ were observed at ~ 6.490 and ~ 7.058 keV, respectively. Elemental mapping (Figs. S1c and S1d) results showed that the nanocomposites contain Fe and Mn elements, which is consistent with the EDS analysis. The SAED pattern (Fig. 1c) exhibits two bright concentric rings, which correspond to the (110) and (111) planes, reveal that the $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs have a composite structure containing iron and manganese. The FT-IR spectra of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs is shown in Fig. 1d.

The absorption peak appearing at 3407 cm^{-1} is associated with the vibrations of OH of the absorbed water molecules. The observed bands at 1627 and 1387 cm^{-1} correspond to the symmetric C-O stretching and the asymmetric stretching of COO^- , respectively. The peak at 575 cm^{-1} corresponds to the Fe-O stretching vibrations in iron oxide. In addition, the peaks at about 505 and 533 cm^{-1} are related to Mn-O from the structure of MnO_2 .^{27,31-33} The crystal structure of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs was further characterized by XRD. As depicted in Fig. 1e, the position and relative intensities of peaks observed at 2θ of $18.45, 30.36, 35.76, 43.47, 63.17^\circ$ match perfectly to Fe_3O_4 with an inverse spinel structure, according to JCPDS data (card no. 75-0449). Diffraction peaks at 2θ of $12.74, 28.74, 37.6, 42.03, 46.09$ and 60.24° correspond well to pure tetragonal phases of $\alpha\text{-MnO}_2$ (JCPDS card no. 72-1982). Furthermore, concerning inheritance of the magnetic property of Fe_3O_4 NPs, the saturation magnetization value of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs was 20.70 emu/g with nearly zero coercivity at room temperature (Fig. 1f). This was sufficient to accomplish a fast and efficient separation with an external magnetic field. All of these results implied that $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs were expectedly synthesized with excellent super magnetic properties.

The mechanism of the sensing system

To investigate the mimetic enzyme activity of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs, TMB was chosen as the chromogenic substrate in the oxidation reaction. Before verifying the mechanism of the

Fig. 1 Characterization of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite. TEM images of (a) Fe_3O_4 nanoparticles and (b) $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite, (c) SAED pattern of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite, (d) FT-IR spectra, (e) X-ray diffraction pattern, (f) magnetic hysteresis loop.

sensing system, we explored the catalytic activity of Fe_3O_4 without MnO_2 . According to Fig. S2, Fe_3O_4 cannot catalyze the TMB color development to form a blue final product with the absence of H_2O_2 . This proved that Fe_3O_4 has no oxidase activity. As shown in Fig. 2, TMB incubated without any oxidizing agent is colorless, with no clear absorption peaks from 420 to 800 nm (curve I). In contrast, $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs can catalyze the oxidation of TMB in the presence of dissolved oxygen and produce a deep-blue color with a strong character absorption peak at 651 nm (curve III), indicating the yield of oxTMB. These results confirmed that $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs possessed the intrinsic oxidase-like activities, owing to the redox properties of MnO_2 . However, $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs could be reduced by GSH directly into Mn^{2+} , while GSH was oxidized to glutathione disulfide (GSSG), as shown in Eq. (1).

Thus, in the presence of GSH, almost no blue color appears, demonstrating that the oxidation of TMB was inhibited by a rapid decomposition of $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs by GSH (curve IV). Besides, there was no significant absorption peak upon incubating $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs with GSH in the sensing system

Fig. 2 UV-Vis spectra of different solutions: (I) 50 μM GSH incubated with 10 mg/mL TMB, (II) 200 $\mu\text{g/mL}$ $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite incubated with 50 μM GSH, (III) 200 $\mu\text{g/mL}$ $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite incubated with 10 mg/mL TMB, (IV) 200 $\mu\text{g/mL}$ $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite incubated with 10 mg/mL TMB and 50 μM GSH, (V) 50 μM GSH incubated with 10 mg/mL TMB after 6 months storage. The inset images correspond to the visual colorimetric response. Error bars represent the standard deviation of three replicates.

Fig. 3 Effect of: (a) the concentration of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs and the amount of TMB; (b) pH; (c) incubation time and the reaction temperature; (d) coloration time. Error bars represent the standard deviation of three replicates.

(curve II). In addition, the long-term stability of the catalytic activity of the $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs was investigated. After 6 months of storage, the $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs exhibited only a slight decrease in the catalytic activity (curve V), indicating their easy storage and excellent long-term stability. Considering the above results, $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs can be used as a turn-off colorimetric sensor for GSH detection based on discerning the color change of oxTMB with bare eyes or recording the maximum absorption peak at 651 nm.

Optimization of sensing conditions

To obtain the best optical sensing performance for GSH detection, the concentrations of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs and TMB, the pH value, the incubation time and the reaction temperature, and the coloration time were optimized by investigating ΔA ($\Delta A = A_0 - A$, where A_0 and A are the absorbance of oxTMB at 651 nm in the absence and presence of GSH, respectively) prior to applying our proposed method. First, a series of different concentrations of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs and TMB was investigated to evaluate their impact on the redox reaction. As shown in Fig. 3a, the final concentrations of 20 $\mu\text{g mL}^{-1}$ of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs and 7.5 μL of TMB (10 mg/mL) were used to reach the maximum ΔA . Subsequently, the pH value is a crucial factor concerning the stability of the catalytic activity of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs. The optimal condition was obtained at pH 7.4 between pH 7.0 to 8.0 (Fig. 3b). Next, the influence of the incubation time and the reaction temperature was monitored, the results are shown in Fig. 3c. It can be seen that 25°C for 20 min is the most suitable reaction condition for the assay. Finally, the coloration time was also optimized by recording ΔA with

different test intervals. The ΔA dramatically increased during the first 10 min incubation, where thereafter, even slight decreasing was observed (Fig. 3d). Based on these results, subsequent experimental conditions were chosen to give the best results: (a) 20 $\mu\text{g mL}^{-1}$ of $\text{Fe}_3\text{O}_4\text{-MnO}_2$ NPs and 7.5 μL of TMB; (b) pH 7.4; (c) incubation time and reaction temperature, 25°C for 20 min; (d) coloration time, 10 min.

Colorimetric detection of GSH

To demonstrate the performance of the detection strategy, varying concentrations of GSH ranging from 0.2 to 25 μM were studied under the aforementioned optimized conditions. As presented by the images in Fig. 4a, with increasing the concentration of GSH, the color response of the sensing system gradually decreased from dark to light blue. The bare-eye limit of detection (LOD) for GSH was determined to be 0.2 μM . The corresponding UV-Vis spectra shows the absorbance progressive decline at 651 nm against the GSH concentration in the range from 0.2 to 25 μM (Fig. 4b). As a result, a good linear relationship can be described as $A_{651} = -0.028C + 0.930$, with a correlation coefficient of $R^2 = 0.998$ (Fig. 4c), where C is the GSH concentration in μM . These consequences indicated that our turn-off colorimetric assay can be successfully applied for GSH detection with high sensitivity.

To prove that the proposed assay has good selectivity for GSH detection, the effects of some possible interfering species were investigated, including Gly, Arg, Trp, Cys and Hcy. As shown in Fig. 4d, most amino acids do not induce any obvious absorbance changes at 651 nm, compared to GSH under the same conditions ($n = 10$). It should be noticed that the other

Fig. 4 (a) Photographs, (b) UV-Vis spectra of the proposed colorimetric assay after incubation with GSH at various concentrations (0, 0.2, 2, 5, 10, 15, 20, 25 μM), (c) the calibration curve for GSH (the absorbance at 651 nm vs. the GSH concentrations), the red point is the value of Blank samples. Error bars represent the standard deviation of three replicates. (d) Selectivity test. The concentration of GSH and all other potential interfering amino acid were 25 μM . All measurements were acquired at 25°C for 30 min in PBS (pH 7.4). Error bars represent the standard deviation of 10 replicates.

thiol-containing amino acids, such as Cys and Hcy, can also cause a slightly decreased absorbance signal. However, the cellular levels of Cys and Hcy are extremely low, whose levels are a thousand-times lower than that of GSH,³⁴ and can be negligible in a cellular GSH evaluation. Besides, the stability of the method was verified by detecting GSH at 25 μM in 1, 4, 7, 14, 21, 28 days. According to Fig. S3, the response of our assay toward to GSH day to day had only a small change difference within 28 days, and the RSD was 2.5%, calculated by the absorbance at 651 nm ($n = 10$). These results showed good stability of our assay. In general, our proposed assay based on $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs as a nanosensor can achieve selective GSH detection without significant interference.

GSH evaluation in cell samples

The applicability of the $\text{Fe}_3\text{O}_4@\text{MnO}_2$ NPs-TMB system was further explored by detecting the GSH concentration in three human cell lines, including one normal cell line (HUVEC) and two cancer cell lines (SMMC-7721 and HepG-2) as the model. We used our approach to evaluate cellular GSH levels and, also employed a commercial GSH assay kit for a comparison. As displayed in Fig. 5, the quantitative results show that there is no significant difference between this assay and the commercial kit ($p > 0.05$), demonstrating the excellent accuracy and reliability of our approach for detecting GSH in cell samples ($n = 10$). Furthermore, all of the experimental results revealed that the overall GSH levels in cancer cells were much higher than that in normal cells, which was quite consistent with previous reports.^{6,35} Thus, our assay developed here can provide a straightforward and reliable protocol to accurately evaluate the cellular GSH

Fig. 5 Comparison of GSH levels in different cell lines evaluated by a commercial GSH assay kit and this assay. All cells were evaluated with equal amounts of cell lysate. Error bars represent the standard deviation of 10 replicates.

Table 1 Comparison of GSH detection by commercial GSH assay kit and our assay

	Commercial kit	Our assay
Total detection time	30 – 65 min	25 min
LOD	1 μM	0.2 μM
Storage conditions	–20°C (NADPH –70°C)	4°C
Reaction temperature	25°C	25°C
Shelf life	3 months	At least 6 months
Detection cost	~\$ 0.59/sample	~\$ 0.014/sample

levels so as to assess whether it is a cancer or normal cell. As listed in Table 1 and Table S1, compared to the commercial GSH assay kit and other approaches, our one-step optical sensing system offers some distinguished advantages in terms of the ease of operation, low-cost and time-saving.

Conclusion

In summary, a $\text{Fe}_3\text{O}_4@\text{MnO}_2$ nanocomposite was synthesized. The possessed a high oxidase-like catalytic activity, and could oxidize TMB to form a blue-colored production oxTMB in a neutral solution. GSH was able to trigger decomposition of the component of MnO_2 , with a remarkable inhibition effect and enzymatic properties, resulting in a decrease of absorbance intensity of the TMB substrate chromogenic system. Therefore, a sensitive and selective colorimetric strategy for GSH sensing was fabricated. The proposed method provides a wide linear range of from 0.2 to 25 μM with a LOD as low as 0.2 μM . It was successfully applied for detecting intracellular GSH. Importantly, our one-step approaches have no need for any procedures of separation and washing, no needs for H_2O_2 , and has no special requirement for the pH and temperature. It can be assumed that this strategy has great potential as an effective tool for clinical diagnosis and even therapy related to GSH.

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Conflicts of Interest

The authors declare no conflict of interests.

Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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