Ratiometric fluorescence and colorimetry dual-mode assay based on manganese dioxide nanosheets for visual detection of alkaline phosphatase activity

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ABSTRACT

Alkaline phosphatase (ALP) activity plays a crucial role in foods and varies greatly in livestock and dairy products; it is quite difficult to compatibly monitor the different activities in various complicated foods. Herein, we proposed a ratiometric fluorescence and colorimetry dual-mode assay based on manganese dioxide (MnO_2) nanosheets for the visualization of ALP activity in livestock serum and pasteurized milk samples compatibly. MnO_2 nanosheets could oxidize dopamine into green fluorescent polydopamine (PDA) nanoparticles, so to quench the red fluorescent quantum dots (QDs), or oxidize colorless 3,3',5,5'-tetramethylbenzidine (TMB) into yellow TMB_{ox}. In the coexistence of ALP and 2-phospho-L-ascorbic acid, MnO_2 nanosheets were reduced into Mn^{2+} ions by the catalyze of ascorbic acid, failing to generate PDA nanoparticles, and therefore, recovering the fluorescence of QDs for ratiometric fluorescence detection of high-activity ALP, or weakening the oxidation of TMB for colorimetric detection of ultralow-activity ALP. The ratiometric fluorescence-colorimetry combination extended the linear range over three orders of magnitude (0.04–80 mU/mL), and lowered the detection limit down to 0.015 mU/mL, along with profuse ALP-dependent (fluorescence) color changes for visual detection of ALP activity. Excellent recognition selectivity for ALP was attained over possibly coexistent reducing substances. Furthermore, the endogenous ALP were detected ranging from 17.32 to 269.54 mU/mL in seven typical livestock sera, consistent with that measured by commercial ALP assay kit; the detection results for ALP in four pasteurized milks matched well with that by test paper. The developed dual-mode assay held great potential for rapid on-site visual determination of reductant-related analytes in complicated matrices.

1. Introduction

Alkaline phosphatase (ALP) is an essential hydrolase and universally exists across a multitude of living organisms. It plays different functions in different tissues by catalyzing the dephosphorylation of various substrates, and promotes the digestion and absorption of calcium and phosphorus [1,2]. Hence, ALP activity assay is necessary for disease diagnosis and nutritional modulation of livestock, which is known as an important food-source. When the ALP activity is elevated or lowered

(normal value: dozens to hundreds mU/mL), the livestock should be diagnosed the problems in the liver, bone, kidney, intestine, stomach, etc., or provided the feeds with suitable calcium and phosphorus content [3–5]. Besides, ALP could be naturally produced by the breast cells and commonly found in milk. During the pasteurization process, ALP could survive better than *Mycobacterium paratuberculosis* which is the most thermo-tolerant bacterium found in milk. Consequently, the activity of ALP higher than 0.35 mU/mL in milk indicates the failed pasteurization [6,7]. As can be seen, the ALP activity assay in livestock

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and dairy products plays a significant role in supervising the food quality that people are paying increasing attention to. Up to now, various methods have been developed to determine the activity of ALP [8], for instance, colorimetry [9], fluorescence [10], chemiluminescence [11], surface-enhanced Raman scattering [12], electrochemistry [13] and electrophoresis [14] methods. Among these, fluorescence is preferred owing to its simplicity and sensitivity as well as tremendous signal providers including the easy-photobleaching organic dyes, toxic semiconductor quantum dots (QDs), expensive metal nanoclusters and synthesis-complicated upconversion nanoparticles [15]. In contrast to these cons, the pros of simple synthesis, non-toxic nature, good biodegradability and photostability as well as low cost make newly emerged intrinsic fluorescent polymer nanoparticles become powerful fluorescence signal providers [16].

Manganese dioxide (MnO_2) nanosheets, one kind of redox-active layered transition-metal dioxide, have attracted much concern owing to the superior physicochemical properties of large surface areas, wide UV–vis absorption band, specific oxidase-like activity, photoelectrochemical and supercapacitor property, and good biocompatibility [16–18]. By virtue of the wide absorption band, MnO_2 nanosheets could act as the potential energy acceptor to quench various fluorescent substances via fluorescence resonance energy transfer (FRET) or inner filter effect (IFE) mechanism [19,20]. In the presence of various antioxidant substances such as glutathione (GSH), cysteine and ascorbic acid (AA), MnO_2 nanosheets are reduced into Mn^{2+} ions and the absorbance is decreased, and thereupon FRET or IFE is restrained and the fluorescence intensity is recovered. This detection principle has been widely applied for determining the antioxidants directly [21], or the antioxidant-related species indirectly like AA's producer of ALP [16], as well as thiocholine's producer of butyrylcholinesterase (BChE) and its inhibitors of organophosphorus pesticides (OPs) [19]. However, the particular oxidase-like activity of MnO_2 nanosheets is rarely applied in the fluorescent sensor construction, especially for ALP detection. Kong et al. [17] and Lin et al. [22] utilized the oxidase-like activity of MnO_2 nanosheets to oxidize dopamine into the polydopamine (PDA) nanoparticles for GSH and alpha-fetoprotein detection, respectively, where the PDA nanoparticles are the typical intrinsic fluorescent polymer nanoparticles. Besides, most of the sensing studies abovementioned (based on wide-absorption or oxidase-like activity) are confined to the single-emission fluorescence [23], which is compromised by various analyte-independent factors, like fluorescent probes concentration, background signal, instrumental and operational fluctuation, etc. The ratiometric fluorescence, alternatively, can eliminate the external interference because of its self-correction function, where the dual or more fluorescence emissions would change out of sync upon the analyte

interaction under the single-wavelength excitation. Furthermore, the ratiometric fluorescence platforms can often provide profuse fluorescence color variation for visualization [24,25].

As known, by the recognition toward AA based on the wide absorption band, the reported MnO_2 -based fluorescence platforms could detect high-activity ALP indirectly ranging from tens to hundreds (mU/mL), and limits of detection (LOD) are rarely lower than 0.35 mU/mL [16,26]. That's why, the reported sensing strategies can be potentially applied for livestock's ALP detection, but are hard to meet the need in pasteurized milk control, compatibly. Hence, another optical strategy of colorimetric detection is expected to cooperate with ratiometric fluorescence for ALP detection both in livestock and dairy products. Thanks to the oxidase-like activity of MnO_2 nanosheets, the colorless 3,3',5,5'-tetramethylbenzidine (TMB) could be directly oxidized into yellow oxidation product (TMB_{ox}) in acid condition without any oxidizing agent (e.g. H_2O_2) [27], which is in contrast to other peroxidase-like nanomaterials [28]. Additionally, TMB is neither mutagenic nor carcinogenic [29], and more sensitive [30] than the traditional substrates of o-phenylenediamine (OPD) and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). This TMB-participating colorimetry wins in short reaction time, simple operation, directly visual outcome and ultrahigh detectability, but remains untouched in MnO_2 -based assays for ALP detection.

Inspired by the aforementioned studies, we decided to propose a ratiometric fluorescence and colorimetry dual-mode assay based on MnO_2 nanosheets for ALP activity detection. As illustrated in route i of Scheme 1, in the presence of trace MnO_2 nanosheets, the non-fluorescent dopamine was oxidized into green fluorescent PDA nanoparticles with a broad absorption over 330–700 nm, overlapping the fluorescence emission of red fluorescent CdTe/ZnS QDs to quench their fluorescence via FRET. Upon the addition of AA, the product of ALP-triggered dephosphorylation of 2-phospho-L-ascorbic acid (AA2P), MnO_2 nanosheets were reduced into Mn^{2+} ions and lost the oxidase-like activity; as a result, the self-polymerization of dopamine failed and FRET was thereupon diminished, expressing as the fluorescence disappearance of PDA nanoparticles and the fluorescence recovery of QDs, along with the fluorescence color changing from green to red. Based on the ratiometric fluorescence mode, ALP in a range of 4–80 mU/mL could be quantitatively detected, which could be used in livestock disease diagnose and feed modulation. Likewise, the oxidation of TMB by MnO_2 nanosheets was held back in the coexistence of ALP and its zymolyte of AA2P, expressing as the less generation of TMB_{ox} and fading of yellow color, as illustrated in route ii of Scheme 1. The ALP within 0.04–2.8 mU/mL could be detected by this colorimetric mode, fully meeting the demand to judge the success or failure of milk

Scheme 1. Illustration of the ratiometric fluorescence and colorimetry dual-mode assay of ALP activity by virtue of the oxidase-like activity and recognizer role of MnO_2 nanosheets.

pasteurization. Significantly, the visualization of ALP activity could be implemented by observing the fluorescence color variation from green to red, or the fading of yellow color. Based on the oxidase-like activity and recognizer role of MnO_2 nanosheets, the combination of ratiometric fluorescence and colorimetric modes enlarged the detection range over 0.04–80 mU/mL and decreased the detection limit down to 0.015 mU/mL, and therefore could be compatibly applied in controlling the quality of the livestock and milk products with precise, sensitive, selective and visual detection results.

2. Experimental

2.1. Reagents and materials

KMnO_4 was provided by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). AA, citric acid, hydrochloric acid (HCl) and ethanol were obtained from XiLong Chemical (Guangdong, China). 4-Morpholineethanesulfonic acid (MES), dopamine, TMB, N-ethylmaleimide (NEM), GSH, trypsin, glucose oxidase (GOx) and peroxidase from horseradish (HRP) were purchased from Aladdin (Shanghai, China). ALP, 2-phospho-L-ascorbic acid (AA2P), lysozyme, lipase and acetylcholinesterase (AChE) were offered by Sigma-Aldrich (Shanghai, China). Cysteine was purchased from Solarbio Science & Technology Co. Ltd. (Beijing, China). Malic acid, tartaric acid and glucose were purchased from Damao Chemical Reagent Factory (Tianjin, China). All reagents were at least of analytical grade and used as received. CdTe/ZnS QDs were synthesized with details given in the Supporting Information. Phosphate buffer (PB) solution (0.1 M, pH 8.0) was used in AA and ALP sensing procedures, and ultrapure water of 18.2 M Ω specific resistance (Millipore, USA) was used in all experiments.

2.2. Apparatus

A fluorescence spectrophotometer (F-7000, HITACHI), a UV–vis spectrophotometer (Tu-1900, PERSEE), a Raman spectrometer (LabRAM HR Evolution, HORIBA) and an X-ray photoelectron spectroscopy (XPS, ESCALAB 250xi, Thermo Scientific) were used to record fluorescence excitation/emission, UV–vis absorption, Raman and XPS spectra, respectively. Morphological evaluation was examined on a transmission electron microscope (TEM, JEOL 2100 F) and an atomic force microscope (AFM, Dimension Edge, Bruker). Elemental mapping was observed by energy dispersive spectroscopy (EDS, X-Max, Oxford). Zeta potential was measured by Malvern Zetasizer Nano-ZS90 (ZEN3590, UK). An UV lamp with 365 nm was used to take fluorescence images.

2.3. Synthesis of MnO_2 nanosheets

MnO_2 nanosheets were synthesized according to the previous work [31] with minor modification. Briefly, 9 mL of KMnO_4 (0.01 M) and 12 mL of MES (0.1 M, pH 6.0) were mixed and sonicated for 5 min. Then, the synthesized black brown colloids were centrifuged and washed with ultrapure water for five times to finally obtain the MnO_2 nanosheets, followed by dispersing in water with a concentration of 1 mg/mL for use.

2.4. Ratiometric fluorescence detection of AA and ALP based on MnO_2 nanosheets

For AA detection, 1 mL of AA at different concentrations (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM) firstly reacted with a mixture of 50 μL of MnO_2 nanosheets (0.2 mg/mL) and 100 μL of PB solution for 10 min. Then, 50 μL of dopamine (200 mM) was added and reacted for 20 min, with adding 60 μL of HCl (0.1 M) to end the polymerization reaction. Finally, 20 μL of CdTe/ZnS QDs were added and the fluorescence emission spectra were recorded (excitation wavelength: 375 nm;

slit widths: 10/10 nm).

For ALP detection, 1 mL of ALP at different activities (0, 4, 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 mU/mL) were initially incubated with 100 μL of AA2P (10 mM, in PB solution) at 37 °C for 50 min; then 50 μL of MnO_2 nanosheets (0.2 mg/mL) were added and incubated for another 10 min. The next steps of adding dopamine, HCl and CdTe/ZnS, as well as recording fluorescence spectra were the same as aforementioned.

2.5. Colorimetric detection of AA and ALP based on MnO_2 nanosheets

For AA detection, 1 mL of AA at different concentrations (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3 and 3.5 μM), 100 μL of PB solution and 50 μL of MnO_2 nanosheets (0.2 mg/mL) were mixed well, and then 50 μL of HCl (1 M) and 50 μL of TMB (0.5 mM) were added. After blending, the UV–vis absorption spectra were recorded.

For ALP detection, 1 mL of ALP at different activities (0, 0.04, 0.08, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 mU/mL) were incubated with 100 μL of AA2P (10 mM, in PB solution) at 37 °C for 50 min, and then 80 μL of MnO_2 nanosheets (0.2 mg/mL) were injected. After adding 50 μL of HCl (1 M) and 50 μL of TMB (0.5 mM), the UV–vis absorption spectra were recorded.

2.6. Selectivity of the dual-mode assay

In the ratiometric fluorescence mode, AA (50 μM) or ALP (50 mU/mL) was replaced by the control reducing substances (malic acid, citric acid, tartaric acid, glucose, cysteine, and GSH) at same concentration of 50 μM and control enzymes (lysozyme, lipase, trypsin, AChE, GOx, and HRP) at same activity of 50 mU/mL, and the as-resulted fluorescence changes at two emission peaks were compared with that toward AA and ALP.

In the colorimetric mode, AA (2 μM) or ALP (2 mU/mL) was substituted by the control reducing substances (their concentration fixed at 2 μM individual) and enzymes (with activity at 2 mU/mL), and the corresponding UV–vis absorption spectra were recorded and the absorbance was observed.

2.7. Practical application of the dual-mode assay to livestock serum and milk products

For livestock serum application, seven serum samples obtained from chicken, duck, rabbit, pig, sheep, goat and cattle, respectively, were tested. In order to ensure the endogenous ALP activities were within the linear range, five serum samples were 4-fold diluted except the duck and pig serum samples. After adding 0.3 mM of NEM [32], all the seven (five diluted and 2 without dilution) livestock serum samples were used for the ratiometric fluorescence tests. And all the test results were compared with that measured by the commercial ALP assay kit (IFCC recommendation method, Nanjing Jiancheng Bioengineering Institute, China). For dairy application, four pasteurized milk products were tested. Firstly, the reducing small molecules such as AA were removed by centrifuging against an ultrafilter (low molecular weight cutoff) [33]. Then, the endogenous and spiked ALP were detected by the proposed colorimetric mode of MnO_2 -TMB and the commercial ALP test paper, respectively.

3. Results and discussion

3.1. Synthesis and characterization of MnO_2 nanosheets

In this work, the ultrathin MnO_2 nanosheets were facilely synthesized via a sonication-induced redox reaction of KMnO_4 using MES as the reducing agent [31]. The TEM (Fig. 1A) and AFM (Fig. 1B) images indicated the typical two-dimensional sheet-like structure with frequent wrinkles and folds, and the average lateral dimension was nearly

Fig. 1. (A) TEM image (inset: HRTEM image), (B) AFM image, (C) UV-vis absorption spectrum, (D) elemental mapping, (E) XPS spectrum and (F) Raman spectrum of the synthesized MnO_2 nanosheets.

100 nm with the ultrathin thickness of approximately 2.5 nm. The clear MnO_2 lattices were meanwhile observed in the high resolution TEM (HRTEM) image (inset of Fig. 1A). The special nanostructure provided a large surface area for full reaction with various substances of AA, dopamine, and TMB. Further, the as-obtained brown MnO_2 nanosheets solution presented a characteristic 372 nm absorption peak located within the 250–700 nm absorption band (Fig. 1C), corresponding to the $d-d$ transition caused by the ligand field of MnO_6 octahedra in MnO_2 crystal lattices [22,31]. Besides, the elemental composition and characteristic functional groups of MnO_2 nanosheets were observed as below. Signals from elements of Mn and O were both observed in the elemental mapping (Fig. 1D) and XPS spectrum (Fig. 1E), where peaks at 654 and 643 eV corresponded to $\text{Mn}_{2p3/2}$ and $\text{Mn}_{2p1/2}$, respectively [34]; and two distinct peaks at 553 and 638 cm^{-1} observed in the Raman spectrum (Fig. 1F) were stemmed from the Mn–O lattice vibrations [35]. All the results demonstrated the successful synthesis of ultrathin MnO_2 nanosheets.

3.2. Possible detection principle of ALP activity by the dual-mode assay based on MnO_2 nanosheets

MnO_2 nanosheets possess the specific oxidizing ability in contrast to other two-dimensional materials [28]. In our work, no significant fluorescence and absorption were observed for the solo MnO_2 nanosheets (Fig. 2A, a and B, a) or solo dopamine (Fig. 2A, b and B, b), while the reaction mixture of MnO_2 nanosheets and dopamine (i.e., PDA nanoparticles) presented obvious fluorescence emission at 485 nm (Fig. 2A, d) and UV-vis absorption over 330–700 nm (Fig. 2B, c). Comparing the TEM images of MnO_2 nanosheets before and after reaction with dopamine (Fig. S1A and B), it could be found that MnO_2 nanosheets were decomposed and replaced by the generation of the spherical nanoparticles with an average diameter of nearly 200 nm. All the experimental results above corroborated that dopamine was dramatically oxidized by MnO_2 nanosheets into its quinone derivative, and further spontaneously polymerized into fluorescent PDA nanoparticles through the covalent bonds, hydrogen bonds, $\pi-\pi$ interaction, etc. [17].

Feasibly, with the broad absorption band over 330–700 nm to overlap the excitation/emission spectra of CdTe/ZnS QDs (Fig. 2B), PDA nanoparticles could act as the energy acceptor to quench QDs (Fig. 2A, c and e) via the possible FRET or IFE. The quenching efficiency of QDs contributed from IFE was corrected on the basis of the cell geometry (Fig. S2A) and the equation [36] at below:

$$\frac{F_{\text{cor}}}{F_{\text{obsd}}} = \frac{2.3dA_{\text{ex}}}{1 - 10^{-dA_{\text{ex}}}} \cdot \frac{10^{gA_{\text{em}}}}{1 - 10^{-gA_{\text{em}}}} \cdot \frac{2.3sA_{\text{em}}}{1 - 10^{-sA_{\text{em}}}}$$

The detailed interpretation for each abbreviation was given in the Supporting Information. Table S1 provides the experimental data with different concentrations of PDA nanoparticles. As can be seen in Fig. S2B, there were no significant differences between the quenching efficiencies for the totally observed and the corrected (i.e., after removing IFE) fluorescence intensities for all the concentrations PDA nanoparticles used. That is, IFE did negligible contribution to the suppressive effect of QDs. Besides, the Zeta potential analysis (Fig. S3) showed that PDA nanoparticles and QDs had the potential values of 2.48 mV and -43.6 mV, respectively. The electrostatic interaction between them was beneficial for their close contact so to guarantee the energy transfer from QDs to PDA nanoparticles. As a contrast, MnO_2 nanosheets only slightly quenched QDs (Fig. 2A, f) because of the little spectrum overlapping (Fig. 2B). Thus, FRET might well account for the fluorescent detection.

As a recognizer, MnO_2 nanosheets could be used to detect various antioxidants and related substances [19,21]. For instance, MnO_2 nanosheets could be effectively reduced into Mn^{2+} ions by AA, which is meanwhile oxidized into dehydroascorbic acid (DAA), as below:

The as-left Mn^{2+} ions were out of oxidizing ability and the generation of PDA nanoparticles was lessened or even failed (Fig. S1E). Evidently, the absorption of PDA was weakened, and FRET was thereupon diminished. On the fluorescence emission spectra, the fluorescence of PDA disappeared but that of QDs was recovered (Fig. 2C, a and b).

Fig. 2. (A) Fluorescence spectra and (B) UV-vis absorption spectra of MnO₂ nanosheets before and after reaction with different substances, and the overlapping with the fluorescence excitation/ emission spectra of CdTe/ZnS QDs. (C) Fluorescence spectra of the ratiometric fluorescence assay (MnO₂-dopamine-QDs) before and after reaction with AA (50 μ M), mixture of ALP (48 mU/mL) and AA2P, and solo ALP (48 mU/mL). (D) UV-vis absorption spectra of MnO₂, TMB and the colorimetric assay (MnO₂-TMB) before and after reaction with AA (2 μ M), mixture of ALP (2 mU/mL) and AA2P, and solo ALP (2 mU/mL). Insets of C and D were the corresponding photographs taken under a 365 nm UV lamp and visible light, respectively.

Typically, ALP could cleave the phosphate functional groups from AA2P to produce AA. With a certain amount of AA2P, ALP at higher activity could produce more AA, and further consume more MnO₂ nanosheets (Fig. S1A–C), generating less PDA nanoparticles (Fig. S1D–F) and recovering the fluorescence of QDs (Fig. 2C, c). Additionally, treating MnO₂ nanosheets with ALP alone had little effect on oxidizing dopamine into PDA (Fig. 2C, d). Hence, the fluorescence decrease and recovery efficiencies could indicate the concentration of AA directly, or the activity of ALP indirectly.

Likewise, the oxidase-like property of MnO₂ nanosheets was demonstrated in the typical chromogenic reaction of TMB, where the colorless MnO₂ nanosheets (Fig. 2D, a) made the colorless TMB (Fig. 2D, b) oxidize into blue oxidation products and the final yellow diamine (TMB_{ox}) in acidic condition (Fig. 2D, c and Fig. S4), with a sharp characteristic peak at 452 nm [37]; meanwhile, MnO₂ nanosheets were broken (Fig. S1G). Similarly, the addition of AA, or a mixture of AA2P and ALP, also led to the destruction of MnO₂ nanosheets (Fig. S1H and I) and further affected the oxidation of TMB with lower absorbance at 452 nm (Fig. 2D, d and e). The control test revealed that the solo addition of ALP had no significant effect on the UV-vis absorption spectrum of TMB_{ox} (Fig. 2D, f).

ALP activity determination by the ratiometric fluorescence and colorimetric modes could be carried out because of the oxidation and recognition ability of MnO₂ nanosheets; the detection range and limit toward ALP, however, were quite different. The ratiometric fluorescence mode of MnO₂-dopamine-QDs covered the ALP activity range in the livestock serum, and the colorimetric mode of MnO₂-TMB sufficed for assessing the successful/failed pasteurization of the milk products. Therefore, ratiometric fluorescence and colorimetry dual-mode assay of

ALP activity based on MnO₂ nanosheets was realized. One of the two optical modes could be flexibly chosen according to the actual need.

3.3. Condition optimization for the ratiometric fluorescence and colorimetry dual-mode assay

Various parameters affecting the sensing performance of MnO₂ nanosheets were investigated, mainly involving the synthesis conditions of PDA nanoparticles, the amount of AA2P in ALP detection, and the oxidation conditions for TMB.

For PDA nanoparticles preparation, the dosage of dopamine, acid-base condition, consumption of MnO₂ nanosheets and the reaction time were all optimized. As can be seen from Fig. S5A, the fluorescence intensity at 485 nm of PDA nanoparticles increased with dopamine from 0 to 200 mM, and then gradually decreased, owing to the formation of a number of large PDA nanoparticles. Then, with dopamine fixed at 200 mM, different pH conditions caused notable distinction in the fluorescence intensity: acidic or neutral condition was adverse to fluorescent PDA nanoparticles production, and excessive alkaline condition made dopamine self-polymerize into PDA nanoparticles although no MnO₂ nanosheets were added, and even with fluorescence intensity decreasing with the addition of MnO₂ nanosheets (Fig. S5B). Given the low self-polymerization and adequate PDA nanoparticles generation, pH 8.0 was chosen as the optimal condition. Combining Fig. S5A and B, it was found that the fluorescence intensity of PDA nanoparticles gradually increased along with the addition of MnO₂ nanoparticles because of the generation of more PDA nanoparticles. However, overmuch MnO₂ nanosheets went against sensitive analyte detection. Considering both the sensitivity and the fluorescence intensity for visualization,

0.2 mg/mL MnO₂ nanosheets was preferred. Finally, as can be seen in Fig. S5C, the fluorescence intensity progressively increased along with the reaction time, then reached a plateau after 20 min and then declined beyond 30 min. Thus, 200 mM of dopamine, pH 8.0, 0.2 mg/mL of MnO₂ nanosheets and 20 min as reaction time were adopted as the optimized conditions for PDA nanoparticles synthesis.

In the presence of ALP, the phosphate group of AA2P was cleaved to produce AA, which participated in the ratiometric fluorescence assay. In this case, the dosage of AA2P and the enzymatic reaction time were optimized. As displayed in Fig. S6A, too small amounts of AA2P (1 mM) was failed to produce sufficient AA even reacting with high activity of ALP, while overmuch AA2P (25 and 50 mM) reacting with low activity of ALP could generate enough AA to completely wiped the fluorescence of PDA nanoparticles. In this work, we aimed to apply the fluorescence sensing system to detect ALP activity in livestock serum, where the ALP activity covers a wide range of dozens to hundreds (mU/mL). Hence, 10 mM of AA2P were selected owing to the appropriately high fluorescence quenching effect. As to the reaction time between ALP and AA2P, as can be seen in Fig. S6B, the decreasing fluorescence intensity or the increasing quenching efficiency reached a balance beyond 50 min. That is, 50 min was long enough for the complete dephosphorylation of AA2P.

Additionally, for the colorimetric assay of MnO₂-TMB, the colorless TMB was transferred into blue products under slightly acidic condition and yellow TMB_{ox} with increasing acidity. So, the addition of adequate HCl was necessary because AA was produced in an alkaline environment (PB solution of pH 8.0) that ensured the hydrolysis capacity of ALP. As can be seen in Fig. S7A, with HCl increasing from 0 to 0.2 M, the absorbance at 652 nm increased but that at 452 nm didn't. This phenomenon was attributed to that a small amount of HCl gradually neutralized the alkaline environment to the weakly acidic so that TMB was oxidized into blue products by MnO₂ nanosheets, accompanying with MnO₂ nanosheets itself being reduced. The blue products were further transferred into yellow diamine with more HCl so the absorbance at 452 nm increased. Since the yellow TMB_{ox} possessed higher molar absorption coefficient (ϵ) than the blue products, yellow TMB_{ox} was determined as the final products and adequate HCl of 1 M was adopted. Besides, absorbance at 452 nm also relied on the addition of TMB. With TMB concentration increasing, absorbance at 452 nm continuously rose until TMB up to 0.5 mM (Fig. S7B), indicating MnO₂ nanosheets were fully decomposed by 0.5 mM of TMB. Hence, 0.5 mM of TMB was used in this colorimetric system. Moreover, the same consumption of MnO₂ nanosheets (50 μ L, 0.2 mg/mL) in AA detection was not suitable for ALP activity determination, where the two absorbance values were quite different, although AA and ALP were at the same concentration/activity of 0. As seen in Fig. S8, when ALP was set to be 0, the colorimetric system containing AA2P presented almost no absorbance at 452 nm in a white transparent state before the usage of MnO₂ nanosheets (0.2 mg/mL) exceeded 30 μ L. It was assumed that some AA molecules were present in AA2P because of their similar structure so to consume a certain amount of MnO₂ nanosheets. Hence, 80 μ L (i.e., 30 μ L plus 50 μ L) of MnO₂ nanosheets (0.2 mg/mL) were used for ALP detection in the colorimetric assay.

3.4. Analytical performance of the dual-mode assay toward ALP activity

In the ratiometric fluorescence mode of MnO₂-dopamine-QDs, ALP catalyzed the dephosphorylation of AA2P to produce AA, which could reduce MnO₂ nanosheets into Mn²⁺ ions and therefore inhibited the synthesis of PDA nanoparticles, and further constrained FRET. As confirmed in Fig. S9, under the optimal conditions, the fluorescence of PDA nanoparticles and QDs regularly decreased and enhanced with increasing AA from 0 to 100 μ M, respectively. As the producer of AA, ALP could deservedly lead to similar experimental phenomena in the presence of AA2P. As can be seen in Fig. 3A, the fluorescence intensity of PDA nanoparticles at 485 nm progressively fell off with the addition of ALP from 0 to 80 mU/mL, while the fluorescence intensity of QDs at

639 nm dramatically increased. This ALP-dependent dual-signal change realized the ratiometric fluorescence assay. As calculated, the declining rate at 485 nm and the enhancing rate at 639 nm, i.e., $\Delta F_{485\text{ nm}}/F_{0, 485\text{ nm}}$ and $\Delta F_{639\text{ nm}}/F_{0, 639\text{ nm}}$, respectively, was linearly or exponentially risen with the increasing activity of ALP ranging from 4 to 80 mU/mL, respectively (Fig. 3B). Additionally, obvious fluorescence color change occurred in the green-yellow-orange-red window (Fig. 3E, a). Obviously, the ratiometric fluorescence sensing system could be applied in ALP activity detection to assess the health of livestock.

As a control, in the single-emission fluorescence sensing system of MnO₂-dopamine without QDs, ALP also triggered the decomposition of MnO₂ nanosheets and failed generation of PDA nanoparticles, so that the fluorescence intensity of PDA nanoparticles was linearly decreased similarly (Fig. 3C), with the dimming of green fluorescence but no color change (Fig. 3E, b). The visual result of this single-emission fluorescence was remarkably inferior to that of the ratiometric fluorescence. Besides, in another single-emission fluorescence sensing system of MnO₂-QDs without adding dopamine, the only fluorescence of QDs was weakly enhanced by ALP (Fig. 3D). The tiny fluorescence intensity enhancement could be ascribed to the blocked energy transfer from QDs to MnO₂ nanosheets, where the trace MnO₂ nanosheets (approximately 0.0078 mg/mL) could have slightly quenched QDs by the wide absorption band over 250–700 nm, but they themselves were reduced. As shown in Fig. 3E, c, fluorescence colors barely changed, owing to the negligible intensity enhancement, even with the presence of ALP. So, the sensing performance, especially the visualization effect, of the ratiometric fluorescence assay was significantly superior to the non-ratiometric assay.

Then, in the colorimetric mode of MnO₂-TMB, AA, or ALP with AA2P, could reduce MnO₂ nanosheets and therefore inhibit the oxidation of TMB. Under the optimal conditions, the generation of TMB_{ox}, manifested as the absorbance at 452 nm, gradually decreased along with AA increasing from 0 to 3.5 μ M (Fig. S10), or with ALP activity increasing from 0 to 2.8 mU/mL (Fig. 3F). Tracing the plots of absorbance versus the different activities of ALP, it could be found that the absorbance decreased linearly with the ALP increase ranging from 0.04 to 2.8 mU/mL (inset of Fig. 3F). And the corresponding yellow color was faded gradually (inset of Fig. 3F). Based on the 3 σ IUPAC criteria of 3 σ /s, where σ is the standard deviation of the blank measurements and s is the slope of the linear calibration, the limit of detection (LOD) was obtained as 0.015 mU/mL, lower than that of commercial ALP test paper (0.02 mU/mL). The LOD value is comparable to or even much lower than that of previous studies [16,26,38]. This ultralow LOD and the linear range covering 0.35 mU/mL ALP allowed the judgment for the success or failure of the pasteurization of milk products.

The combination of ratiometric fluorescence and colorimetric modes expanded the quantitative range from 0.04 to 80 mU/mL and lowered the LOD down to 0.015 mU/mL. The proposed MnO₂-based dual-mode ALP assay with such satisfactory sensing performance could compatibly meet the determination of totally different ALP activities in various complex food products. One of the two modes could be chosen flexibly according to actual needs.

3.5. Selectivity of the dual-mode assay toward ALP activity

The selectivity of the two optical modes was investigated by comparing the change of fluorescence intensity and absorbance of AA and ALP with that of the control reducing substances and various enzymes. As can be seen in Fig. 4, some reducing substances of cysteine and GSH caused the obvious fluorescence/absorbance signal's change so that it could interfere with the detection of ALP, owing to their relatively strong reducing ability. However, the interference effect of cysteine and GSH could be blocked by NEM, which is a masking agent that can specifically react with the thiol group of GSH and cysteine [32]. Except for GSH and cysteine, other reducing substances didn't induce remarkable fluorescence/absorbance response because of the low reducing ability. Additionally, the impact on fluorescence/absorbance

signals caused by ALP was far apparent compared with the control enzymes, stemming from its specific catalytic action for the dephosphorylation of AA2P, and the recognition role of MnO_2 nanosheets toward the product of AA. Thus, both the MnO_2 -dopamine-QDs ratiometric fluorescence and MnO_2 -TMB colorimetric assays presented excellent selectivity for ALP detection.

3.6. Application of the dual-mode assay to livestock and milk samples

The applicability of the proposed MnO_2 -based dual-mode assay toward ALP activity was investigated in seven livestock serum samples and four pasteurized milk products. Before the ratiometric fluorescence determination, five serum samples were diluted appropriately to ensure

Fig. 3. Fluorescence spectra (A) and the corresponding intensity decreasing/enhancing efficiencies fitting curves (B) and fluorescence images (E, a) of the ratiometric fluorescence assay of MnO_2 -dopamine-QDs toward 0–80 mU/mL ALP; fluorescence spectra (C) and fluorescence images (E, b) of the single-emission fluorescence assay of MnO_2 -dopamine toward 0–80 mU/mL ALP, and inset of C shows the corresponding fitting curve; fluorescence spectra (D) and fluorescence images (E, c) of the single-emission fluorescence assay of MnO_2 -QDs toward 0–80 mU/mL ALP; (F) UV-vis absorption spectra of MnO_2 -TMB assay with increasing ALP from 0 to 2.8 mU/mL, and insets show the corresponding images under visible light (upper) and the corresponding fitting curve of absorbance (below) versus the varying activity of ALP. Related parameters for the fitting curves (B, inset of C, inset of F) were listed in Table S2.

Fig. 4. (A) Fluorescence intensity decreasing or enhancing at 485 and 639 nm of the ratiometric fluorescence mode of MnO_2 -dopamine-QDs toward various reducing substances and enzymes (AA and other reducing substances: 50 μM ; ALP and other enzymes 50 mU/mL; NEM: 150 μM), respectively. (B) Absorbance at 452 nm of the colorimetric mode of MnO_2 -TMB toward various reducing substances and enzymes (AA and other reducing substances: 2 μM ; ALP and other enzymes 2 mU/mL; NEM: 6 μM).

Table 1

ALP activities measured by the proposed dual-mode assay and the commercial ALP assay kit or test paper.

Sample		Proposed dual-mode assay (mU/mL) ^a $\pm$ RSD (%)	Commercial ALP assay kit or test paper (mU/mL) ^a $\pm$ RSD (%), $n = 3$	Relative error (%) ^b or match or not ^c
Livestock serum	Rabbit	181.35 $\pm$ 2.89 ^d	175.82 $\pm$ 3.85	3.13
	Chicken	267.32 $\pm$ 3.64 ^d	275.06 $\pm$ 2.07	-2.81
	Duck	17.32 $\pm$ 7.32	15.62 $\pm$ 14.41	10.88
	Pig	63.52 $\pm$ 5.46	59.12 $\pm$ 6.00	7.44
	Goat	224.29 $\pm$ 2.34 ^d	226.97 $\pm$ 2.71	-1.18
	Sheep	269.54 $\pm$ 3.58 ^d	256.88 $\pm$ 1.15	4.93
	Bovine	170.22 $\pm$ 4.35 ^d	162.34 $\pm$ 4.17	4.85
Pasteurized milk	Sample 1	LOD < ALP < 0.04 $\pm$ 5.13	Successful pasteurization	Match
	Spiked sample 1 ^e	1.03 $\pm$ 4.62	Failed pasteurization	Match
	Sample 2	0.06 $\pm$ 6.25	Successful pasteurization	Match
	Spiked sample 2 ^e	1.08 $\pm$ 5.49	Failed pasteurization	Match
	Sample 3	0.05 $\pm$ 4.23	Successful pasteurization	Match
	Spiked sample 3 ^e	1.06 $\pm$ 5.12	Failed pasteurization	Match
	Sample 4	LOD < ALP < 0.04 $\pm$ 4.76	Successful pasteurization	Match
	Spiked sample 4 ^e	1.04 $\pm$ 3.98	Failed pasteurization	Match

^a Average value from three parallel determinations.

^b Relative error (%) of the test results measured by present assay against the commercial ALP assay kit.

^c The test results measured by present assay matched or not that by commercial ALP test paper.

^d Multiply the test value of the 4-fold diluted serum by 4. Namely, $41.3376 \times 4 \approx 181.35$ mU/mL ALP for rabbit serum, $66.8311 \times 4 \approx 267.32$ mU/mL ALP for chicken serum, $56.0721 \times 4 \approx 224.29$ mU/mL ALP for goat serum, $67.3851 \times 4 \approx 269.54$ mU/mL ALP for sheep serum, and $42.5549 \times 4 \approx 170.22$ for bovine serum.

^e spiked with ALP at 1 mU/mL.

the endogenous ALP activities were within the linear range, that is, all were 4-fold diluted except the duck and pig serum. Accordingly, the test results of ALP activity in the diluted five serum samples were as follows: 41.34 mU/mL for rabbit, 66.83 mU/mL for chicken, 56.07 mU/mL for serum, 67.39 mU/mL for sheep, and 42.56 mU/mL for bovine. Then, the ALP activity results in all the seven livestock serum samples were listed in Table 1. As seen, the ALP activity was detected at 17.32–269.54 mU/mL, with relative standard deviations (RSDs) between 2.34 and 7.32%. The results were in accordance with that measured by the commercial ALP assay kit, considering the low relative errors ranging from -2.81 to 10.88%.

In addition, the ALP activities in four pasteurized milk products were measured by the colorimetric assay. The reducing small molecules such as AA existing in milk could interfere with the test results and, therefore, were removed from the four milk products by centrifuging against an ultrafilter [33]. The eliminating effect could be self-demonstrated by the MnO_2 -TMB platform: the samples without AA did not make a decrease in absorbance at 452 nm in the absence of AA2P. Then,

the ALP activities in undiluted pasteurized milk measured by our colorimetric method were given in Table 1, and all were less than 0.35 mU/mL with RSDs of 3.98–6.25%, demonstrating that ALP in the milk products was denatured and the four milk products were pasteurized successfully. In addition, the spiked ALP in milk higher than 0.35 mU/mL to stand for the failed pasteurization were also correctly measured and provided in Table 1. The results agreed well with those afforded by the commercial ALP test paper for milk, which could only judge the success or failure of the pasteurization but not provide the exact values of ALP activity.

The successful ALP determination in different complex matrices demonstrated the developed dual-mode assay based on MnO_2 nanosheets was reliable and could resist various interference. Moreover, the present assay could provide visual outcomes conveniently via (fluorescence) color change, allowing the untrained people to readily perform analysis but without learning about the sophisticated sensing principles involved.

4. Conclusions

In conclusion, ratiometric fluorescence and colorimetry dual-mode assay based on MnO₂ nanosheets was developed for the visual detection of ALP activity in livestock serum and pasteurized milk samples. MnO₂ nanosheets played the role of oxidizing agent to oxidize dopamine or TMB into fluorescent PDA nanoparticles or yellow TMB_{ox}, respectively, and also acted as the recognizer to specifically react with antioxidants like AA. Therefore, in the presence of AA2P, ALP at high activity in livestock serum reduced the MnO₂ nanosheets, and resulted in the fluorescence disappearing of PDA nanoparticles and fluorescence recovery of CdTe/ZnS QDs in a ratiometric fluorescence mode; the ultralow-activity ALP in pasteurized milk decomposed the MnO₂ nanosheets and therefore caused less TMB_{ox} production and lower absorbance at 452 nm in a colorimetric mode. The two optical modes provided an obvious (fluorescence) color variation in an ALP-dependent manner, available for visualization of ALP. The dual-mode assay based on MnO₂ nanosheets resisted the interference in real samples and provided reliable test results which were consistent with that afforded by the commercial ALP assay kit and test paper. As a successful proof of concept, we believe that the MnO₂ nanosheets based dual-mode assay can be extended to detect wider types of antioxidant-related target analytes in more fields and contribute to the development of rapid on-site visual detection.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.snb.2019.127176>.

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