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Journal Pre-proof

Bioinspired Nanozyme for Portable Immunoassay of Allergenic Proteins

Based on A Smartphone

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Abstract

Inspired by the structure of the catalytically active center of natural laccase, a novel laccase mimics (named LM nanozymes) with a superior catalytic activity was successfully prepared by using glutathione (GSH) and copper (II) chloride as precursors via a facile hydrothermal method. The catalytically active center structure of LM nanozymes was revealed, which was constructed based on the numerous copper (I) and copper (II) coordinating with thiol/amino group. The possible catalytic mechanism of LM nanozymes was also proposed. Similar to natural laccase, the prepared LM nanozymes can catalyze the oxidative coupling reaction between 2,4-dichlorophenol (2,4-DP) and 4-aminoantipyrine (4-AP) to produce an obvious red product. Compared with natural laccase, the LM nanozymes showed many outstanding advantages such as robust stability, lower cost, stronger catalytic activity and substrate affinity. Based on its excellent performances, LM nanozymes were employed as a powerful alternative to the natural enzyme in a traditional enzyme-linked immunosorbent assay (ELISA) to establish a nanozyme-based ELISA towards alpha-lactalbumin (allergenic protein). Impressively, a high-throughput and portable detection method was established by the integration of the nanozyme-based ELISA with a smartphone. The portable detection strategy achieved a limit of detection as low as 0.056 ng/mL with high specificity, and also showed excellent applicability in food sample analysis. This work not only enriches the diversities of nanozymes, but also broadens the promising applications of nanozymes in the biosensing area.

Keywords: Nanozymes, Enzyme-linked immunosorbent assay, Biosensing, Smartphone, Allergenic protein assay

1 Introduction

Enzyme-linked immunosorbent assay (ELISA) is established based on the highly specific interaction between antibody and antigen. Due to its merits of good sensitivity, simplicity, and easy operation, ELISA as an extremely powerful tool has been extensively applied in multiple fields(Wu et al. 2019b; Yalow and Berson 1959; Zhdanov et al. 2018). In conventional ELISAs, natural enzymes (e.g., alkaline phosphatase (ALP) and horseradish peroxidase (HRP)) as labels play a key role on signal generation/amplification for the sensitive detection of trace-level targets(Chen et al. 2018b). However, natural enzymes used in traditional ELISAs usually suffer from some inherent disadvantages such as low catalytic stability, high manufacturing costs, production complexity, and low catalytic activity under harsh conditions, which hinder the applicability of ELISAs to a certain extent(Farka et al. 2017; Wang et al. 2018; Wu et al. 2019b). To overcome the shortcomings of nature enzymes, great efforts have been devoted to the development of enzyme mimics with lower cost and higher stability to improve the performances of traditional ELISAs.

Nanomaterials with intrinsic enzyme-mimicking catalytic activity, namely nanozymes, have provided great opportunities for boosting the development of analytical science(Huang et al. 2019; O'Mara et al. 2019; Wu et al. 2019a). Compared with natural enzymes, nanozymes exhibit many notable virtues (e.g., robust catalytic activity, simple preparation strategies, low cost, and higher stability), which as a promising alternative to natural enzyme in traditional ELISAs greatly improve the detection performances of ELISAs, including sensitivity, stability, and accuracy(Liu et al. 2020; Wang et al. 2018; Zhang et al. 2018; Zhang et al. 2019d; Zhang et al. 2017). Up to now, a great many nanozymes such as noble metal, carbon materials, and metal organic frameworks (MOFs) have been successfully developed(Han et al. 2017; Jiang et al. 2019; Niu et al. 2019; Wu et al. 2020; Zhang et al. 2019a; Zhang et al. 2019b; Zhang et al. 2019c; Zhang et al. 2017). Although nanozyme studies have achieved great progresses over the past decade, the categories of existing nanozyme catalysis activity are relatively monotonous compared with natural enzymes, mainly including hydrolases and oxidoreductase, and especially nanozymes with oxidoreductase-mimicking catalytic activities (e.g., peroxidase and oxidase) were extensively applied in nanomaterials-based ELISAs(Wang et al. 2018). Thus, the development of novel

nanozymes with other catalytic activities is extremely significant for enriching nanozyme diversities and broadening their promising applications in analytical science. In previous studies, several laccase mimics such as Cu-containing carbon dots(Ren et al. 2015), Cu/GMP nanozyme(Liang et al. 2017), and CH-Cu nanozymes(Wang et al. 2019) have been developed. However, these laccase mimics are rarely studied as an alternative to natural enzymes in traditional ELISAs to construct an immunosensor. In this study, we successfully synthesized a new laccase mimics (namely LM nanozymes) with an excellent laccase-like catalytic activity through a convenient hydrothermal method. The strategy for the preparation of LM nanozyme was inspired via the structure of the catalytically active sites (the coordination between Cu (I)/Cu (II) and a cysteine-histidine dipeptide) in the natural laccase. On this basis, LM nanozymes were synthesized by employing the CuCl_2 and GSH as precursors. The structure of LM nanozyme was characterized by using SEM, TEM, HRTEM, FTIR, XRD, and XPS. The LM nanozyme performances including catalytic activity, substrate specificity, catalytic stability, and recyclability were assessed and compared with that of natural laccase. Furthermore, the possible catalytic mechanism of LM nanozymes was proposed. The prepared LM nanozyme exhibited an excellent laccase-like catalytic activity. And the abundant $-\text{NH}_2$ group on the surface of LM nanozymes endow them with a good biocompatibility, facilitating the modification of antibodies on nanozymes. Compared with the natural enzymes used in traditional ELISAs, the prepared LM nanozymes showed the many merits, including high catalytic stability, low manufacturing costs, easy production, and good catalytic performances under harsh conditions. Inspired by these, a novel nanozyme-mediated ELISA (nano-ELISA) was successfully established.

Food allergy is a cell- or IgE-mediated type I hypersensitivity responses caused by the humoral immune system exposed to certain life-threatening food allergens. Food allergy affects about 1-10% of the ordinary population, and has been considered as a major global public health concern and brought forward an enormous challenge to food industry(Campuzano et al. 2020; Khedri et al. 2018). Among these food allergy, milk allergy has been regarded as one of the most common incentives of allergic reactions(Høst 2002). Milk allergy is an adverse humoral immune response caused by milk proteins. The Voluntary Incidental Trace Allergen Labelling (VITAL) framework has indicated that the relatively low levels of allergens in milk still can lead to adverse immune reactions in allergic individuals(Allen et al. 2014). By now, the strict avoidance of the

milk allergens is still the most effective strategy for protecting allergic individuals from the allergic responses due to no present vaccines or treatments against hypersensitive immune reactions caused by food allergens. Though several countries have the legislation on the obligation to mark the existence of certain allergenic ingredients on the food labels, unconscious cross contamination of allergenic substances in the process of food manufacturing and transformation is still a tremendous risk factor for causing allergenic responses (Amaya-González et al. 2013). Therefore, the sensitive and reliable determination of food allergens, especially in cross-contaminated foods is of great importance for the effective management of food allergen risk. Currently, ELISA is the most common method for the determination of milk allergenic proteins (Su et al. 2019).

Herein, the prepared LM nanozymes with an intrinsic laccase-like catalytic activity were employed as a catalytic label for the construction of nano-ELISA towards alpha-lactalbumin (α -LA) (**Scheme 1**). In this nano-ELISA, LM nanozyme modified by detection antibodies played dual roles: specific recognition and signal generation. Due to its excellent laccase-like catalytic activity, LM nanozyme can catalyze the oxidation of the substrate 2,4-DP to produce an oxidation product in the presence of O_2 , facilitating a chromogenic reaction through the oxidative coupling of the generated oxidation product with 4-AP. During the process of oxidative coupling reaction, an obvious red product with the absorption band at 510 nm was produced (Wang et al. 2014). Accordingly, a novel immunoassay was established based on LM nanozymes-mediated chromogenic reaction between 2,4-DP and 4-AP. Due to its easy-to-operate, ubiquitous, and portable characteristics, smartphone have been widely used as a convenient analytical device to develop smartphone-based optical assays for the food safety field in recent years (Nelis et al. 2020). To achieve a rapid and portable detection of α -LA, a smartphone was employed as a detector and processor to couple with the nano-ELISA for establishing a new and portable integration system.

2 Experimental section

Materials and apparatus, Characterization and measurements, Preparation of the LM nanozymes, Fabrication of antibody-modified LM nanozymes, Assessment of the catalytic activity of LM nanozymes and laccase, Measurements of catalytic kinetic parameters, Evaluation of

catalytic stability, Establishment of nanozyme-based ELISA ,and Analysis of milk samples were described in detail in the Supporting Information.

3 Results and discussion

The illustration for the preparation of LM nanozymes was shown in **SI, Figure S-1**. Inspired by the structure of the active sites (the coordination between $\text{Cu}^+/\text{Cu}^{2+}$ and a cysteine-histidine dipeptide) in natural laccase, Cu^{2+} and GSH were employed as precursors to synthesize the LM nanozyme with an inherent laccase-like catalytic activity through a simple hydrothermal method. Herein, GSH serves as metal ligands to coordinate metal ions (Cu^{2+}) for the construction of LM nanozymes due to the contributions from the thiol (-SH) group and $-\text{NH}_2$ group in GSH. Due to the abundant $-\text{NH}_2$ group on their surface, the prepared LM nanozymes possess a good biocompatibility, facilitating the modification of detection antibodies on them (**SI, Figure S-1**).

3.1 Characterization of LM nanozymes

The morphology of LM nanozymes was characterized via SEM and TEM. As **Figure 1A** showed, SEM images of LM nanozymes showed its morphologies and sizes, and LM nanozymes possessed an average diameter of ~600nm. **Figure 1B** and **Figure 1C** further showed that the nanoparticles of LM nanozymes were nearly ellipsoidal. The crystal structure of LM nanozymes was further studied by using XRD characterization. As shown in **SI, Figure S-4**, the result of XRD indicated that structure of LM nanozymes was noncrystalline. And the structure of LM nanozymes was different from that of GSH, which may be ascribed to the highly flexible coordination of copper ions and the asymmetric chemical structure of GSH ligand. As **Figure 1E, F, G, H, and I** showed, EDX mappings showed that LM nanozymes contained various elements (including C, N, O, S, and Cu), which indicated that LM nanozymes were formed and the distribution of Cu element was highly dispersed. Furthermore, to further study the surface area and the pore size distribution of LM nanozymes, the nitrogen adsorption was performed and the result was calculated through the Barrett-Joyner-Halenda model (**SI, Figure S-5**). Based on the N_2 adsorption/desorption isotherms, the pore structure parameters of LM nanozymes could be well

known. The specific surface area of LM nanozymes was $9.24 \text{ m}^2/\text{g}$, and the dried LM nanozymes possessed the certain porosity larger than 25 nm (**SI, Figure S-6**). Due to its larger specific surface area and numerous pores in comparison with natural laccase, LM nanozymes have better access to the substrate and show a strong adsorption that increase the substrate concentrations around the LM nanozymes, thereby leading to the enhancement of catalytic activity.

To study the valence state of Cu elements in the prepared LM nanozymes, XPS was employed for the determination. As shown in the full scan spectrum (**SI, Figure S-7**), LM nanozymes include C, N, O, S, and Cu elements, which are consistent with results of EDX mappings, as well as the elements of the GSH ligands and Cu ions. As shown in the high-resolution XPS spectrum (**Figure 2A**), the peaks at 954.05 eV and 934.20 eV separately belong to the Cu $2p_{1/2}$ and Cu $2p_{3/2}$ electrons of Cu^{2+} , while the lower binding energy peaks at 952.07 eV ($2p_{1/2}$) and 932.28 eV ($2p_{3/2}$) demonstrated the presence of Cu^+ . To further explore the oxidation state of Cu elements in the LM nanozymes, the Cu LMM Auger spectrum was performed. As shown in **Figure 2B**, the peak fitting at 572.56 eV indicated the existence of Cu^+ , and the peak fitting at 569.08 eV showed the presence of Cu^{2+} . Moreover, the peak fitting at 566.39 eV suggested that Cu^0 atoms also existed in LM nanozymes, which may be attributed to the reduction of $\text{Cu}^+/\text{Cu}^{2+}$ via GSH. The existence of these reduced copper species may lead to the higher catalytic activity of the LM nanozymes. In addition, based on the Cu $2p$ XPS spectrum and Cu LMM Auger spectrum, it was estimated that about 55% of copper in LM nanozymes was Cu^+ and approximately 43% was Cu^{2+} . In addition, the coordination of Cu with S was also further studied by recording the S $2p$ XPS spectrum of LM nanozymes. As **Figure 2C** shows, the peak at 161.37 eV belongs to S-Cu, which demonstrated that the coordination of Cu with S exists in the LM nanozymes. According to the peak at 162.35 eV , it was found that $\sim 44\%$ of -SH kept the original chemical state. Moreover, S^0 was also discovered in the LM nanozymes, which may be produced by the oxidation of Cu^{2+} . Due to the higher content of Cu^+ (55%) in the LM nanozymes, the content of S- Cu^+ is speculated to be higher than that of S- Cu^{2+} .

To study the bonding structure status in the LM nanozymes, LM nanozymes and GSH were determined by using FTIR spectral. The results of FTIR spectral analysis were shown in **Figure 2D and SI, Table S1**. The stretching vibrations at 3347.6 cm^{-1} and 3435.4 cm^{-1} indicate the presence of an $-\text{NH}_2$ group in GSH and LM nanozymes, respectively. The existence of peptide

bonds is indicated via the stretching vibration of C=O at 1623.6 cm^{-1} and 1605.3 cm^{-1} , C-N at 1245.3 cm^{-1} and 1185.4 cm^{-1} , and -NH- at 3141.2 cm^{-1} and 3242.6 cm^{-1} in the GSH and LM nanozymes, respectively. The carboxyl group in GSH and LM nanozymes is separately demonstrated by the -OH stretching vibration at 2863.2 cm^{-1} and 2908.3 cm^{-1} . Furthermore, the stretching vibrations at 2574.6 cm^{-1} and 2608.5 cm^{-1} demonstrate the presence of -SH in the GSH and LM nanozymes, respectively. The results of FTIR spectral analysis were roughly consistent with the study previously reported (Wang et al. 2019). Based on the FTIR spectra analysis results, it is found that the FTIR spectra of LM nanozymes have a slight difference with that of GSH, which may be attributed to the combination of Cu ions with GSH molecules during the preparation of LM nanozymes.

3.2 Catalytic mechanism of LM nanozymes

Natural laccase can catalyze the single-electron oxidation reaction of substrates, and O_2 molecular is reduced to H_2O through four-electron reduction reaction. In this process, the redox reactivity of Cu cluster active sites in laccase plays an extremely important role on transferring the electrons from the substrate to O_2 (Solomon et al. 1996). Laccase as one of multi-copper oxidases can oxidize some environmentally important substrates such as phenolic compounds. Similar to the natural laccase, LM nanozymes can also catalyze the phenolic compound (e.g., 2,4-DP). By now, the catalytic mechanism of natural laccase has been proposed by Solomon's group (Hadt et al. 2014). Briefly, laccases include four copper sites (i.e., Type 1 (T1), Type 2 (T2), and binuclear Type 3 (T3) Cu sites), the substrate firstly combines to the active sites of natural laccase and subsequently the substrate oxidation occurs near the T1 Cu, and the electrons transfer to the T2/T3 trinuclear copper clusters (where the reduction of dioxygen occurs) through Cys-His pathway. Moreover, Cys-His has been considered as the electron-transfer (ET) bridge in the laccase. Furthermore, it was proven that the electrons probably transfer from the Cu^+ to Cu^{2+} through the Cys-His pathway in natural laccase due to the higher redox potential of Cu^+/Cu^0 than $\text{Cu}^{2+}/\text{Cu}^+$. On this basis, we proposed that the Cu^+ is mainly bound to the thiol group (-SH) by a chemical binding and the Cu^{2+} is mainly bound to the $-\text{NH}_2$ group via the coordination in LM nanozymes.

To further explore the intrinsic catalytic mechanism of LM nanozymes, the 2,4-DP was

employed as a phenolic substrate and the catalytically active center of the LM nanozymes was used. In the active center of LM nanozymes, the Cu^{I} is bound to the -SH group via chemical binding due to the reducibility of -SH and the Cu^{II} is bound to the $-\text{NH}_2$ group via a coordinate bond. Significantly, a possible catalytic mechanism of LM nanozymes was proposed according to the catalytic mechanism of natural laccase. As shown in **Figure 3**, the catalytic mechanism of LM nanozymes mainly includes four processes (i.e., (i) the substrate binding with LM nanozymes, (ii) the substrate oxidation around the Cu^{I} -S sites, (iii) electron transfer, and (iv) O_2 reduction around the Cu^{II} -N sites.). Briefly, the phenolic substrate 2,4-DP is oxidized via the Cu^{I} owing to the higher oxidation ability of Cu^{I} than Cu^{II} . During the substrate oxidation, the phenolic hydroxyl radical produced in this process was confirmed by the EPR spectra with 5, 5-dimethyl-1-pyridine N-oxide (DMPO) as a spin trap (**SI, Figure S-8**). Meantime, Cu^{I} serves as the primary electron acceptor site with single-electron oxidation in the LM nanozymes, and the electrons are transferred from Cu^{I} to Cu^{II} by the electron transfer pathway containing through-space interactions (saturated bonds), covalent bonds, and H-bonds. Moreover, the highly covalent Cu^{I} -S bonds in LM nanozymes facilitate a fast electron transfer. After getting electrons, the Cu^{II} is converted to Cu^{I} . However, due to the lack of protection from the circumambient chemical groups, the Cu^{I} is oxidized into Cu^{II} via O_2 . During this process, O_2 molecular as the final electron acceptor is reduced to H_2O through a four-electron reduction reaction, and simultaneously the LM nanozymes are regenerated.

3.3 Assessment of the catalytic activity of LM nanozymes

As previously reported, the catalytic activity of LM nanozymes and laccase were measured through the chromogenic reaction generated from the oxidative coupling of phenolic compounds with 4-AP (**Figure 4A**) (Liang et al. 2017; Wang et al. 2019). As the UV-Vis spectra shown in **Figure 4B**, neither 2,4-DP nor 4-AP has no absorption peak at 510 nm (in the visible region). Moreover, the mixture of 2,4-DP and 4-AP also have no absorption peak at 510 nm. Upon the addition of LM nanozymes or laccase, the oxidation product of 2,4-DP (the real substrate) reacts with 4-AP to produce a red product with the absorption band at 510 nm.

The free Cu^{2+} could also catalyze the chromogenic reaction between 2,4-DP and 4-AP

(**Figure 4C**), and thus a control experiment was simply designed (Liang et al. 2017). In brief, the aqueous dispersion of LM nanozyme was separated by the centrifugation (10,000 rpm, 5 min) to obtain the precipitants and supernatant. The supernatant and sediment were added into the mixture of 2,4-DP and 4-AP to react for 1h, respectively. As shown in **SI, Figure S-9**, the mixture reacted with supernatant showed a light pink color, which demonstrated that free Cu^{2+} has a moderate catalytic activity. While the mixture reacted with precipitants exhibited a dark red color, which indicated LM nanozyme possessed a high catalytic activity. The above results confirmed that the higher laccase-like catalytic activity originated from LM nanozymes instead of the free Cu^{2+} . Effect of different pH values on the catalytic activity of LM nanozymes was measured. According to the absorption values at different pH shown in **SI, Figure S-10**, LM nanozymes possess the highest activity at pH=6.8, and laccase also exhibits a high catalytic activity at the same pH value. Thus, MES buffer (pH=6.8) was chosen for subsequent catalytic reactions, facilitating the comparison of LM nanozymes with natural laccase.

Obviously, laccase can directly catalyze the reduction of O_2 molecular into H_2O without the generation of H_2O_2 . Similarly, LM nanozymes was also confirmed to have the same performances with natural laccase (**SI, Figure S-11**). In comparison, some oxidases like glucose oxidase (GOD) oxidize the substrates by reducing O_2 to H_2O_2 . To confirm the generated reduction products of O_2 catalyzed by LM nanozymes, the 2,4-DP was reacted with LM nanozymes (4-AP was not added for avoiding the interference from the red color). Then, the ABTS and HRP were rapidly added into the supernatant, and there were no color variation or absorption observed. Subsequently, with the addition of H_2O_2 into the above mixture, the solution immediately turned to be green color, as **SI, Figure S-12** showed. Thus, LM nanozymes is a laccase mimic rather than other oxidase mimics that can generate H_2O_2 .

To further study the catalytic activity of LM nanozymes, the catalytic kinetic parameters of laccase and LM nanozyme were measured with various substrate concentrations. With the Michaelis-Menten model, K_m and v_{max} values of laccase and LM nanozymes were obtained (**SI, Table S2**). The value of K_m demonstrates the affinity of enzymes towards the substrate, and a lower value of K_m shows that enzymes possess a stronger affinity for substrates. The K_m value of LM nanozymes was 0.15 mM and lower than that of laccase (0.43 mM), which may be attributed to the numerous pores on the LM nanozymes. The v_{max} value of LM nanozyme was 8.26×10^{-3} mM

min⁻¹, which was higher than that of laccase (6.63×10^{-3} mM min⁻¹). The v_{max} and K_m values demonstrated that the LM nanozymes possessed a higher catalytic activity compared with laccase. The values of K_{cat} and K_{cat}/K_m indicate the catalytic efficiency of the nanozymes or enzyme (Jiang et al. 2018). The LM nanozymes possessed much higher K_{cat} (3.06×10^4 min⁻¹) and K_{cat}/K_m (2.04×10^5 mM⁻¹min⁻¹) values than that of laccase, which demonstrated that LM nanozymes had a higher catalytic efficiency and possessed much more catalytically active sites than laccase. As previously reported, CH-Cu nanozymes with laccase-like activity were also confirmed with a similar result ($K_{cat}=1.91 \times 10^4$ min⁻¹) (Wang et al. 2019).

3.4 Evaluation of catalytic stability and recyclability

Due to the high catalytic activities and outstanding substrate specificity, enzymes have been extensively applied in multiple areas (Liang et al. 2020). However, harsh catalysis conditions (e.g., high temperature, extreme pH, and organic solvents) severely restricted their practical applications (Lian et al. 2017). By now, great efforts have been devoted to improving their stability and recyclability. The immobilization of enzymes onto a support (MOFs, covalent organic frameworks (COFs), mesoporous silica, etc.) has been used for the enhancement of stability and recyclability (Kandambeth et al. 2015; Li et al. 2016; Sun et al. 2018). Furthermore, biomineralization has also been widely applied in the preparation of hybrid organic-inorganic nanoflowers to improve the stability and recyclability of natural enzymes (Kong et al. 2018). In addition, emerging nanozymes have been proved to possess a high stability and good recyclability (Wu et al. 2019a; Zhang et al. 2020). After evaluating the catalytic activity of LM nanozymes, the stability at various pH, temperature, ionic strength, organic solvent, and storage time, as well as the recyclability were also evaluated.

First of all, LM nanozymes and laccase were separately incubated in the buffers with a wide pH ranging from 3.0 to 9.0 at 25 °C for 8 h. Subsequently, the activities of LM nanozyme and laccase in the reaction buffer (pH=6.8) at 25 °C for 1 h were individually determined (**SI, Figure S-13A**). After incubating in the buffers (pH=3.0 and pH=9.0), laccase lost about 70% of catalytic activity. However, LM nanozymes retained at least 65% of activity and approximately 85% catalytic activity in pH=3.0 and 9.0 buffers. Then, influence of temperature on the activities of LM

nanozymes was studied. LM nanozymes and laccase were incubated in different temperatures ranging from 0 to 100 °C for 1 h. As shown in **(SI, Figure S-13B)**, laccase almost lost its whole catalytic activity at 90 °C, while LM nanozymes maintained approximately 80% of catalytic activity at same conditions. Moreover, LM nanozymes exhibited excellent thermal stability from 0 to 90 °C.

Effects of ionic strength on the activities of LM nanozymes and laccase were also further studied **(SI, Figure S-13C)**. With the increase of NaCl concentration, the activity of laccase showed a dramatic decline. Moreover, the catalytic activity of laccase could be completely suppressed by the NaCl solution with high concentrations. The results were attributed to the influence of high ionic strength of the spatial structure and charge distribution of laccase, which lead to the salting-out effect and thereby reduced the solubility of the enzyme. In addition, chloride ions can bind to the type 2/type 3 (T2/T3) trinuclear copper sites of laccase, resulting in the loss of its catalytic activity (Xu 1996). However, the activity of LM nanozymes continuously increased with the enhancement of NaCl concentration, and showed an enhancement of approximately 300% in 600 mM NaCl. For exploring the role NaCl played on the catalytic activity of LM nanozymes, the adsorption values of substrate with LM nanozymes with the existence and absence of NaCl were measured. In short, the same amount of substrate (2,4-DP) aqueous dispersion was added into 1 mg/mL of LM nanozyme aqueous dispersion or 1 mg/mL of LM nanozyme aqueous dispersion containing 4 M NaCl. The mixture was incubated for 5 h at 25 °C, and the adsorption at 284 nm of the supernatant was measured after centrifugation. As **SI, Figure S-14** showed, the absorbance value of 2,4-DP at 284 nm in LM nanozymes dispersion was slightly below that of 2,4-DP in aqueous solution. However, the absorbance value for LM nanozyme dispersion containing the NaCl is approximately 67% of that without NaCl, demonstrating the adsorption of about 33% of 2,4-DP on the LM nanozymes with the existence of NaCl. The results indicated that the presence of NaCl can promote the adsorption of 2,4-DP on the LM nanozymes and accordingly improve its catalytic activity. In addition, **SI, Table S3** showed that the zeta potential of LM nanozymes increased from -0.85 mV to -3.41 mV after adding NaCl, which indicated that the addition of NaCl increased the charge of LM nanozymes and thus promoted the improvement of catalytic activity of LM nanozymes.

To study the influence of solvent on the activities of LM nanozymes and laccase, different

amounts of ethanol (0, 30%, 50%, 70%, and 100% v/v) were added into the reactions. As shown in **SI, Figure S-13D**, the activities of LM nanozymes and laccase decreased with the enhancement of the amount of ethanol. When 100% ethanol was mixed, the catalytic activity of laccase was completely suppressed. After that, O₂ was pumped in the mixed solution after reaction to verify that the ethanol hindered the catalytic reaction instead of the absence of O₂. However, LM nanozymes kept approximately 70% of catalytic activity at same conditions.

The long-term storage stability of enzyme is extremely important in practical application(Chen et al. 2018a). As **SI, Figure S-13E** shows, the activity of laccase continuously declined after being stored in aqueous solution at 25 °C, and completely lost its catalytic activity in the 10th day. Nevertheless, LM nanozymes maintained more than 80% of catalytic activity after being stored in water for 14 days. To investigate the recyclability of the LM nanozymes, 4-AP and 2,4-DP were added into the MES buffer (pH=6.8), and then incubated at 25 °C for 1 h after LM nanozymes (1 mg/mL) was mixed with the mixture. The absorbance of supernatant at 510 nm was tested after 1 h of the chromogenic reaction for per cycle. The LM nanozymes were collected for the next reaction cycle through centrifugation (10,000 rpm, 5 min) and washed three times with deionized water after per cycle. As **SI, Figure S-13F** shows, LM nanozymes retained approximately 70% of catalytic activity after ten cycles. Compared with the natural laccase, LM nanozymes exhibited a higher catalytic stability and recyclability.

3.5 Optimization of nano-ELISA conditions

To achieve the best detection performances of nano-ELISA, the immunoassay conditions were optimized. The amount of anti α -LA (pAb) integrated with LM nanozymes can influence the catalytic activity of LM nanozymes, which thereby affects detection performances of nano-ELISA. Thus, the effects of amount of anti α -LA integrated with LM nanozymes on detection performances of nano-ELISA were studied. First of all, the successful integration of the anti α -LA with LM nanozymes was verified. **SI, Figure S-15A** showed that it had no absorbance when no anti α -LA was integrated with LM nanozymes and it possessed a remarkable absorbance at 510 nm when the anti α -LA was integrated with LM nanozymes, indicating that the anti α -LA was successfully modified on LM nanozymes. According to **SI, Figure S-15A**, it was found that the absorbance

values significantly enhanced with the increase of anti α -LA concentration, while the absorbance slightly down when anti α -LA concentration reach 200 $\mu\text{g/mL}$. The enhanced absorbance may be owing to the existence of much more anti α -LA that can endow anti α -LA@LM nanozymes with the higher capture efficiency towards targets. However, the slight descent of absorbance values may be attributed to the excess anti α -LA that hinders the binding between the catalytically active sites of LM nanozymes and substrate. To obtain the best sensitivity of nano-ELISA, the anti α -LA@LM nanozymes prepared with 200 $\mu\text{g/mL}$ of anti α -LA were applied in the following immunoassay. For further exploring immunoassay conditions, the immunoassay with antigen was set as experimental group and the immunoassay in the absence of antigen served as a control group. As shown in **SI, Figure S-16A**, the absorbance values of the experimental group increase with the enhancement of capture antibody concentration. When the capture antibody concentration attained 10 $\mu\text{g/mL}$, the absorbance values in the experimental group almost kept constant. Nevertheless, the absorbance was not observed in the control group at the same conditions. Above results demonstrated that the 10 $\mu\text{g/mL}$ of capture antibody can meet the microplate coating for the immunoassay.

In addition, to avoid generating false-positive results, the conditions including blocking agent amount, anti α -LA@LM nanozyme concentration, and incubation time were optimized. **SI, Figure S-16B** and **16C** indicated that the false-positive results can be obviously observed when the anti α -LA@LM nanozyme concentration and blocking agent amount exceed 12 $\mu\text{g/mL}$ and 1% BSA, respectively. Above results may be owing to the nonspecific binding of detection antibody with the blocking agent. Therefore, 12 $\mu\text{g/mL}$ of anti α -LA@LM nanozymes and 1% BSA were employed in the following experiments. Similarly, **SI, Figure S-16D** indicated the false-positive result occurred when the incubation time attained 1 h, which may be attributed to that the longer incubation time can cause much more nonspecific binding. Thus, the optimal incubation time was set as 1 h.

Highly catalytic activity of nanozyme is very critical for the improvement of nano-ELISA performances(Zhang et al. 2019d). To achieve the excellent detection performances of the immunoassay, the parameters (i.e., reaction time and temperature) of the catalytic reaction are optimized using response surface methodology (RSM) based on a Central Composite Design approach. RSM can be used to evaluate the interactive effects among the tested factors, which can

offer more suitable catalytic reaction conditions for the LM nanozymes during immunoassay compared with a commonly used single factor experiment (Su et al. 2019). According to the experimental data, a regression equation was established for predicting the optimum points. The predicted second-order polynomial model is shown as follows:

$$Y = 0.84 + 0.3x_1 - 0.026x_2 - 0.042x_1x_2 - 0.20x_1^2 - 0.25x_2^2$$

where Y represents the absorbance value, x_1 and x_2 represent the catalytic reaction time and temperature during the immunoassay, respectively. **SI, Figure S-15B** showed three-dimensional response surface curves. The analysis of variance (ANOVA) exhibited that the fitness was significant at the level of $p < 0.0001$. Moreover, the R^2 value that expresses the variability of the response was 0.9682, which demonstrated that the experimental value is almost consistent with the predicted value. Based on the RSM analysis, the optimal conditions were set as the 30 min of catalytic reaction time at 65 °C. With the proposed optimal conditions, the absorbance value maximum was predicted to be 0.842. To verify the suitability of the proposed conditions, six experiments were carried out and the average absorbance value was 0.836, which showed the feasibility that the response model for reflecting the expected optimal conditions. Based on above results, the parameters (i.e., reaction time (30 min) and temperature (65 °C)) are used in the following experiments.

3.6 Evaluation of nano-ELISA performances

Under the optimal conditions, the performances of the established nano-ELISA were evaluated by employing α -LA as a target analyte. With the existence of α -LA with various concentrations, the absorbance of the nano-ELISA based on LM nanozymes was tested by using UV-Vis spectra. As shown in **Figure 5A**, the absorbance gradually increased with the increased concentration of α -LA from 0 to 1000 ng/mL, which indicated that the developed nano-ELISA can be used for the detection of α -LA. As **Figure 5B** showed, the calibration curve indicated a linear relationship ($R^2=0.9956$) between the absorbance at 510 nm and the concentration (a wide range of 0.06-400 ng/mL) of α -LA. The limit of detection (LOD) toward α -LA was estimated as 0.01 ng/mL (S/N=3:1). The newly developed method was compared with the previously reported method for α -LA assay in terms of linear detection range and LOD. As shown in **SI, Table S4**, the

linear detection range and LOD of the established method showed superior performances, indicating a great potential in the detection of α -LA.

The specificity of the established nano-ELISA towards various non-target proteins was also evaluated by measuring the cross-reactivity with various allergenic proteins (i.e., β -LG, κ -CN, α -CN, β -CN, soybean protein, wheat protein, peanut protein, and egg protein) at the concentrations of 0.1, 1, and 10 μ g/mL. **Figure 6A** indicated that soybean proteins, wheat proteins, peanut proteins, egg proteins, and BSA exhibited no cross-reactivity with α -LA. Nevertheless, β -LG and caseins (κ -CN, α -CN, and β -CN) showed the cross-reactivity with the detection and capture antibodies, which may be owing to the residue of α -LA in β -LG and caseins. To evaluate the storage stability of the as-prepared nanozyme labels, 10 μ g/mL of α -LA was employed as the target. **Figure 6B** showed that the biological activity of anti α -LA@LM nanozymes still maintained about 80% of the initial biological activity when stored at 4 °C for 35 days, indicating the high stability of the nanozyme labels.

Recovery test was carried out via separately spiking 1 and 5 mg/mL of α -LA in raw cow milk, UHT cow milk, and yogurt and separately spiking 1 and 5 mg/g in the candy, hydrolyzed infant formula (EHF), and partial hydrolyzed infant formula (PHF). Calculation of recovery was achieved according to following formula:

$$R = \frac{C_2 - C_1}{C_3} \times 100\%$$

where, R, C_1 , C_2 , and C_3 represent the recovery, sample concentration, the spiked sample concentration, and the standard concentration of spiked α -LA, respectively.

According to the **SI, Table S5**, it can be seen that the average recoveries of α -LA from spiked raw cow milk, UHT cow milk, yogurt, candy, EHF, and PHF were 100.04, 101.17, 99.24, 97.64, 96.51, and 99.48%, respectively. Moreover, the relative standard deviation (RSD) ranged from 0.47% to 4.12%. Based on above results, it is confirmed that the established nano-ELISA can be used to determine the trace-level α -LA in real food samples with a high recovery.

3.7 Smartphone-based integration system for the α -LA detection

To achieve a portable detection towards α -LA, nanozymes-mediated ELISA was integrated with a smartphone. The α -LA immunoassay in microplates was analyzed by using the

Spotxcel[®] Reader (Sicasys Software GmbH, Germany) installed on a smartphone. The camera and touch-screen of smartphone served as an image capturing device and a control interface, respectively. As shown in **SI, Figure S-17A**, smartphone-based immunosensor was performed mainly including the following three steps: (i) Image capture; (ii) Signal detection and quantification; (iii) Assay analysis. Briefly, firstly, the nanozymes-mediated immunosensor was put into a light box with two LED lamps. Then, smartphone was fixed with a distance (15 cm) from the microplate using a holder. Image was captured by using a smartphone built-in camera at the highest possible resolution and then the image of the microplate was saved to a file. Herein, the pixel of Iphone 6s plus could meet the requirements of the detection, and thus the smartphone with a better camera pixel could also be used for the detection. Subsequently, the color signal in each well was determined by image analysis and the measured color intensity was reported. The normalized color intensity (the value ranging from 0 to 100) reported by the app was employed for supporting various color scales and measures, and the maximum color intensity value of assay was set as 70. After detecting and quantifying the signal of wells, assay setup was carried out, including the microplate annotation, the review of the measured color intensity, and standard concentration setting. Finally, the assay analysis was performed with standard curve. The proposed smartphone-based integration system was employed for the detection of α -LA with a concentration range of 0-1000 ng/mL. As shown in **SI, Figure S-17B**, six repetitions of readout results showed that the α -LA could be detected in a liner range of 0.12-3.46 ng/mL ($R^2=0.9916$) with a LOD of 0.056 ng/mL by using the established smartphone-based integration system.

3.8 Determination of allergenic proteins in food samples

Inspired by the outstanding detection performances of the developed nano-ELISA, various food samples were used for the analysis of α -LA. As shown in **SI, Table S5**, the contents of α -LA in spiked raw cow milk, UHT cow milk, yogurt, and candy were 1.13 mg/mL, 0.48 mg/mL, 0.23 mg/mL, and 0 mg/g, respectively. The content of α -LA in raw cow milk was found to be 1.13 mg/mL by using the developed method, which was almost consistent with the previous literature that reported the α -LA content in the mature bovine milk (approximately 1.2 g/L)(Kamau et al. 2010). However, the lower content of α -LA was found in UHT cow milk, which was due to the

α -LA molecule unfolding caused by the heat-denaturation during the processing (Ruiz-Valdepeñas Montiel et al. 2016). Thus, the determination of α -LA content was also used to evaluate the heat treatment of milk. In addition, the allergenic residues (α -LA) in protein hydrolyzed infant formulas were also determined. According to the proteolysis degree, protein hydrolyzed infant formulas were divided into two types (i.e., EHF and PHF). **SI, Table S5** indicated that the contents of α -LA in EHF and PHF were 0 mg/g and 0.34 mg/g, respectively. Compared with PHF, EHF is more suitable for the infants who are allergic to cow milk proteins and suffer from severe enteritis because it contains the mixture of short peptides, dipeptides, tripeptides, and free amino acids which produced from the deep hydrolysis of protein. Overall, the above detection results of α -LA in food samples (**SI, Table S5**) showed that the proposed LM nanozyme-mediated ELISA had the reliable accuracy and outstanding applicability in the determination of α -LA residue in real food samples.

4 Conclusions

To sum up, a new LM nanozymes with excellent laccase-like activity has been successfully prepared through a simple strategy inspired by the structure of the catalytic activity center of natural laccase. The prepared LM nanozymes exhibits higher catalytic activity and better stability than natural laccase, and shows a good recyclability. Moreover, by employing 2,4-DP as a substrate, a possible catalytic mechanism involving LM nanozymes was proposed. GSH and CuCl_2 serve as precursors for the synthesis of LM nanozymes, which are cost-efficient, easily available, and environmentally friendly. Furthermore, the LM nanozymes prepared using GSH and CuCl_2 have abundant $-\text{NH}_2$ group on their surface, which endows them with a good biocompatibility and thus facilitates the modification of antibodies on nanozymes to construct a nanozymes-based ELISA. Utilizing the superior performances of LM nanozymes, a novel nanozyme-based ELISA for the sensitive and specific detection of α -LA was developed through a LM nanozymes-mediated chromogenic reaction between 2,4-DP and 4-AP. The integration system of nanozyme-based immunoassay with a smartphone provided a rapid, reliable, and sensitive detection of α -LA with a LOD as low as 0.056 ng/mL, which also performed well in food samples. In addition, this work provides a general strategy (i.e., key peptides coordinating metal ions) for

the development of new nanozymes by mimicking the structure of the active center of other natural enzymes, and broadens their applications in the portable assay of trace analytes.

Acknowledgments

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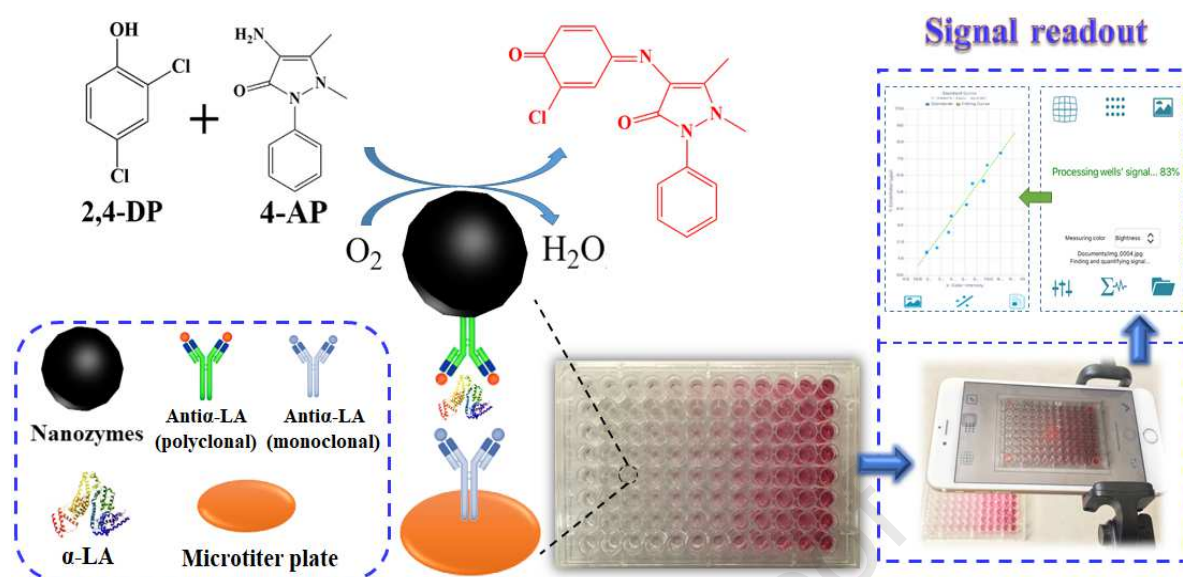
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Scheme 1. Schematic illustration of the integration system of nanozyme-based immunoassay with a smartphone for the detection of α-LA.

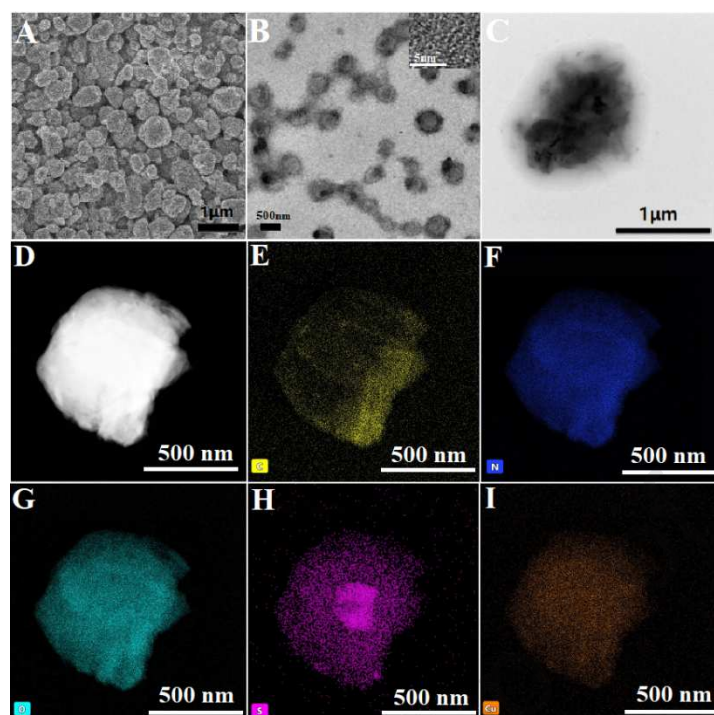


Figure 1. SEM images (A), TEM images (B, C), HAADF image (D), and elemental mapping (E, C element; F, N element; G, O element; H, S element; and I, Cu element.) of LM nanozymes. The inset in (B) was the HRTEM image of LM nanozymes.

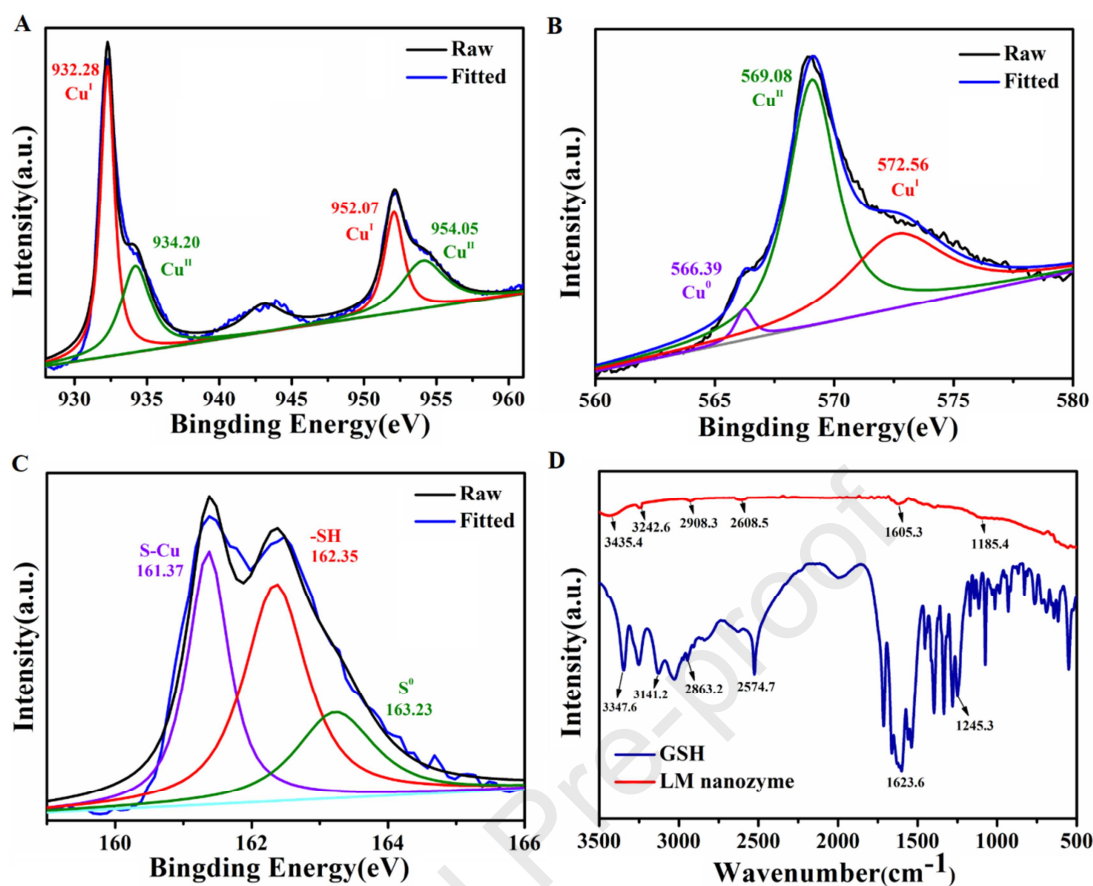


Figure 2. For LM nanozymes: (A) Cu 2p XPS spectrum, (B) Cu LMM Auger spectrum, (C) S 2p XPS spectrum, (D) FTIR spectrum and one for GSH. In (A) and (B), green is peaks of Cu^{2+} and the red is peaks of Cu^+ . In (C), green is peaks of S^0 , the red is peaks of -SH, and the purple is peaks of S-Cu. These peaks were obtained using XPS peak-differentiating-imitating analysis.

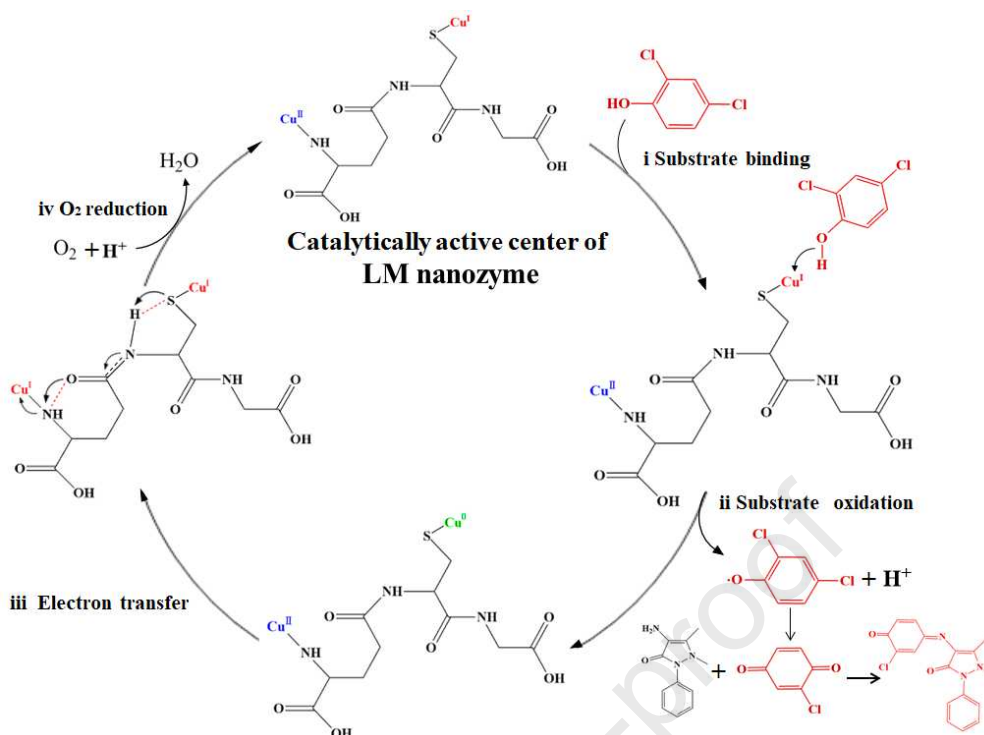


Figure 3. Possible catalytic mechanism of the LM nanozymes (red dotted lines represent H-bonds and black dotted lines represent through-space interactions).

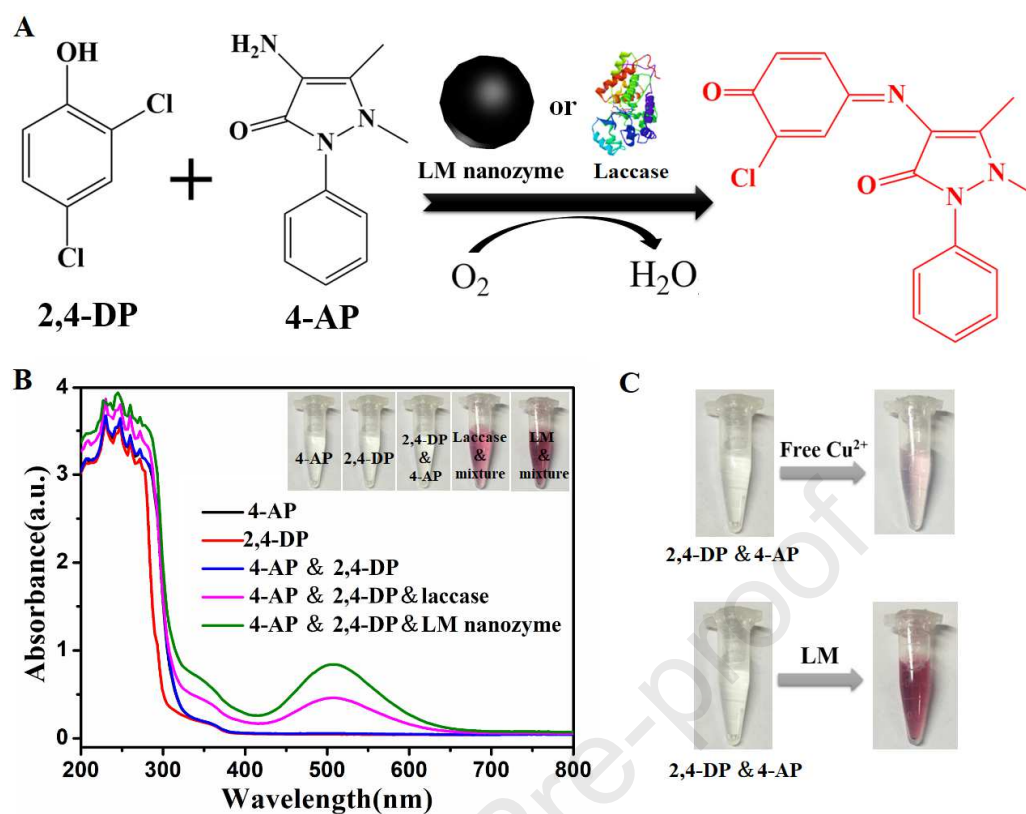


Figure 4. (A) Reaction of 2,4-DP and 4-AP catalyzed by LM nanozymes or laccase. (B) UV-Vis spectra of the 4-AP, 2,4-DP, two substrate mixture, and their reaction product in the presence of laccase or LM nanozymes. (C) Control experiment for the comparison of free Cu^{2+} with LM nanozymes in laccase-like catalytic activity.

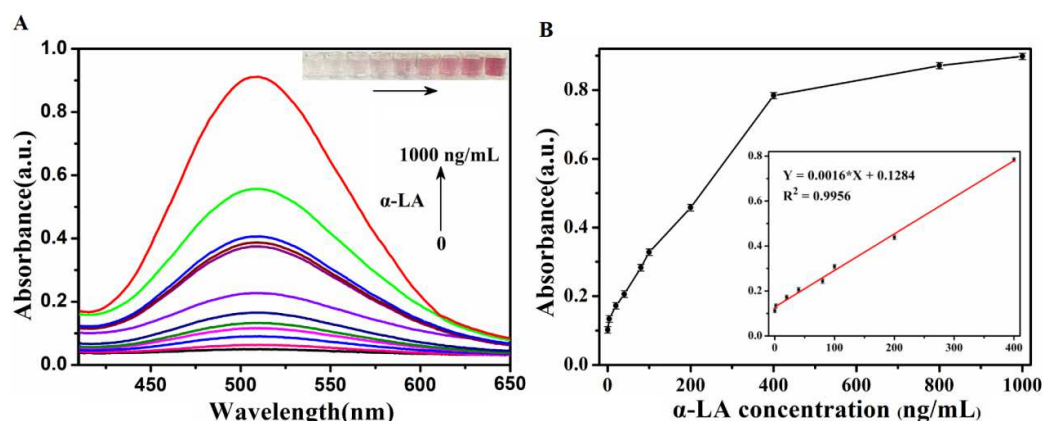


Figure 5. Absorption spectra (A) and absorption intensities at 510 nm (B) of chromogenic reaction in the LM nanozymes-based immunoassays towards α -LA at different concentrations. The inset in (A) showed the photographs of the LM nanozymes-based immunoassays with the existence of α -LA at the concentrations of 0, 0.2, 2, 20, 40, 80, 100, 200, and 400 ng/mL. The inset in (B) showed the calibration curve of the absorbance at 510 nm at versus α -LA concentration.

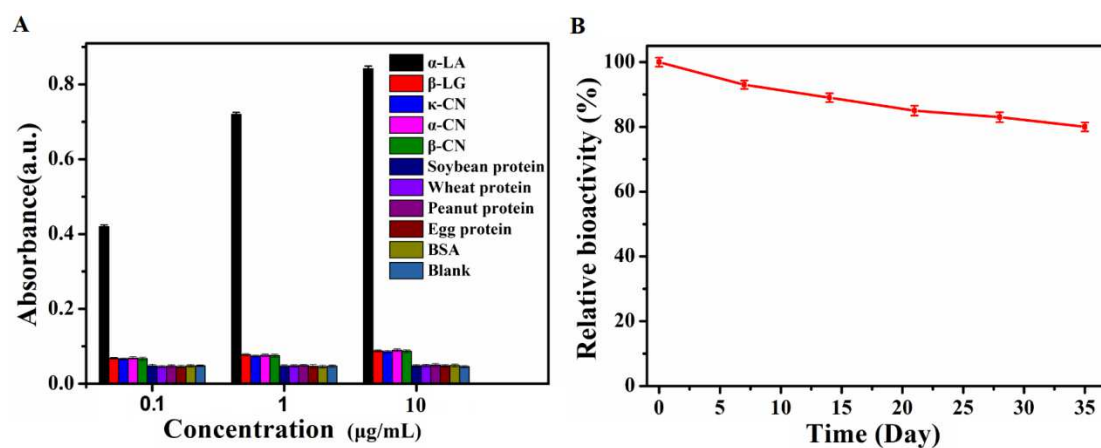


Figure 6. Cross-reactivity of the established method towards α -LA with β -LG, κ -CN, α -CN, β -CN, soybean proteins, wheat proteins, peanut proteins, egg proteins, and BSA. (B) Changes in the bioactivities of anti- α -LA@LM nanozymes after being stored for different time at 4 °C.

Highlights

Bioinspired nanozymes with laccase-like catalytic activity were prepared

The catalytic mechanism of laccase-mimicking nanozymes was proposed

Nanozyme-based enzyme-linked immunosorbent assay was constructed

A portable smartphone-based allergenic protein detection system was established

The authors declared that they have no conflicts of interest to this work.

Journal Pre-proof

Supporting Information

Bioinspired Nanozyme for Portable Immunoassay of Allergenic Proteins Based on A Smartphone

Materials and apparatus

Bovine serum albumin (BSA), alpha-lactalbumin (α -LA), β -lactoglobulin (β -LG), κ -casein (κ -CN), α -casein (α -CN), wheat protein, soybean protein, egg protein, peanut protein, β -casein (β -CN), laccase, and horseradish peroxidase (HRP) were purchased from yuanye Bio-Technology Co. Ltd. (Shanghai, China). Polyclonal antibody (pAb) to α -LA was purchased from Cloud-Clone Corp (Wuhan, China), and monoclonal antibody (mAb) to α -LA was obtained from Bioss Antibodies (Beijing, China). N, N-dimethylformamide (DMF), 4-aminoantipyrine (4-AP), 2, 4-dichlorophenol (2,4-DP), 2-(N-morpholino) ethanesulfonic acid (MES) monohydrate, glutathione (GSH), Tween 20, N-ethyl-N'-(3-(dimethylamino) propyl)-carbodiimide hydrochloride (EDC), N-Hydroxysuccinimide sodium salt (NHS), copper (II) chloride dehydrate, and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Aladdin Industrial Corp (Shanghai, China). Raw cow milk, UHT cow milk, yogurt, infant formulas, and candy were obtained from the local supermarkets. The 96-well polystyrene plate was obtained from Jincanhua (Shenzhen, China). The other reagents were all of analytical grade and used without further purification. All solutions and buffers were prepared with Milli-Q water.

Characterization and measurements

Transmission electron microscope (TEM) images were recorded by using a Talos F2100X G20 (Thermo Scientific, USA). SEM images were obtained with a JSM-7500F (JEOL, Japan). The energy dispersive X-ray spectroscopy (EDX) mapping was carried out using an X-Max (Oxford, UK). X-ray photoelectron spectroscopy (XPS) was performed on an Escalab 250Xi (Thermo Fisher, USA). The X-ray diffraction (XRD) curve was obtained with a Bruker D8 (Bruker, Germany). Mapping was carried out with X-Max (Oxford, UK). FTIR spectra were performed using a Nicolet-iS10 (Thermo Scientific, USA). Brunauer-Emmett-Teller (BET)

specific surface area was measured at 77 K under N₂ sorption using a TriStar II Plus 2.02 physical equipment (Micromeritics, USA). Zeta potentials were measured using a Litesizer 500 (Anton Paar, Austria). Ultraviolet (UV) measurement was carried out with a Thermo evolution 201 (Thermo Scientific, USA). **EPR spectra were measured with a Bruker E500 (Bruker, Germany).** The absorbance of the wells of 96-well polystyrene plate was measured with a Multiskan (Thermo Scientific, USA). **Images were obtained with an Iphone 6s plus smartphone camera, which could meet the requirements of the detection.** Signal detection, quantification, and assay analysis were achieved using a commercially-available Spotxel® Reader (Sicasys Software GmbH, Germany), which was purchased from the App store and installed on an Iphone 6s plus smartphone.

Preparation of the LM nanozymes

LM nanozymes were prepared using a convenient hydrothermal method, and synthesis conditions (i.e., temperature and time) were further optimized (**Figure S-2 in Supporting Information (SI)**). In addition, the CuCl₂ concentration for the nanozyme synthesis was also optimized (**SI, Figure S-3**). Under the optimized conditions, 1 mL of aqueous CuCl₂ solution (0.24 M), 2 mL of aqueous GSH solution (0.12 M), and 4 mL of DMF were continuously stirred for 5 min at room temperature, and then the mixture of solution was transferred into a 10 mL teflon-lined stainless-steel autoclave. The autoclave was heated at 140 °C for 4.5 h, and subsequently it was cooled to room temperature. After that, LM nanozymes were collected through centrifugation (10,000 rpm, 5 min), and the process of collection and washing with DMF and ultrapure water was repeated three times for the removal of the redundant reactant. The obtained product was dried at 45 °C under vacuum for further use.

Fabrication of antibody-modified LM nanozymes

To prepare LM nanozyme@anti α -LA (pAb), the coupling reagents EDC (1 mg) and NHS (1 mg) were added into 1 mg/mL LM nanozymes under vortexing, and the mixture were incubated with a vigorous shaking (200 rpm, 30 min) at 37 °C. Afterward, LM nanozymes were separated by centrifugation (10,000 rpm, 5 min) and washed with deionized water twice to remove the excess EDC and NHS. The amino-functionalized LM nanozymes were dispersed in 1 mL phosphate

buffered saline (PBS, 10 mM, pH=7.2). Subsequently, the pAb was added into the above dispersion and then incubated in a shaker (200 rpm, 30 min) at 37 °C. After that, 1% BSA was added into the solution to block the residual binding sites on the surface of LM nanozymes. Finally, the unconjugated pAb was removed through centrifugation (10,000 rpm, 5 min), and the nanozyme tags were re-dispersed in PBS solution (PBS, 10 mM, pH 7.2) for further use.

Assessment of the catalytic activity of LM nanozymes and laccase

The catalytic activity of LM nanozymes and laccase was evaluated through the chromogenic reaction produced from the oxidative coupling of phenolic compounds with 4-AP. In brief, 100 µL of aqueous 2,4-DP solution (1 mg/mL) and 100 µL of aqueous 4-AP solution (1 mg/mL) were added into the 700 µL of MES buffer (30 mM, pH 6.8) and then were homogeneously mixed. Subsequently, the 100 µL of LM nanozyme aqueous dispersion or laccase solution (1 mg/mL) was added into the above mixture. **The reaction was kept for 1 h at 25 °C, and the mixture was separated by centrifugation (10,000 rpm, 5 min).** Finally, the absorbance of the supernatant was measured at 510 nm.

Measurements of catalytic kinetic parameters

To further evaluate the catalytic activities of LM nanozyme and laccase, the certain amount of LM nanozyme and laccase was added into the mixture solution of 2,4-DP (10, 20, 40, 60, 80 and 100 µg/mL) and 4-AP (150 µg/mL, in excess for all reactions) in MES buffer (30 mM, pH 6.8). Time-dependent absorbance at 510 nm was measured at 25 °C. Above experiments were repeated three times for the reproducibility. The initial velocities (v) were calculated using Eq. (1) and Eq. (2).

$$C_p = A_{510} / \varepsilon L \quad (1)$$

$$v = dC_p / dt \quad (2)$$

where C_p is the concentration of 2,4-DP, A_{510} is the absorbance at 510 nm, ε represents the extinction coefficient of reaction product ($\varepsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$), L represents the 1 cm of optical path length.

To calculate the catalytic kinetic parameters (K_m and v_{max}) of LM nanozymes and laccase, various concentrations of 2,4-DP (10, 20, 40, 60, 80 and 100 $\mu\text{g/mL}$) were reacted with LM nanozymes and laccase and 4-AP (150 $\mu\text{g/mL}$). The kinetics constants (K_m and v_{max}) were calculated through the Michaelis-Menten Eq. (3):

$$v = (v_{max} \times [S]) / (K_m + [S]) \quad (3)$$

where v represents the initial reaction velocity, v_{max} is the maximum reaction rate, $[S]$ represents the concentration of the substrate, K_m is the Michaelis-Menten constant. K_m can reflect the affinity of the LM nanozymes and laccase for the substrate and is defined as the concentration of substrate at half the maximum catalytic rate. Furthermore, based on the method proposed by Yan's group (Jiang et al. 2018), K_{cat} of LM nanozymes and laccase was calculated by Eq. (4):

$$K_{cat} = v_{max} / [C] \quad (4)$$

where K_{cat} is rate constant that defined the maximum number of substrate molecule transformed into product per unit of time. $[C]$ is the molar concentration of LM nanozymes or laccase.

$$[C] = \rho / (m_a \times N_A) \quad (5)$$

where the ρ represents the mass concentration of LM nanozymes, m_a represents the average mass of a single LM nanozyme particle, and N_A represents the Avogadro's constant ($N_A = 6.02 \times 10^{23} \text{ mol}^{-1}$). The m_a of LM nanozyme was calculated based on a previous report (Wang et al. 2019).

Evaluation of catalytic stability

The effects of pH, temperature, ionic strength, organic solvent, and storage time on the stability of LM nanozyme and laccase were studied. To evaluate the effect of pH on the catalytic activity, Laccase and LM nanozyme was incubated for 8 h at a variety of pH (3-9) before the catalytic activity determination, respectively. The relative activity of laccase and LM nanozyme were compared with that at pH=6.8. Temperature stability of laccase and LM nanozyme was measured by storing laccase and LM nanozyme at various temperatures (from 0 to 100 $^{\circ}\text{C}$) for 1 h.

The catalytic activity measured at 25 °C was chosen as reference. Laccase and LM nanozyme was incubated in different concentrations of NaCl (0, 150, 300, 450, and 600 mM) to study the effect of ionic strength. The effect of organic solvent was investigated by adding different amounts of ethanol (0, 30%, 50%, 70%, and 100% v/v) into the reactions to measure the catalytic activity. The storage stability was studied by measuring the daily residual activity of laccase and LM nanozyme dispersed in deionized water and stored at room temperature. The recyclability of LM nanozyme was investigated by using the assessment method of the catalytic activity of LM nanozyme to measure the catalytic activity. The LM nanozyme collected for the next reaction cycle through centrifugation (10,000 rpm, 5 min) and washed three times with deionized water after per cycle. For all these experiments, the absorbance of supernatant at 510 nm was monitored after 1 h of the chromogenic reaction.

Establishment of nanozyme-based ELISA

The 250 μ L of capture antibody was added into each well of a 96-well microliter plates and incubated overnight at 4 °C. After that, the wells were washed three times with PBST (10 mM, pH 7.2 PBS containing 0.1% Tween 20), followed by the addition of 250 μ L of PBSA (10 mM, pH 7.2 PBS containing BSA) to block unbound sites. **The blocking reaction was kept at 37 °C for 1 h, and then wells were washed with PBST three times.** Afterwards, 250 μ L of α -LA or food samples were added into wells, and were incubated with the immobilized capture antibody for 1 h at 37 °C. Meanwhile, PBST containing BSA was employed as a control. After washing three times to remove unbound α -LA, the diluted nanozyme tags were added and incubated for 1 h at 37 °C. Subsequently, unbound nanozyme tags were removed by washing with PBST three times. Finally, aqueous solutions of 4-AP (1 mg/mL, 75 μ L) and 2,4-DP (1 mg/mL, 75 μ L) was mixed with MES buffer (30 mM, pH 6.8, 100 μ L), and the mixture was added. The reaction was kept for 30 min at 65 °C, and the absorbance at 510 nm of wells was measured by using a UV-Vis spectrophotometer or the results were obtained using a smartphone with Spotxcel® Reader.

Analysis of food samples

The established nano-ELISA were employed for the detection of α -LA in in different food

samples, including UHT cow milk, raw cow milk, yogurt, candy, and infant formulas. The sample treatment was performed according to a previous report with minor modification (Ruiz-Valdepeñas Montiel et al. 2016). To make the α -LA concentration in the food samples correspond with the linear portion of the calibration graph, the food samples were treated through an appropriate dilution with the PBST buffer (pH=7.2). All food samples were treated using above sample pretreatment method for further usage.

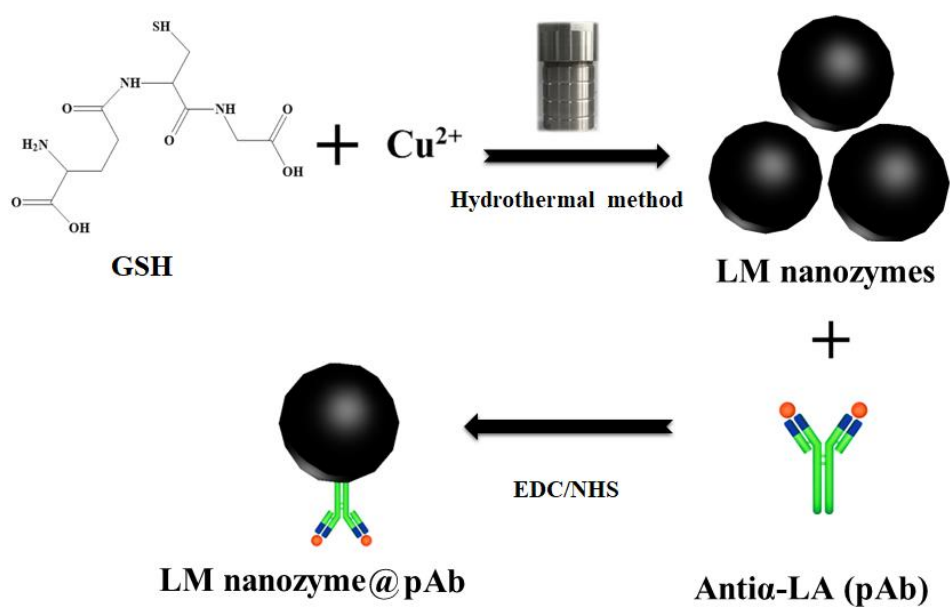


Figure S-1. Schematic illustration of the preparation of LM nanozymes and LM nanozymes@pAb.

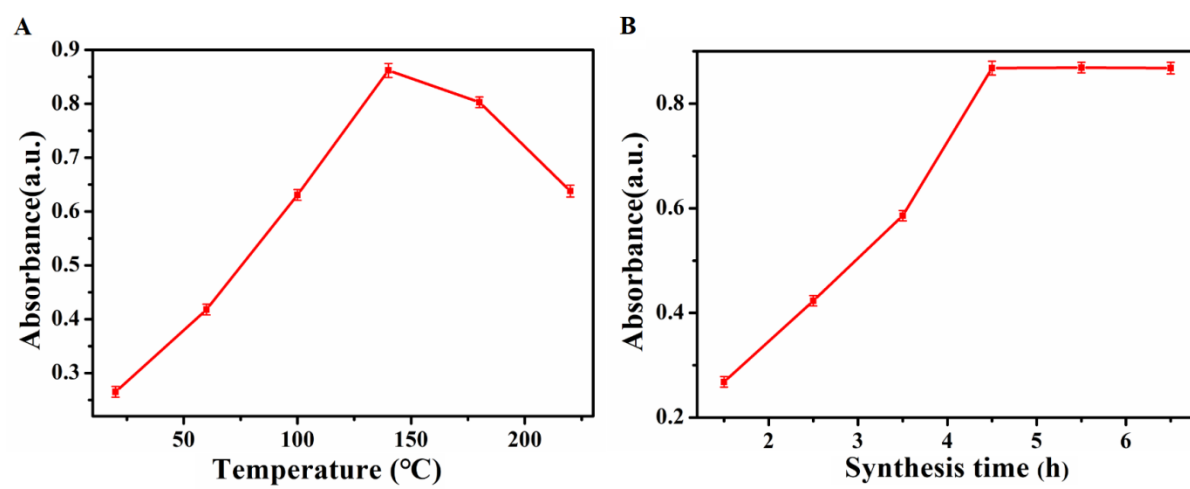


Figure S-2. Optimization on the synthesis conditions (i.e., (A) temperature and (B) time.) of LM nanozymes.

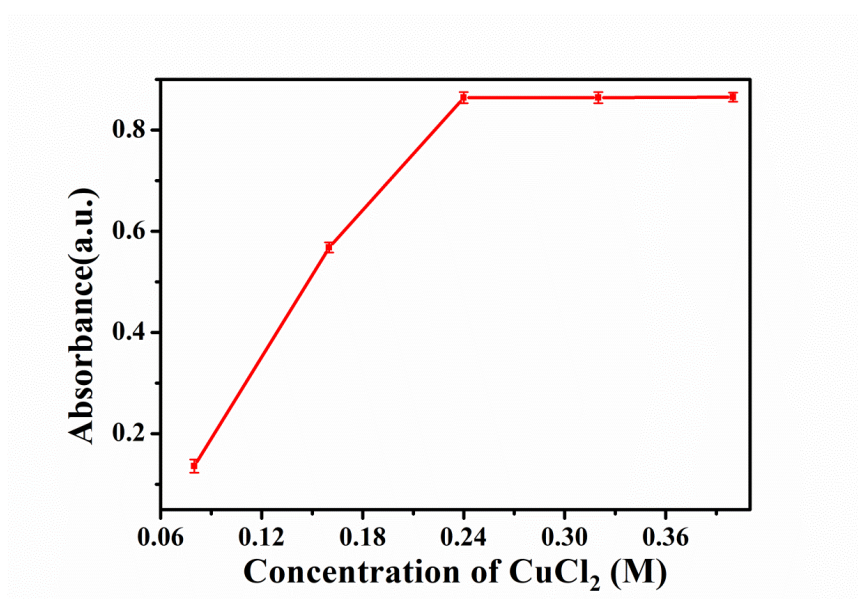


Figure S-3. The LM nanozyme synthesis using the various concentrations of aqueous CuCl_2 solution.

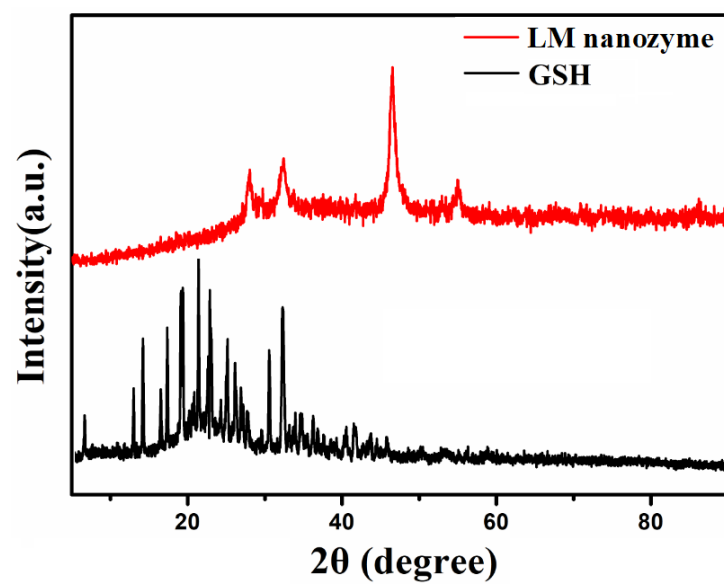


Figure S-4. XRD patterns of LM nanozymes and GSH.

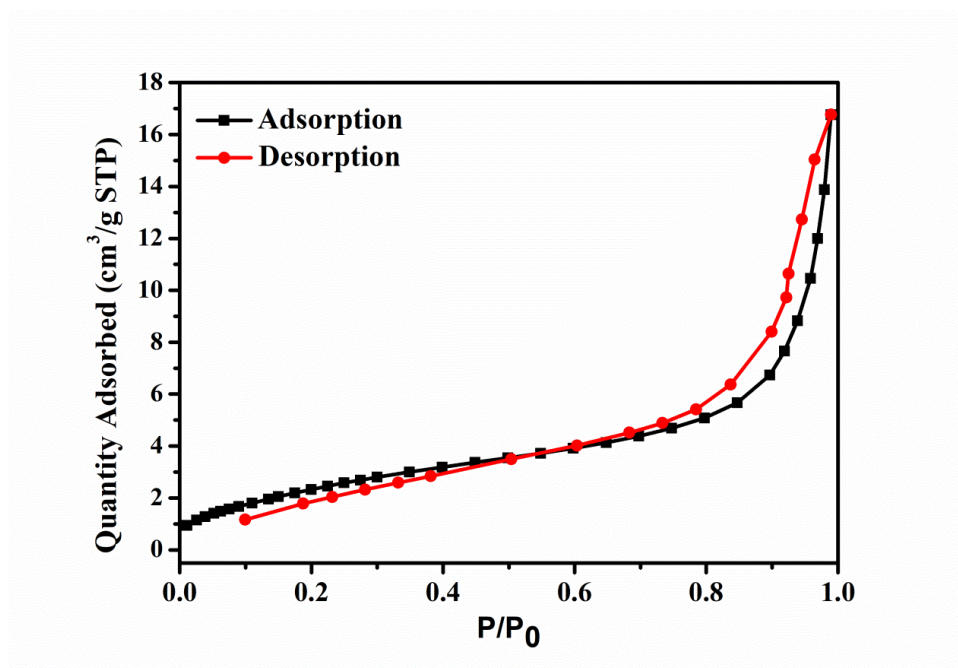


Figure S-5. N_2 adsorption-desorption curves of LM nanozymes. (The black is adsorption curves and the red is desorption curves).

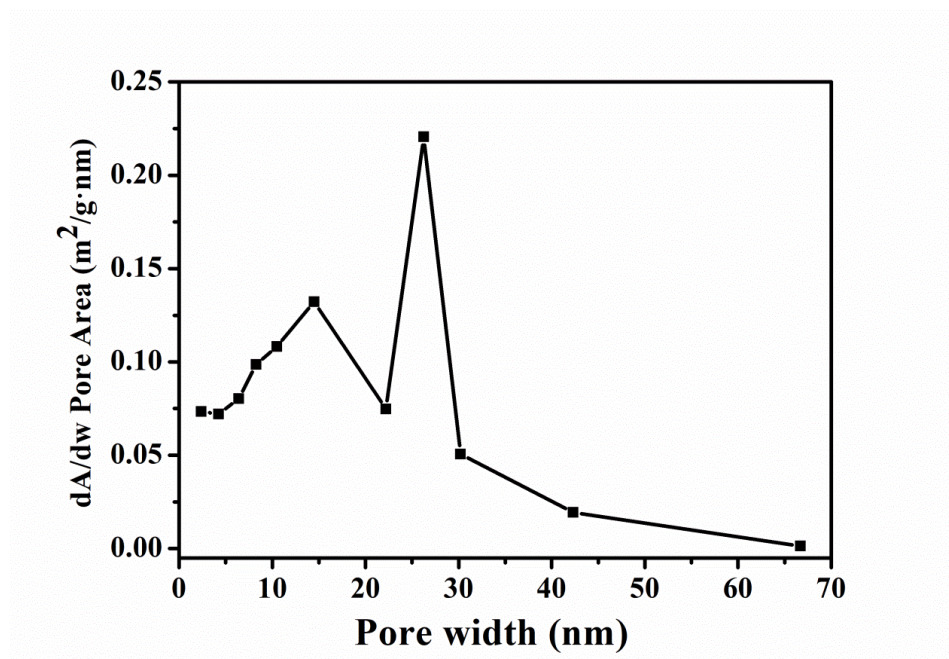


Figure S-6. Pore size distribution of the dried LM nanozymes from the N_2 adsorption.

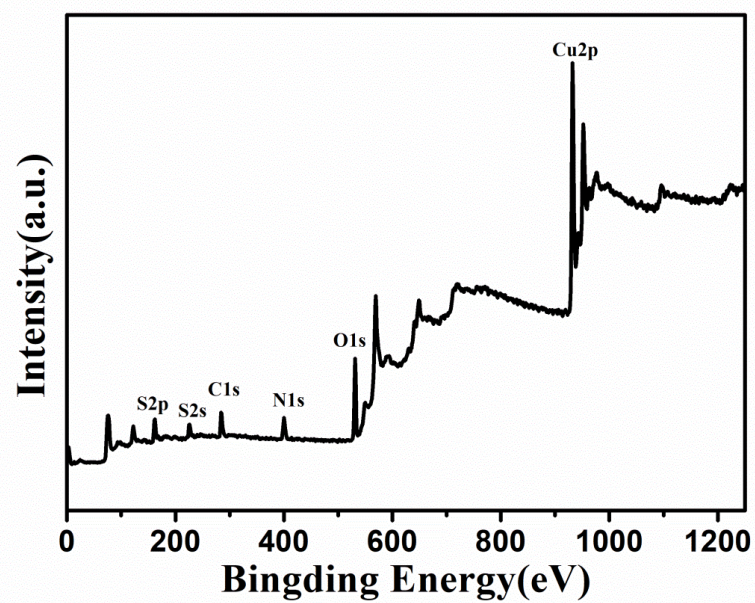


Figure S-7. XPS fully scanned spectrum of the LM nanozymes.

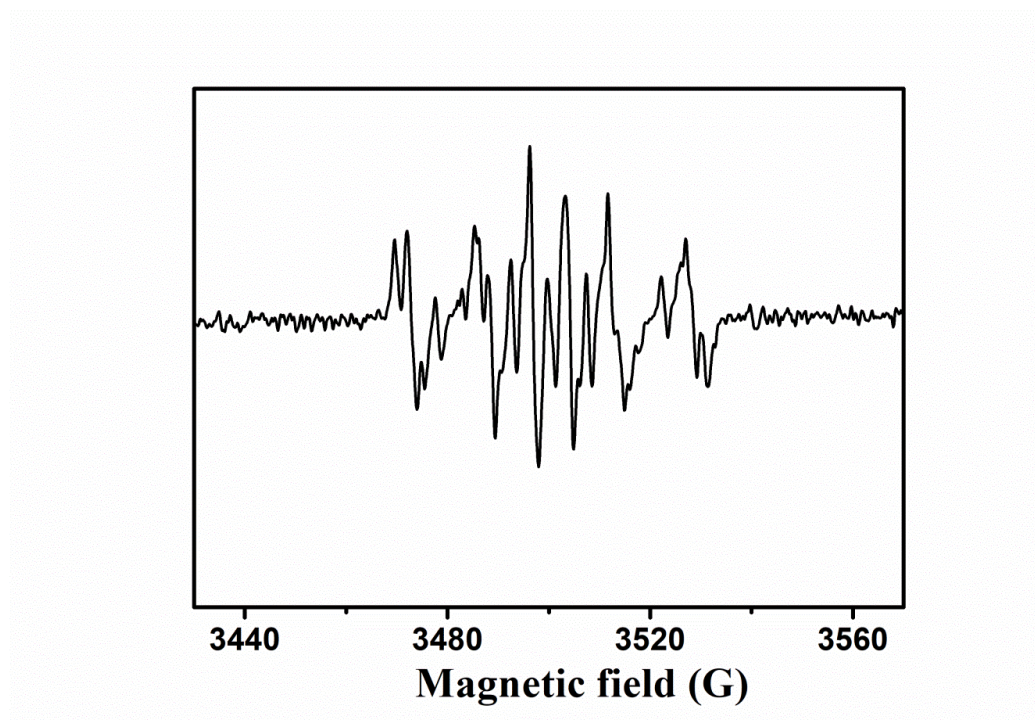


Figure S-8. EPR spectra of the substrate (2,4-DP) catalyzed by LM nanozymes.

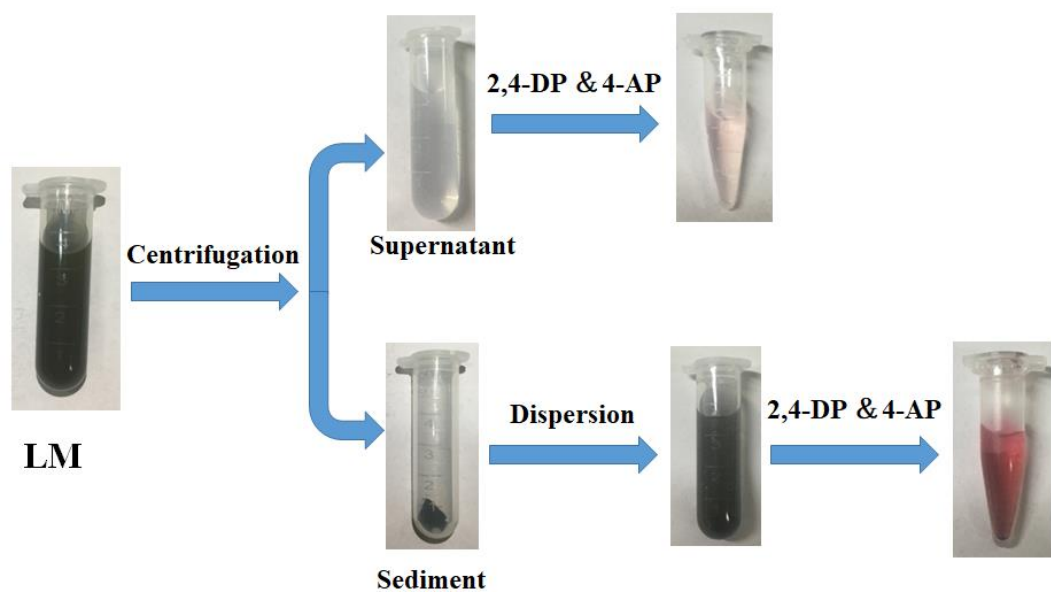


Figure S-9. (A) Control experiment for comparing the laccase-like activity of the precipitated LM nanozymes and the supernatant.

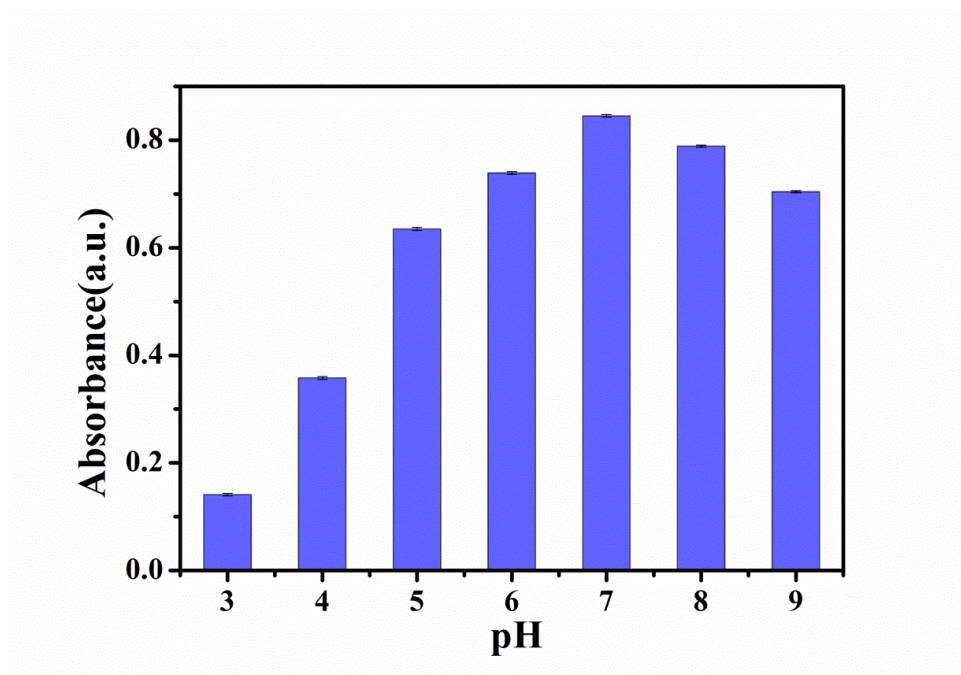


Figure S-10. pH-dependent catalytic activity of the LM nanozymes by determining the absorbance of products at 510 nm. (C) Photographs of (a) LM nanozymes reacted with 2,4-DP in MES buffer (pH=6.8), (b) ABTS and HRP added into the supernatant of (a), and (c) after the addition of H_2O_2 into (b). UV-Vis absorbance at 414 nm of (a), (b), and (c).

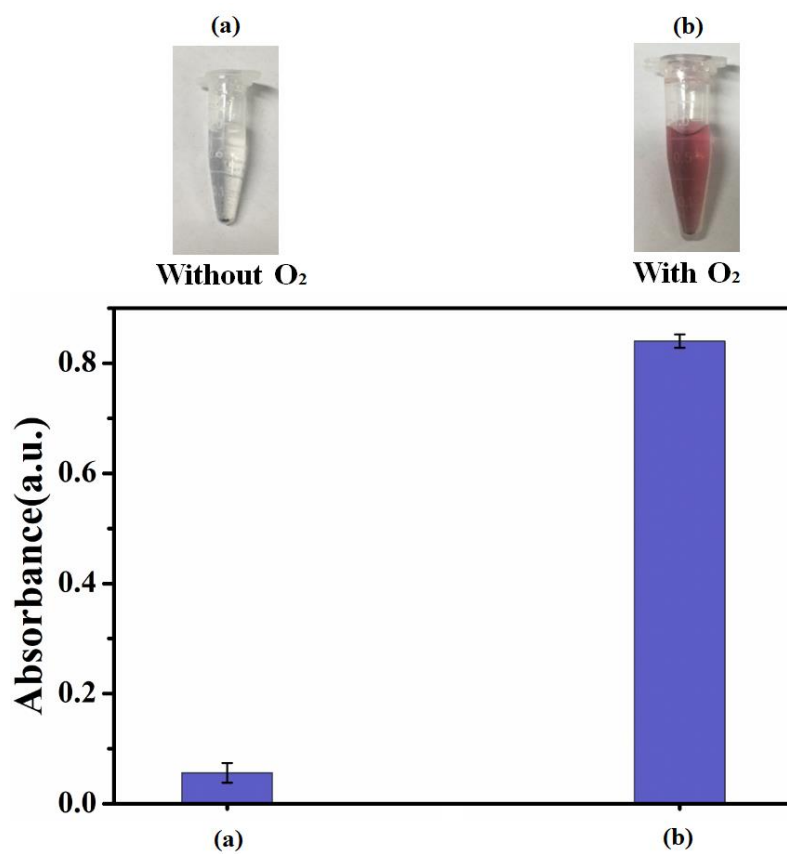


Figure S-11. Photographs and UV-Vis absorbance at 510 nm of reaction of 2,4-DP and 4-AP catalyzed by LM nanozyme in the absence of O₂ (a) and in the presence of O₂ (b).

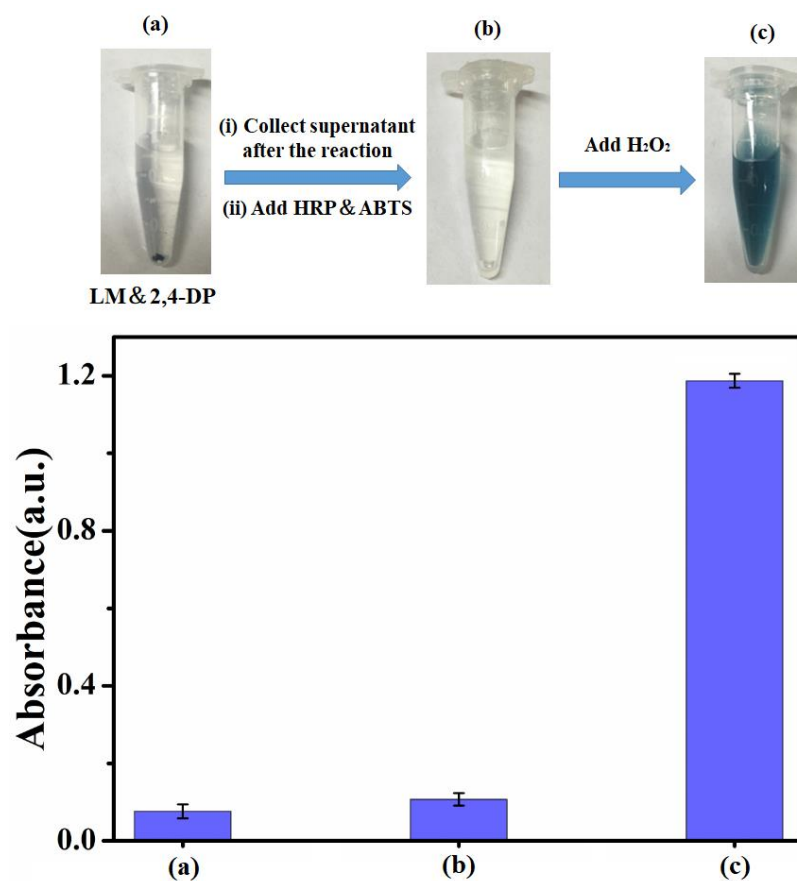


Figure S-12. Photographs of (a) LM nanozymes reacted with 2,4-DP in MES buffer (pH=6.8), (b) ABTS and HRP added into the supernatant of (a), and (c) after the addition of H₂O₂ into (b). UV-Vis absorbance at 414 nm of (a), (b), and (c).

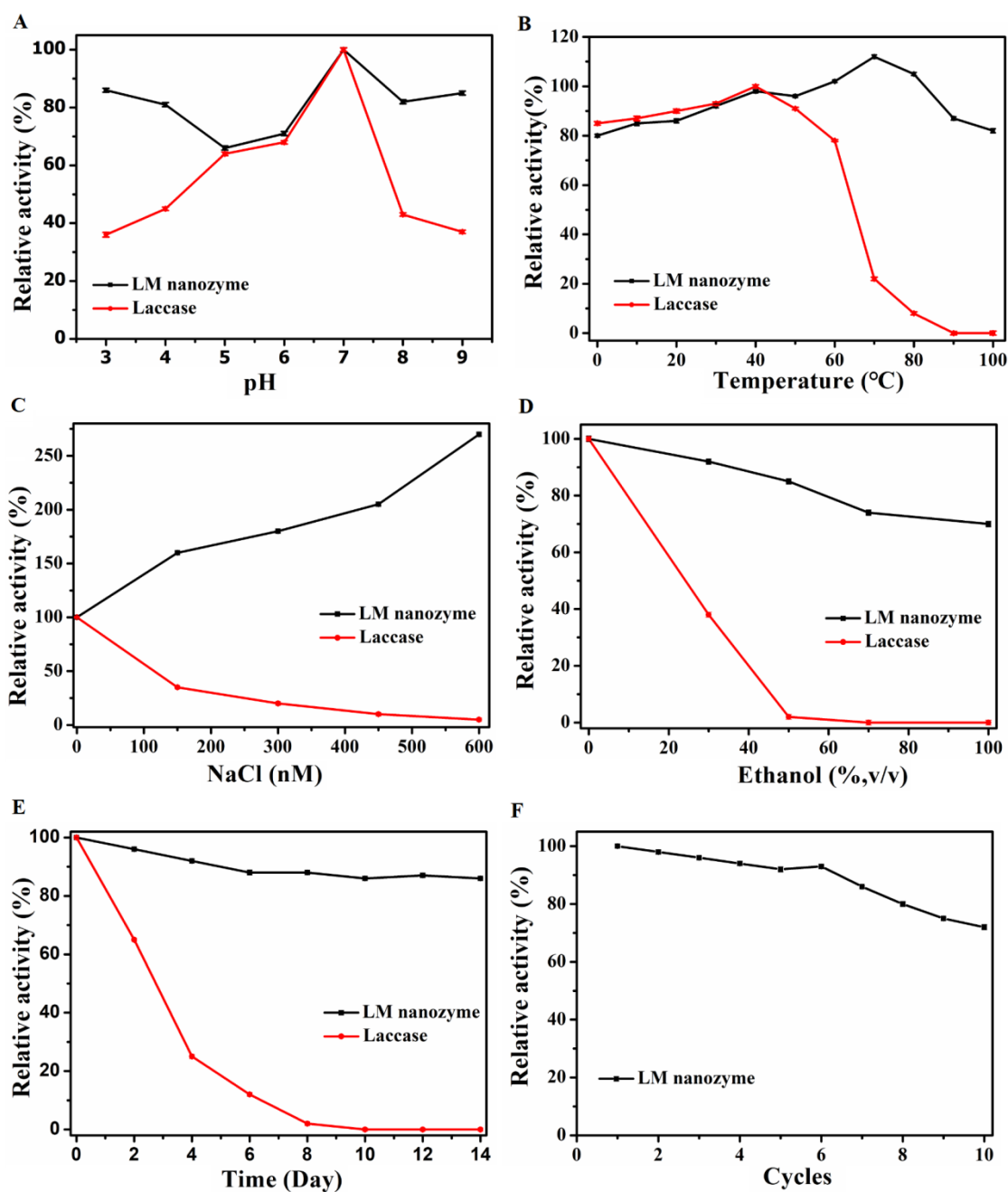


Figure S-13. Catalytic stability of laccase and LM nanozymes with the same mass concentration at various (A) pH, (B) temperature, (C) NaCl concentration (D) ethanol content, and (E) storage time, (F) Recyclability of LM nanozymes in the chromogenic reaction after recycling and reusing.

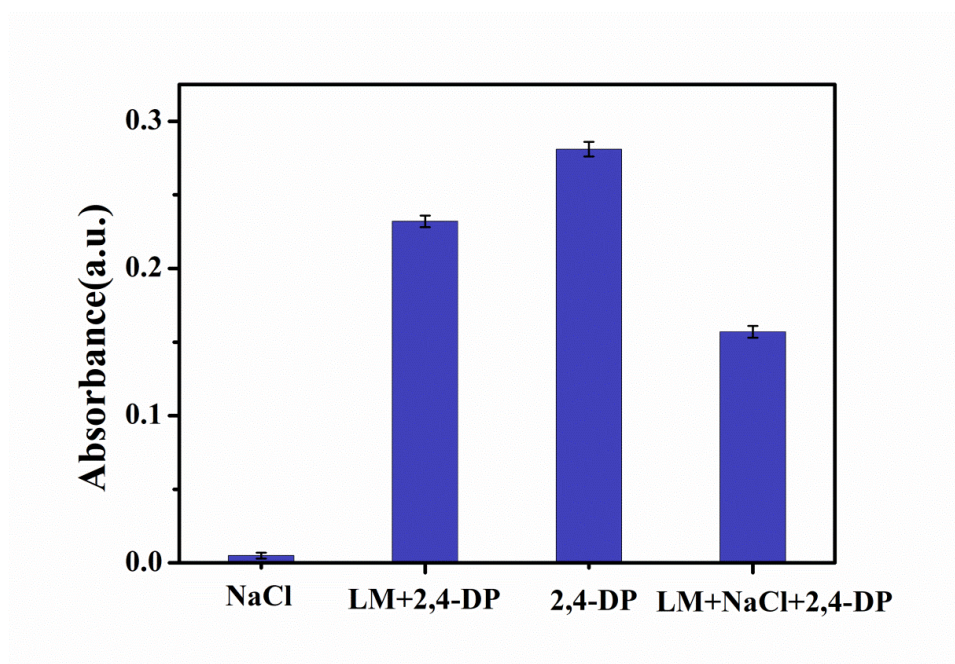


Figure S-14. Effects of NaCl on the 2,4-DP adsorption on LM nanozymes.

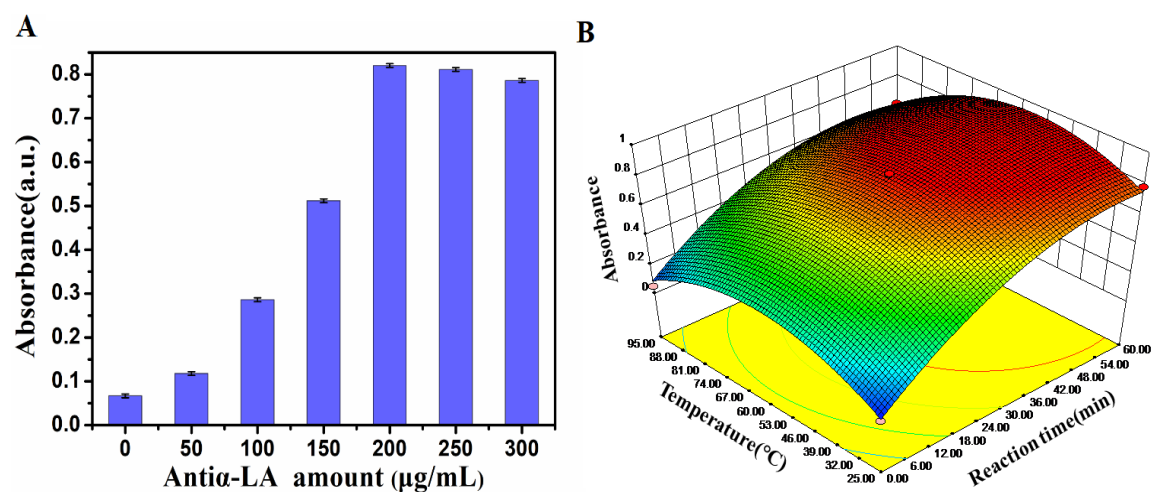


Figure S-15. (A) UV-Vis absorbance at 510 nm in the immunoassays based on anti-α-LA@LM nanozymes containing various amounts of anti-α-LA. (B) Optimization of catalytic reaction time and temperature of LM nanozymes-based immunoassays by response surface methodology.

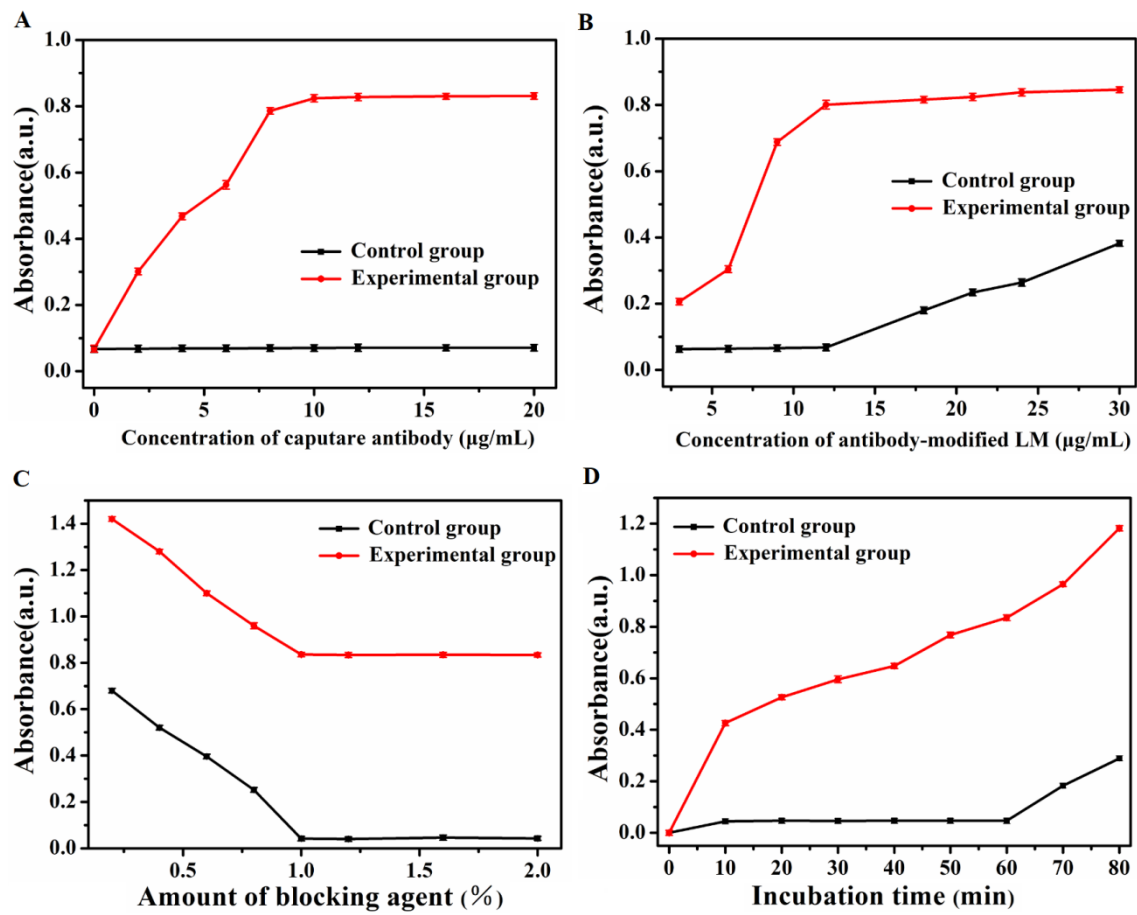


Figure S-16. Effects of (A) capture antibody concentration, (B) anti-α-LA@LM nanozyme concentration, (C) blocking agent, and (D) reaction time on the LM nanozyme-based immunoassays with (the red is experimental group) and without (the black is control group) antigens.

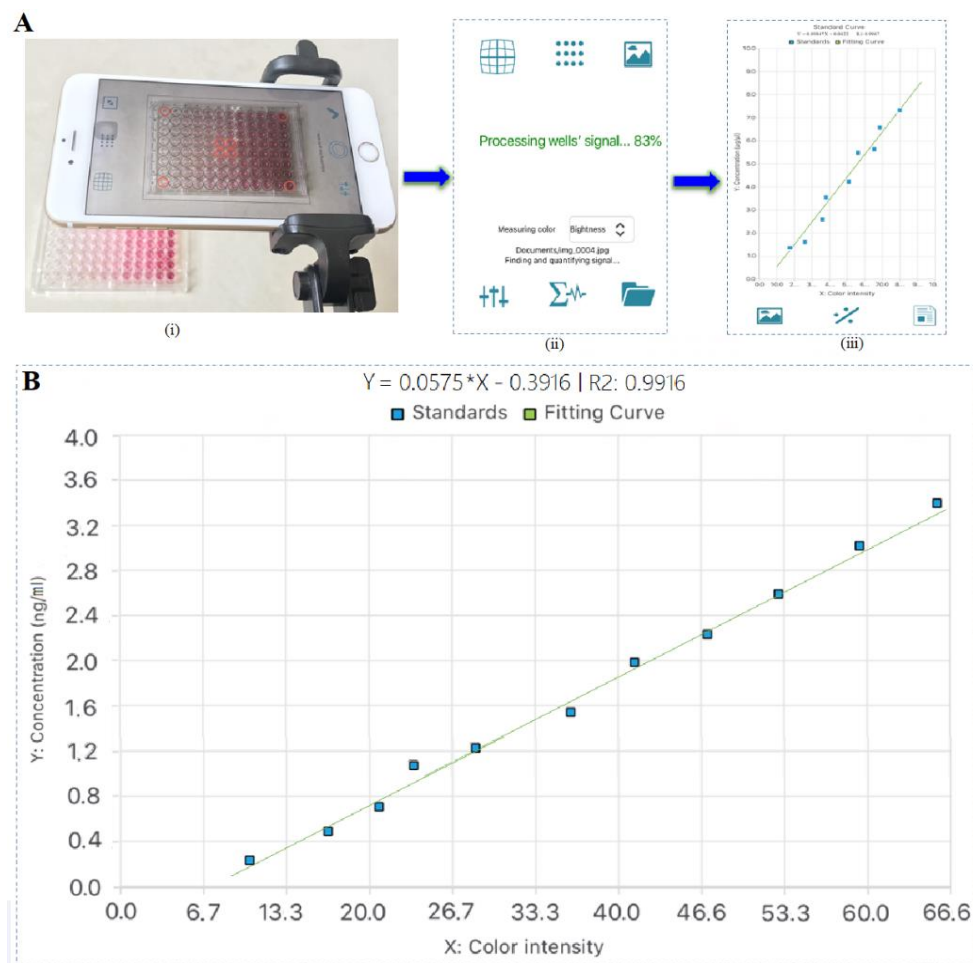


Figure S-17. Integration system of the nanozymes-mediated immunoassay with a smartphone. (A) Illustration of smartphone-based integration system for the α -LA detection. (i) Image capture; (ii) Signal detection and quantification; (iii) Assay analysis. (B) The standard curve of α -LA immunoassay based on a smartphone.

Table S1. The FT-IR spectral summary of GSH and LM nanozymes.

Chemical bond	GSH (cm ⁻¹)	LM nanozyme (cm ⁻¹)
$\nu(\text{C-N})$	1245.3	1185.4
$\nu(\text{C=O})$	1623.6	1605.3
$\nu(\text{-OH})$	2863.2	2908.3
$\nu(\text{-NH}_2)$	3347.6	3435.4
$\nu(\text{-NH-})$	3141.2	3242.6
$\nu(\text{-SH})$	2574.6	2608.5

Table S2. Catalytic kinetics of LM nanozyme and laccase towards the reaction between 2, 4-DP and 4-AP at room temperature.

Catalyst	$v_{max}(\text{mM min}^{-1})$	$K_m(\text{mM})$	$K_{cat}(\text{min}^{-1})$	$K_{cat}/K_m(\text{mM}^{-1}\text{min}^{-1})$
Laccase	6.63×10^{-3}	0.43	4.47	10.39
LM	8.26×10^{-3}	0.15	3.06×10^4	2.04×10^5

Table S3. Zeta potential of LM nanozymes in the MES buffer and LM nanozymes in the MES buffer with 500 mM NaCl.

Test samples	ζ -potential (mV)
LM nanozymes in the MES buffer	-0.85 ± 0.04
LM nanozymes in the MES buffer with 500 mM NaCl	-3.41 ± 0.26

Table S4. Comparison of the present study with the previously reported methods for α -LA assay

Analytical methods	linear range (ng/mL)	LOD (ng/mL)	Ref.
Electrochemical immunosensing	0.037-5ng/mL	0.011 ng/mL	(Ruiz-Valdepeñas Montiel et al. 2016)
Electrochemical sensor	20-80 ng/mL	9.7 ng/mL	(Raouafi et al. 2017)
Fluorescent immunosorbent assay	0.1-1000 ng/mL	0.1 ng/mL	(Yang et al. 2014)
Colloidal gold immunochromatographic strip assay	/	0.128 ng/mL	(Tao et al. 2016)
Electrochemical immunoassay	0.75-630 ng/mL	0.07 ng/mL	(Mars et al. 2018)
Nanozymes-mediated ELISA (using UV spectrophotometer)	0.06-400 ng/mL	0.01 ng/mL	This study
Nanozymes-mediated ELISA (using smartphone-based integrated system)	0.12-3.46 ng/mL	0.056 ng/mL	This study

Note:/, not mentioned.

Table S5. Results of the proposed method for the detection of α -LA in food samples

Food samples	Spiked concentration (mg/mL or mg/g) ^a	Dilution factor	Detected concentration (mg/mL or mg/g) ^a	Recovery (%)	RSD (%)
Raw cow milk	0	1,000,000	1.13 ± 0.06		1.63
	1	1,000,000	2.12 ± 0.11	99.67	2.45
	5	2,000,000	6.15 ± 0.01	100.41	1.72
UHT cow milk	0	1,000,000	0.48 ± 0.02		0.86
	1	1,000,000	1.47 ± 0.03	99.62	2.14
	5	2,000,000	5.61 ± 0.02	102.72	3.12
Yogurt	0	1,000,000	0.23 ± 0.07		2.51
	1	1,000,000	1.21 ± 0.08	98.21	3.47
	5	2,000,000	5.24 ± 0.11	100.27	2.78
Candy	0	1,000,000	ND ^b		
	1	1,000,000	0.95 ± 0.05	95.46	2.92
	5	2,000,000	4.99 ± 0.01	99.82	4.12
EHF	0	1,000,000	ND ^b		
	1	1,000,000	0.93 ± 0.07	93.36	2.30
	5	2,000,000	4.98 ± 0.02	99.65	1.64
PHF	0	1,000,000	0.34 ± 0.06		1.83
	1	1,000,000	1.35 ± 0.01	101.34	0.47
	5	2,000,000	5.22 ± 0.12	97.61	3.41

Note: ^aThe “mg/mL” represented the unit of raw cow milk, UHT cow milk, and yogurt, and “mg/g” represented the unit of candy, EHF, and PHF. ^bND, not detected.

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