

Bio-inspired Nanoenzyme Synthesis and Its Application in A Portable Immunoassay for Food Allergy Proteins

Xianlong Zhang, Guoliang Li,* Jianghua Liu, and Zhuoqun Su

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ABSTRACT: Nanozymes as a cost-effective and robust enzyme mimic have attracted widespread attention in the development of novel analytical methods. Herein, a new nanozyme-enhanced surface-enhanced Raman scattering (SERS) immunoassay platform was successfully developed using a peroxidase-mimicking nanozyme to replace the natural enzymes as a catalytic label of the enzyme-linked immunosorbent assay for the detection of allergy proteins. In this platform, the peroxidase-mimicking nanozymes as a catalytic label could catalyze the oxidation of the Raman-inactive reporter [i.e., leucomalachite green (LMG)] to generate Raman-active malachite green (MG) with H_2O_2 . Moreover, the produced MG Raman signal was further enhanced by the formed Raman “hot spot” through MG-induced gold nanoparticle aggregation, which could be recorded by a portable Raman spectrometer. On this basis, the established nanozyme-enhanced SERS immunoassay showed improved accuracy, high sensitivity, and good selectivity and was used for accurate quantification of α -lactalbumin (α -LA). With this method, α -LA could be detected with a limit of detection as low as 0.01 ng/mL. Moreover, the method was also verified by performing in food samples and showed satisfactory recoveries and high reliability. This study not only provides insight into the use of a nanozyme to establish new analytical methods but also broadens the applications of nanozymes in a food safety assay.

KEYWORDS: nanozymes, surface-enhanced Raman scattering, immunoassay, food allergy proteins, biosensing

INTRODUCTION

Food allergy, defined by the European Academy of Allergology and Clinical Immunology (EAACI), is usually caused by the immunoglobulin E (IgE)/cell-mediated humoral immune type I hypersensitivity responses to the exposure to some life-threatening food allergens.^{1,2} Food allergy affects approximately 5% of adults and 8% of children and has been an enormous global health burden and a great challenge to both food industry and clinical allergology over the past decades as a result of its widespread prevalence around the world.^{3,4} Currently, nine types of common food allergens that trigger the majority of allergic reactions have been reported by the Food Allergen Research & Education (FARE), including milk, peanuts, soybeans, eggs, sesame, wheat, fish (e.g., tuna, cod, salmon, and catfish), shellfish (such as shrimp, clams, lobster, crab, mussels, octopus, oysters, and scallops), and tree nuts (such as walnut, cashew, almond, pistachio, pecan, and hazelnut).⁵ Among these food allergens, milk allergy has been regarded as one of the most common incentives of allergic reactions.⁶ Milk allergy is an adverse humoral immune response caused by milk proteins. Milk contains over 25 various proteins, while only four caseins and whey proteins [i.e., α -lactalbumin (α -LA), β -lactoglobulin (β -LG), lactoferrin, and bovine serum albumin (BSA)] have been identified as milk allergens.¹ Moreover, the Voluntary Incidental Trace Allergen Labeling (VITAL) framework has indicated that the relatively low levels of allergens in milk can lead to adverse immune reactions in allergic individuals.⁷ At present, the strict avoidance of the milk allergens is still the most effective strategy for protecting allergic individuals from the allergic

responses as a result of no present vaccines or treatments against hypersensitive immune reactions caused by food allergens. Despite several countries have legislated to the obligation to label the presence of certain food allergenic ingredients on the product labels, the food allergen from adulterated foods, the possible existence of hidden food allergens, and unconscious cross-contamination of food processing are still great risk factors to induce allergenic responses.^{2,8} To address this issue, plenty of analytical methods [such as polymerase chain reaction (PCR),⁹ ultra-liquid chromatography tandem mass spectrometry (LC-MS/MS),¹⁰ and immunoassays^{11,12}] have been developed for the determination of food allergens. However, the food allergen detection using LC-MS/MS and PCR may suffer from the typical shortcomings, such as the requirement of a well-trained operator, complicated operations, and high costs.¹¹ Immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA), are the most commonly used for the reliable determination and identification of food allergy proteins to effectively manage the food allergen risk.¹¹

ELISAs are constructed mainly dependent upon the high specificity recognition of the antibody for antigen and have achieved a series of applications in the fields of food safety

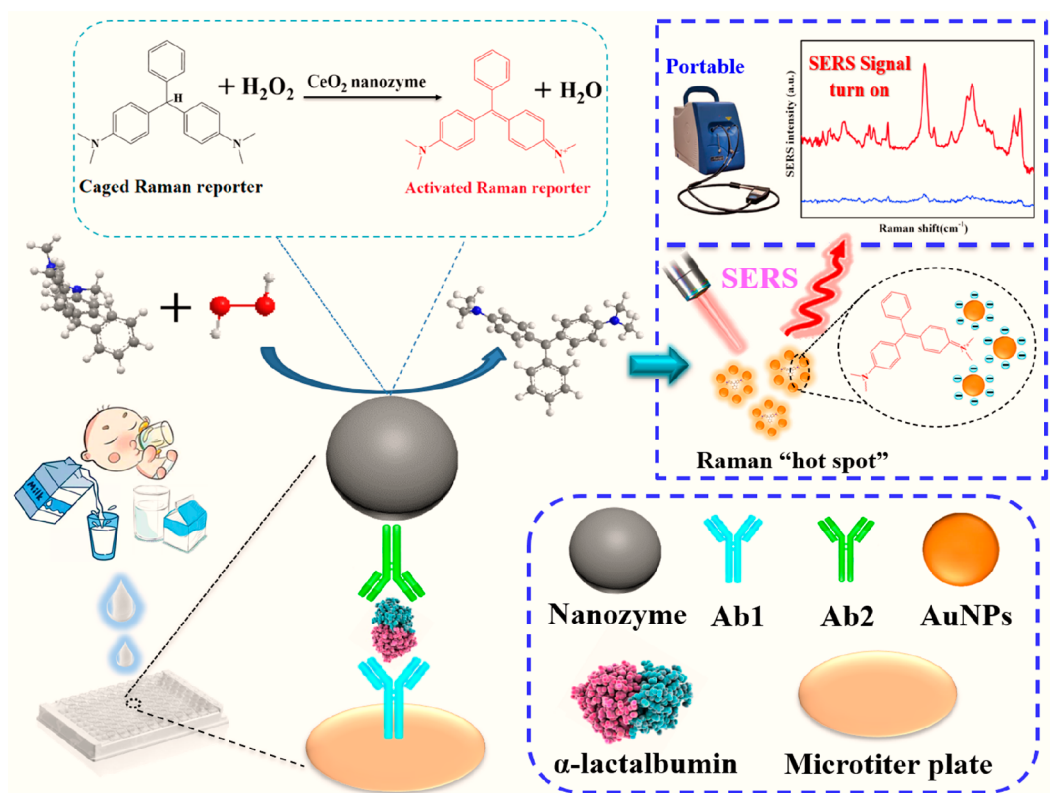
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Scheme 1. Schematic Illustration of Peroxidase-Mimicking Nanozyme-Catalyzing Signal Amplification for A Portable SERS Immunoassay of Food Allergy Proteins



detection, clinical diagnosis, and environmental monitoring because of their easy operation, high sensitivity, simplicity, and rapidness.¹³ In conventional ELISAs, biological enzymes [such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP)] have been widely used as the signal production/amplification labels for the analysis of trace-level targets.^{14,15} Nevertheless, biological enzymes used in the conventional ELISAs usually have some intrinsic shortcomings, such as complex preparation and purification, high costs, low stability, and poor catalytic activity under extreme conditions, which may limit their widespread applicability in highly sensitive and reliable assays in a way.^{13,16,17} To solve these problems, many researchers have devoted enormous efforts to exploring and developing the low-cost and highly stable enzyme mimics to increase the detection capabilities of traditional ELISAs.^{18,19} Nanomaterials with intrinsic enzyme-like catalytic activities (named as nanozymes), such as noble metal, metal oxides, metal–organic frameworks (MOFs), covalent–organic framework (COFs), and carbon materials, have been successfully developed and widely applied in the establishment of analytical methods.^{11,20–25} In comparison to biological enzymes, nanozymes have shown numerous fascinating advantages (such as simple synthesis method, good stability, robust catalytic activity, and low cost).^{16,26} Encouraged by its high stability and catalytic activity, nanozymes have been widely used as an exciting alternative to biological enzymes in conventional ELISAs for the signal production/amplification [e.g., colorimetry, fluorescence, and surface-enhanced Raman scattering (SERS) signal] to boost their detection performances, including stability, accuracy, sensitivity, etc.^{16,27,28} In comparison to the conventional ELISAs, nanozyme-based ELISAs show numerous merits, such as better stability and lower cost.

SERS as a promising and powerful optical sensing technology has been extensively used in the development of detection methods, owing to its advantages of high signal enhancement, *in situ* non-invasive assay, single-molecule-level sensitivity, and unique molecular fingerprint spectrum.^{29,30} Inspired by its superior performances, SERS has been successfully applied in the establishment of immunoassay methods, achieving widespread applications in the virus assay, detection of disease biomarkers, bacteria assay, etc.^{31,32} Currently, most SERS-based immunoassays usually employed the antibody-functionalized SERS label to directly readout the SERS signal, which exhibited good sensitivity.^{33,34} However, the background interferences of the antibody may lead to the different characteristic peaks in the final Raman spectrum, and the resultant complicated and unrecognizable Raman signals are not conducive to perform trace-level analysis. Furthermore, the lack of enzyme-catalyzed secondary amplification of analyte signals may also limit their ultratrace-level assays.¹¹ For SERS-based immunoassays, reducing background interferences and purifying Raman signals is of great significance to boost an ultrasensitive detection. Meantime, the secondary amplification of analyte signals also facilitates the establishment of the ultratrace-level assays. Therefore, nanozyme used as an antibody label for catalyzing the production of new Raman signals is very promising to improve the detection performances of SERS-based immunoassays.

In the present study, we successfully developed a nanozyme-enhanced SERS immunoassay platform for the portable and sensitive detection of food allergy proteins using a peroxidase-mimicking CeO₂ nanozyme. Herein, the CeO₂ nanozyme with peroxidase-like activity can catalyze the oxidation of a Raman-inactive reporter [i.e., leucomalachite green (LMG)] in the

presence of H_2O_2 to produce active malachite green (MG).³⁵ SERS possesses the superior sensitivity and can boost the weak Raman signals as high as 10^{15} , which is highly sensitive for recognizing the unique SERS spectral fingerprints of MG.³⁶ Synthetic gold nanoparticles (AuNPs) with negative charges were used as the SERS substrates for enhancing the Raman signals of generated MG. Moreover, as-produced MG with the positive charges could be adsorbed on the surface of negatively charged AuNPs by electrostatic force to form an intense Raman “hot spot” to further significantly amplify the SERS signal of MG.^{37,38} Encouraged by these, the CeO_2 nanozymes were employed as a catalytic tag for the Raman signal “turn-on” and amplification to establish a portable SERS-based immunoassay of food allergy proteins (Scheme 1). As a proof of concept, the allergy protein α -LA was selected as a target analyte. To perform the immunoassay of α -LA, the polyclonal antibody (Ab2) against α -LA was modified on the surface of CeO_2 nanozymes, and prepared CeO_2 nanozymes@Ab2 played two roles: the specific recognition toward α -LA and the Raman signal “turn-on”. In the presence of α -LA, CeO_2 nanozymes could catalyze the production of MG with H_2O_2 , and the Raman signal of newly formed MG was further enhanced by the MG-induced Raman “hot spot”. The enhanced Raman signals of MG could be captured by a portable Raman spectrometer to achieve an accurate quantification of α -LA. The proposed nanozyme-enhanced SERS immunoassay can not only reduce the background interferences through the nanozyme-catalyzed Raman signal “turn-on” but also improve the detection sensitivity via a secondary Raman signal amplification and the formed Raman “hot spot”. In addition, the established immunoassay also exhibited many merits, such as convenience, high sensitivity and specificity, and excellent applicability, for the detection of food allergy proteins.

MATERIALS AND METHODS

Reagents and Chemicals. Cerium(III) nitrate hexahydrate, polyvinylpyrrolidone (PVP), *o*-phenylenediamine (OPD), polyethylenimine (PEI), glutaraldehyde, and LMG were purchased from Macklin Biochemical Technology Co., Ltd., Shanghai, China. BSA, α -LA, α -casein (α -CN), β -LG, soybean protein, κ -casein (κ -CN), egg protein, wheat protein, β -casein (β -CN), and peanut protein were supplied by Yuanye Bio-Technology Co., Ltd., Shanghai, China. Monoclonal antibody (mAb) against α -LA was purchased from Bioss Antibodies (Beijing, China), and polyclonal antibody (pAb) against α -LA was supplied by Cloud-Clone Corp., Wuhan, China. Tween 20, chloroauric acid tetrahydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$), sodium citrate tribasic dehydrate, 3,3',5,5'-tetramethylbenzidine (TMB), citric acid, and sodium phosphate monobasic monohydrate were purchased from Aladdin Industrial Corp., Shanghai, China. Raw cow milk, ultra-heat-treated (UHT) cow milk, peanut milk drink, and walnut milk drink were bought in the local supermarkets. The 96-well polystyrene plates were supplied by Jincanhua, Shenzhen, China. Other reagents and chemicals were all of analytical grade, and all reagents and chemicals were used without further purification. The preparation of all solutions and buffers used in this study were performed using Milli-Q water.

Instrumentation and Measurements. Scanning electron microscopy (SEM) images were acquired using a JSM-7500F (JEOL, Japan). Transmission electron microscopy (TEM) images were obtained on a Talos F2100X G20 (Thermo Scientific, Waltham, MA, U.S.A.). The X-ray diffraction (XRD) curve was recorded using a Bruker D8 (Bruker, Germany). Brunauer–Emmett–Teller (BET) was carried out under N_2 sorption at 77 K with TriStar II Plus 2.02 physical equipment (Micromeritics, Norcross, GA, U.S.A.). The absorbances of the 96-well polystyrene plate were recorded using a

Multiskan (Thermo Scientific, Waltham, MA, U.S.A.). Ultraviolet (UV) measurement was performed using a Thermo evolution 201 (Thermo Scientific, Waltham, MA, U.S.A.). SERS spectra were measured using a portable Raman spectrometer (BWS465-785, B&W Tek, Inc., Newark, DE, U.S.A.), and all of the measurements were carried out with a 785 nm laser line.

Preparation of the CeO_2 Nanozymes. CeO_2 nanozymes were synthesized according to a classical preparation method, with a minor modification.³⁹ Briefly, cerium nitrate (2 mmol) and PVP (4 mmol) were added to 40 mL of ultrapure water and continuously stirred for 5 min at room temperature. The resulting solution was transferred to a 50 mL Teflon-lined stainless-steel autoclave. Then, the autoclave was heated for 2.5 h at 200 °C, and subsequently, it was slowly cooled at room temperature. Afterward, the obtained CeO_2 nanozymes were collected via centrifugation (8000 rpm for 5 min), and the collection process and washing process with ultrapure water were repeated 3 times to remove the redundant reactant. Finally, the collected products were dried through vacuum freeze drying for further use.

Synthesis of Antibody-Modified CeO_2 Nanozymes. To prepare CeO_2 nanozymes@Ab2, 2 mg of CeO_2 nanozymes was dissolved in 1 mL of ultrapure water and treated for 5 min using the ultrasound. After that, 1 mL of PEI (0.25%) was added to CeO_2 solution, and the resulting mixture solution was continuously ultrasound for 2 h. The resultant composites were collected via centrifugation (8000 rpm for 5 min), and the washing process was performed with ultrapure water 3 times to remove excess PEI molecules. Afterward, the obtained products were mixed with 1 mL of glutaraldehyde solution (0.25%), incubated overnight under continuous oscillation, and then washed 3 times using ultrapure water. Ab2 was added to the nanozyme suspension and incubated at 4 °C overnight under continuous oscillation for covalently immobilizing Ab2 onto the nanozyme surface. Finally, unbound Ab2 was removed by centrifugation (8000 rpm for 5 min) 3 times, and CeO_2 nanozymes@Ab2 was redispersed in phosphate-buffered saline (PBS) solution (pH 7.2) for further use.

Preparation of AuNPs. The preparation of AuNP seed solutions was performed on the basis of a previously reported sodium citrate reduction method with minor modification.⁴⁰ Typically, 300 μL of 1% (w/v) HAuCl_4 and 30 mL of ultrapure water were added to a 250 mL Erlenmeyer flask. The mixed solutions were quickly heated to boiling while stirring at the maximum speed using a clean stir rod for the solution to not splash. Then, 180 μL of 1% (w/v) sodium citrate tribasic dihydrate was added when the solution was boiling. The Erlenmeyer flask was moved away from the heating apparatus after 15 min. The AuNP maturation was completed as indicated by the color transition. The obtained AuNP seed solutions were kept in the dark for further use.

Evaluation of Catalytic Activity, Stability, and Recyclability of Nanozymes. To evaluate catalytic activity of CeO_2 nanozymes, OPD and H_2O_2 were used as the nanozyme substrates. The catalytic activity of CeO_2 nanozymes was measured through a colorimetric reaction induced by CeO_2 nanozyme catalyzing the oxidation of OPD. Briefly, 200 μL of CeO_2 nanozymes (2 mg/mL) was added to 800 μL of the freshly prepared OPD solution (4 mM) containing H_2O_2 . After incubation for 1 h at 25 °C, and the mixture was separated by centrifugation (8000 rpm for 5 min). In the end, the absorbance of the supernatant was measured at 432 nm. Currently, CeO_2 NPs have been reported to have multi-enzyme-like activities, such as ALP, oxidase, catalase, and superoxide dismutase.^{21,41} Thus, the peroxidase-like activity of CeO_2 nanozymes was further confirmed by a classical colorimetric reaction that employed TMB as the substrate in the presence of H_2O_2 . After evaluation of the catalytic activity, the stability of CeO_2 nanozymes was evaluated by studying the effects of the pH, temperature, and storage time on the catalytic activity of CeO_2 nanozymes. First of all, the effects of pH on the catalytic activity of CeO_2 nanozyme were evaluated. CeO_2 nanozymes were incubated for 8 h in different pH before measuring their catalytic activity. The CeO_2 nanozymes showed the highest activity at approximately pH 5, and thus, the activity of CeO_2 nanozymes at pH 5 was used as a reference. To study the temperature stability, CeO_2 nanozymes were

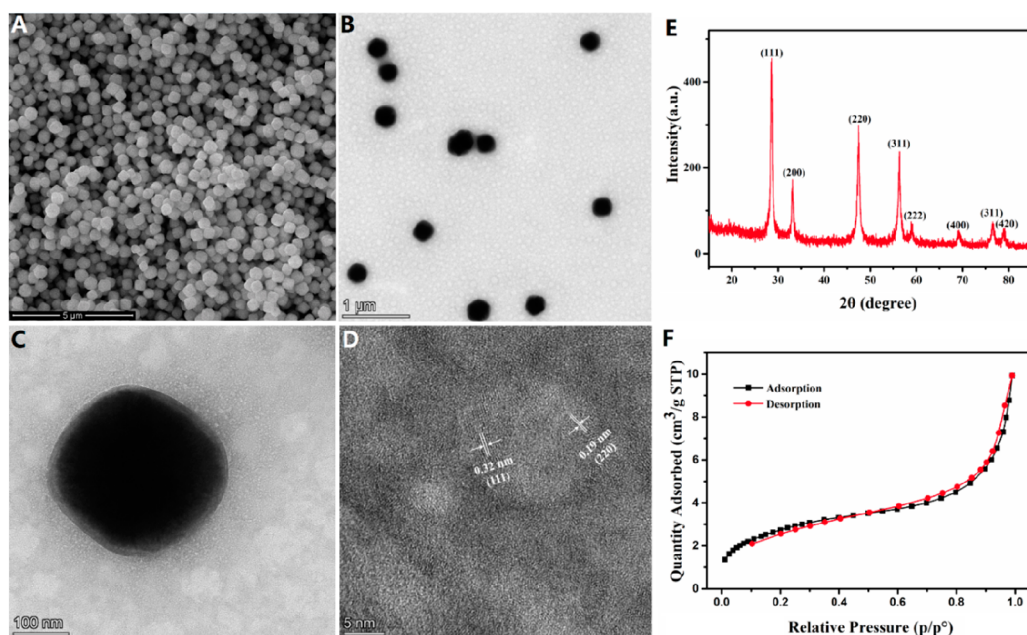


Figure 1. (A) SEM image, (B and C) TEM images, (D) HR-TEM image, (E) XRD pattern, and (F) N₂ adsorption/desorption isotherms of CeO₂ nanozymes.

stored at different temperatures (ranging from 4 to 100 °C) for 1 h. The catalytic activity of CeO₂ nanozyme stored at 40 °C was selected as a reference. To study storage stability of CeO₂ nanozyme, the CeO₂ nanozymes were stored at room temperature to measure their daily residual activity. In addition, the CeO₂ nanozyme recyclability was studied using the catalytic activity evaluation method of CeO₂ nanozyme for measuring its catalytic activity. After one cycle, the CeO₂ nanozymes were centrifuged (8000 rpm for 5 min) for the next reaction cycle.

Nanozyme-Catalyzed Raman Signal Amplification. As reported by previous studies, nanozymes with peroxidase-like activity have been confirmed to catalyze the Raman-inactive reporter (i.e., LMG) into Raman-active reporters (i.e., MG) in the presence of H₂O₂.^{35,42} To study the Raman signal amplification catalyzed by the peroxidase-like CeO₂ nanozymes, LMG and H₂O₂ were selected as the CeO₂ nanozyme substrates. A total of 50 μ L of CeO₂ nanozyme (10 mg/mL) and 400 μ L of LMG (100 mmol/L) were added to the Tris–HCl buffer (50 mmol/L, pH 7.0) and uniformly mixed. Then, 50 μ L of H₂O₂ (100 mmol/L) was added, and the uniformly mixed solution was incubated in a shaker at 37 °C for 1 h. After incubation, CeO₂ nanozymes were removed via centrifugation (8000 rpm for 5 min). The absorbance spectroscopy of obtained supernatants was measured by ultraviolet–visible (UV–vis) spectroscopy. Moreover, 100 μ L of supernatants was taken out to mix with AuNP solution and slowly stirred for 3 min, and then 10 μ L of the mixed solution was taken to drop on the aluminum-foil-wrapped glass slide for the SERS measurement using a laser power (10 mW).

Construction of the Nanozyme-Based Immunoassay. To establish the nanozyme-based immunoassay, 200 μ L of Ab1 was first added in the 96-well microliter plate and incubated at 4 °C overnight. Then, each 96-well microliter plate was washed with PBS containing 0.1% Tween 20 (PBST, 10 mM, pH 7.2) 3 times, and subsequently, unbound sites of the 96-well microliter plate were blocked with the addition of 200 μ L of PBS containing BSA (PBBSA, 10 mM, pH 7.2). After incubation for 1 h at 37 °C, the 96-well microliter plate was washed by PBST 3 times to remove redundant BSA. Subsequently, 200 μ L of α -LA or food samples was added to the 96-well microliter plate and incubated with Ab1 immobilized on the wells at 37 °C for 1 h. At the same time, BSA-contained PBST was used as a control. Unbound α -LA was removed by washing 3 times, and then diluted CeO₂ nanozymes@Ab2 was added to wells and incubated at 37 °C for 1 h. The unbound nanozyme labels were removed using PBST to

wash 3 times. At last, 100 μ L of LMG (100 mmol/L), 100 μ L of Tris–HCl buffer (50 mmol/L, pH 7.0), and 20 μ L of H₂O₂ (100 mmol/L) were added to the wells and incubated in a shaker at 37 °C for 1 h. To perform the SERS analysis, 100 μ L of the reaction solution taken from wells was mixed with AuNP solution and slowly stirred for 3 min. Finally, 10 μ L of the mixed solution was taken to drop on the aluminum-foil-wrapped glass slide for the SERS measurement through a laser power (10 mW).

Analysis of Food Samples. The proposed nanozyme-based SERS immunoassay platform was used for the determination of α -LA in milk samples. The milk samples were treated on the basis of a previously reported method with minor modification.⁴³ In brief, the milk samples were appropriately diluted by the PBST buffer (10 mM, pH 7.2) to make the concentration of α -LA in the milk samples correspond with the linear portion of the calibration graph. All samples were appropriately diluted before the measurement with the above sample pretreatment for further use.

RESULTS AND DISCUSSION

Characterization of Materials. The CeO₂ nanozymes were successfully prepared through a simple procedure (Figure S1 of the Supporting Information). The morphologies of as-prepared CeO₂ nanozymes were characterized using SEM and TEM. As the SEM image (Figure 1A) showed, the nanoparticles of CeO₂ nanozymes had nearly spherical morphology and showed a narrow particle size distribution. Moreover, TEM images of CeO₂ nanozymes in panels B and C of Figure 1 further demonstrated their sizes and morphologies, which possessed a nearly spherical morphology with an average diameter of approximately 250 nm. The morphologies of the prepared CeO₂ nanozymes in this study are nearly consistent with sphere CeO₂ prepared using a similar strategy in a previous study, and the size of the former was slightly less than that of the later.³⁹ As revealed in Figure 1D, the high-resolution TEM (HR-TEM) image of CeO₂ nanozymes showed that CeO₂ nanozymes possessed obvious distinct lattice fringes. Two lattice parameters of 0.19 and 0.32 nm in the HR-TEM image corresponded to the (220) and (111) planes in the XRD pattern, respectively. The (220) and (111)

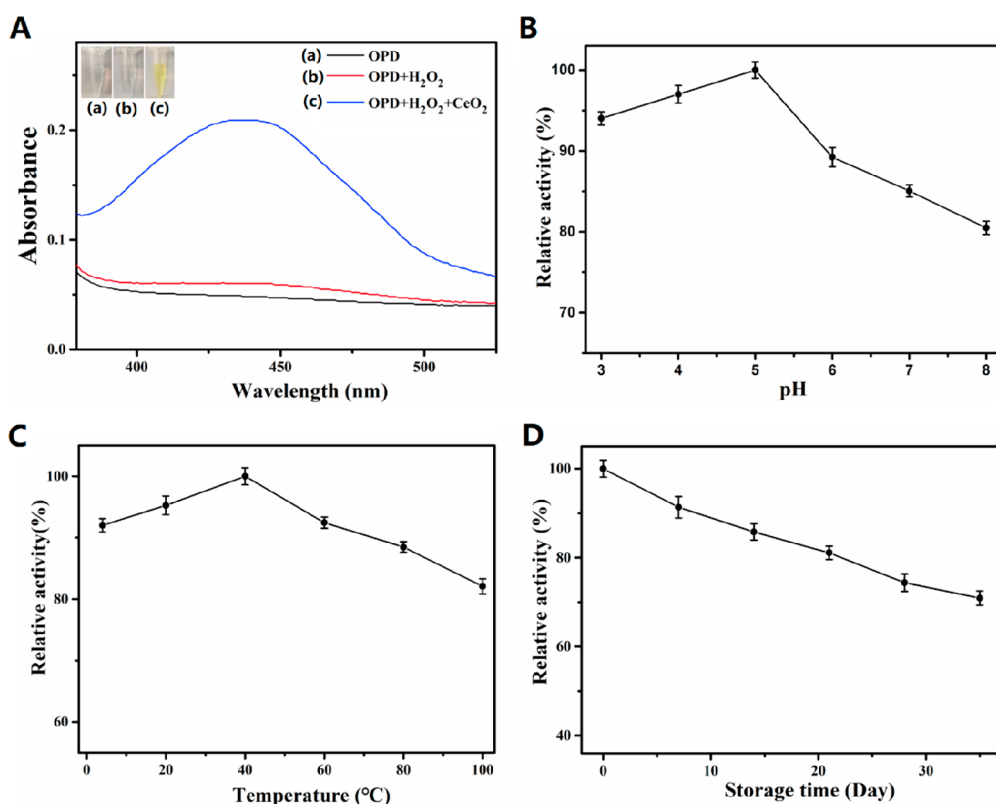


Figure 2. (A) Oxidation of OPD catalyzed by CeO₂ nanozymes with the existence of H₂O₂. UV-vis spectra of (a) OPD, (b) OPD + H₂O₂, and (c) OPD + H₂O₂ + CeO₂ nanozyme at 432 nm. Effects of different (B) pH, (C) temperature, and (D) storage time on the catalytic activity of CeO₂ nanozymes.

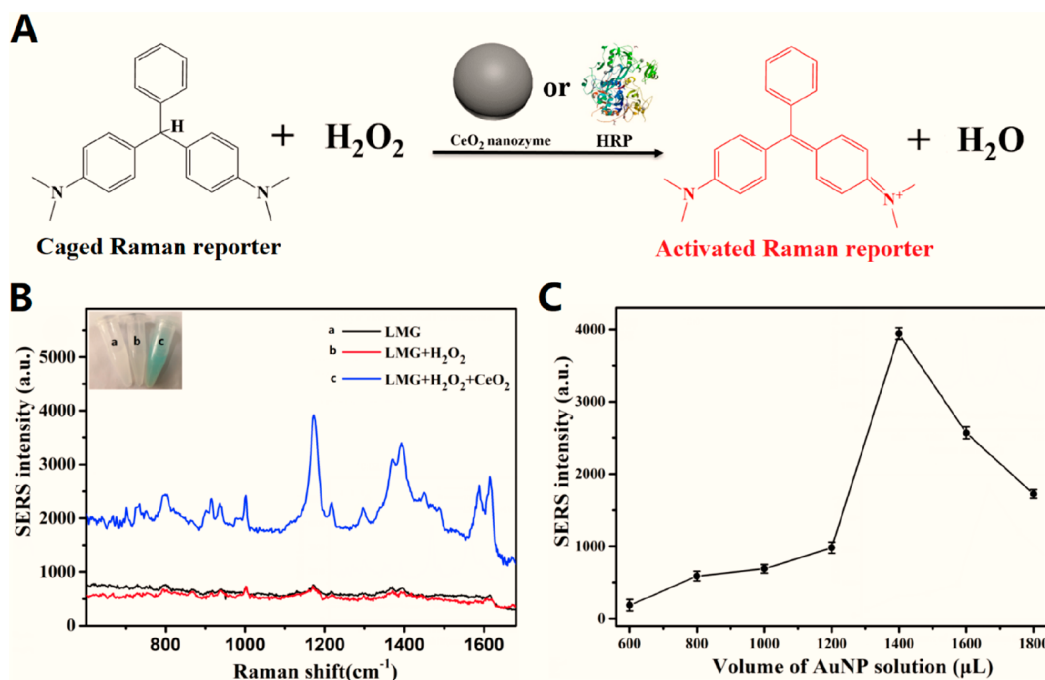


Figure 3. (A) Schematic illustration of the peroxidase-like CeO₂ nanozymes or peroxidase catalytically transform LMG (Raman-inactive reporters) into MG (Raman-active reporters) with the existence of H₂O₂. (B) Corresponding SERS spectra of (a) LMG, (b) LMG + H₂O₂, and (c) LMG + H₂O₂ + CeO₂ nanozyme. (C) Optimization of the volume of AuNP solution to enhance the SERS signals. The inset in panel B showed the corresponding photographs of LMG, LMG + H₂O₂, and LMG + H₂O₂ + CeO₂ nanozyme.

planes possessed relatively high intensity in the XRD pattern. To further study the crystal structure of CeO₂ nanozymes, the XRD characterization was performed. As Figure 1E showed,

eight characteristic peaks (i.e., $2\theta = 28.63^\circ, 33.09^\circ, 47.35^\circ, 56.33^\circ, 59.00^\circ, 69.22^\circ, 76.53^\circ, \text{ and } 78.87^\circ$) could be observed to correspond to the prepared CeO₂ nanozymes in 2θ ranging

from 30° to 80°, and these peak positions were able to be identified as the lattice planes [i.e., (111), (200), (220), (311), (222), (400), (331), and (420)]. Furthermore, the nitrogen physical adsorption/desorption measurements were carried out to study the surface area and pore size distribution of CeO₂ nanozymes. The results were calculated via the Barrett–Joyner–Halenda model. The N₂ adsorption/desorption isotherms (Figure 1F) showed that CeO₂ nanozymes possessed a surface area of 10.16 m²/g. Moreover, CeO₂ nanozymes were measured with an average pore size of 6.05 nm. The above results showed that CeO₂ nanozymes possessed a mesoporous structure. Owing to their larger surface area and mesoporous structure, CeO₂ nanozymes obtained better access to the substrate and exhibited a strong adsorption ability that increased the substrate concentrations near the CeO₂ nanozymes, thus resulting in the improvement of catalytic activity.

Assessment of Catalytic Performances. The catalytic activities of CeO₂ nanozymes were studied using OPD and H₂O₂ as substrates. Moreover, CeO₂ nanozymes have been reported with peroxidase-like activity for catalyzing the OPD oxidation to produce OPD_{OX} in the presence of H₂O₂.³⁹ As shown in UV–vis spectra (Figure 2A), the absorbance of OPD_{OX} at 432 nm could be observed. Moreover, Figure S2 of the Supporting Information also confirmed that CeO₂ nanozymes possessed a good peroxidase-like activity. After assessment of its catalytic activity, the stability of CeO₂ nanozymes at different pH, temperature, storage time, and recyclability was also further studied. First, the catalytic activity of CeO₂ nanozymes incubated in different pH was measured. Figure 2B showed that CeO₂ nanozymes maintained approximately 94% activity and about 80% catalytic activity after being incubated in pH 3.0 and 8.0 buffers, respectively. The results indicated that CeO₂ nanozymes possessed good stability in various pH buffers. Subsequently, the effects of different temperatures on the catalytic activity of CeO₂ nanozymes were studied. As Figure 2C showed, CeO₂ nanozymes still kept about 82% catalytic activity at 100 °C, which indicated that it possessed high thermal stability in the range of 4–100 °C. The good storage stability of catalysts is of great importance for practical applications.⁴⁴ Thus, the catalytic activity of CeO₂ nanozymes stored at room temperature for different days was measured. As shown in Figure 2D, CeO₂ nanozymes still retained about 70% catalytic activity after being stored for 35 days. To study the recyclability of CeO₂ nanozymes, the catalytic activity of CeO₂ nanozymes at each reaction cycle was measured. As shown in Figure S3 of the Supporting Information, CeO₂ nanozymes still maintained about 60% activity after 6 cycles. In general, the prepared CeO₂ nanozymes possessed good stability and recyclability.

As a benefit from its good catalytic activity and high stability, the peroxidase-like CeO₂ nanozyme was used to catalyze LMG into MG in the presence of H₂O₂ (Figure 3A). As shown in Figure 3B and Figure S4 of the Supporting Information, caged LMG is white in color and has no UV–vis absorbance at 620 nm and SERS signal, while activated MG is green in color and possesses a significant UV–vis absorbance at 620 nm and a strong SERS signal. In addition, the volume of AuNP solution for enhancing the SERS signals was also further optimized (Figure 3C). Encouraged by the outstanding nanozyme-catalyzed Raman signal amplification performances, a sensitive SERS-based immunoassay method was successfully estab-

lished. In comparison to the some existing CeO₂ nanozyme-based colorimetric methods, the established SERS-based methods can efficiently avoid the potential interferences from the sample background color and achieve an ultrasensitive assay as a result of its unique molecular fingerprint spectrum, high signal enhancement, and single-molecule-level sensitivity.

Optimization of Nanozyme-Based SERS Immunoassay Conditions. To obtain the best immunoassay performances, the detection conditions of the established method need to be further optimized. Ab2 was attached on the surface of CeO₂ nanozymes to obtain Ab2@CeO₂, of which the amount may affect the activity of CeO₂ nanozymes and, thus, influence the immunoassay performances. Therefore, to study the effects of the amount of Ab2 modified on CeO₂ nanozymes on immunoassay performances, the immunoassay was performed in the presence of targets. As shown in Figure S5 of the Supporting Information, Ab2 was verified to successfully integrate with CeO₂ nanozymes. The significant Raman intensity of MG could be observed when Ab2 was successfully modified on CeO₂ nanozymes. In contrast, no Ab2 was attached on the CeO₂ nanozymes, which resulted in a weak Raman peak intensity. In addition, Figure 4A also showed that

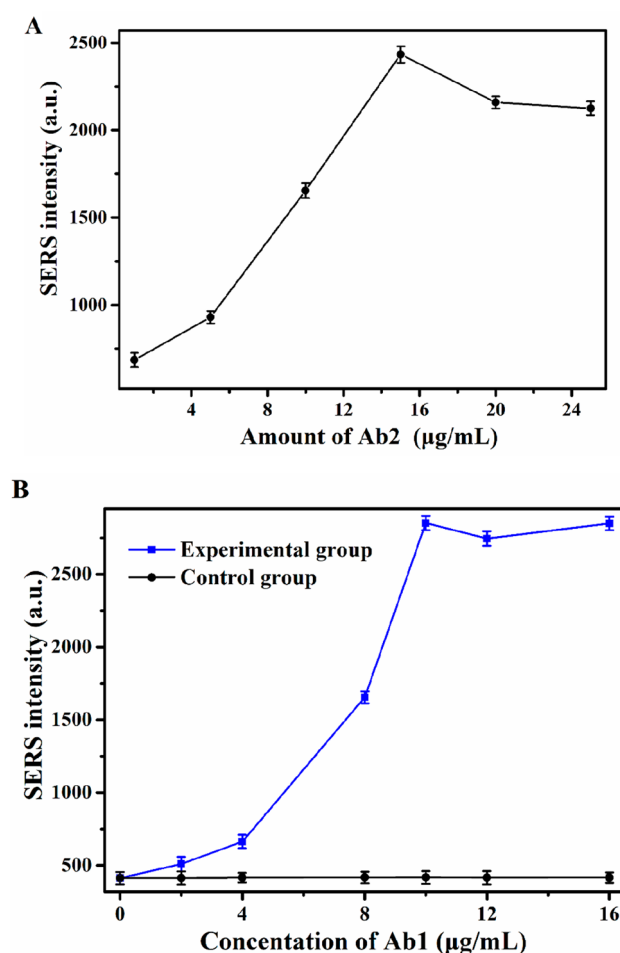


Figure 4. (A) SERS intensity in the SERS immunoassays based on CeO₂@Ab2 containing different amounts of Ab2. (B) Effect of the capture antibody (Ab1) concentration on the CeO₂ nanozyme-based SERS immunoassay in the presence (the blue represents the experimental group) and absence (the black represents the control group) of antigens.

Raman peak intensity of MG significantly increased with the enhancement of Ab2 concentrations, yet the Raman peak intensity was slightly down when the Ab2 concentration exceeded 15 $\mu\text{g/mL}$. The increased Raman peak intensity may be attributed to the modification of much more Ab2 on the CeO_2 nanozymes that improved the capture efficiency of Ab2@ CeO_2 for targets, while the slight decrease of the Raman peak intensity may be owing to excessive Ab2 modified on CeO_2 nanozymes that may limit the contact of its active sites with the substrate.

To further study the SERS immunoassay conditions, the immunoassay performed in the presence of a target was selected as an experimental group and the experiment without a target was set as a control group. First of all, the concentration of Ab1 was optimized. As Figure 4B showed, the SERS intensity in the experimental group have a gradual increase with the increase of Ab1 concentrations. The SERS intensity of the experimental group was almost kept constant when the concentrations of Ab1 attained 10 $\mu\text{g/mL}$. However, no obvious SERS intensity change in the control group was observed at the same conditions. The results indicated that 10 $\mu\text{g/mL}$ of Ab1 could meet the microplate coating for the following experiments. Furthermore, to avoid producing the false-positive results, the concentrations of CeO_2 @Ab2, the amount of blocking agent, and the incubation time in the process of immunoassay were also further optimized, respectively. As shown in Figures S6 and S7 of the Supporting Information, both of the concentrations of CeO_2 @Ab2 exceeded 10 mg/mL and the blocking agent amount below 1% caused the false-positive results, which may be attributed to the non-specific binding of Ab2 with the blocking agent or the microplate. In addition, the false-positive result would also be observed after the incubation time exceeded 1 h, which may be owing to the much more non-specific binding caused by the long incubation time (Figure S8 of the Supporting Information). After optimization of immunoassay conditions, the detection performances of nanozyme-based SERS immunoassay conditions were further evaluated.

Assessment of Analytical Performances. The analytical ability of the nanozyme-based SERS immunoassay was further studied. Under optimized conditions, the analytical performances of the nanozyme-based SERS immunoassay were assessed by selecting α -LA as a target analyte. In the presence of α -LA with the varying concentrations ranging from 0 to 1200 ng/mL , the SERS spectrum intensity of the nanozyme-based immunoassay was recorded with a portable Raman spectrometer. As Figure 5A showed, the measured SERS spectrum intensity gradually enhanced with an increase in the α -LA concentrations from 0 to 1200 ng/mL , demonstrating that the established nanozyme-based SERS immunoassay could be applied for detecting α -LA. As shown in Figure 5B, the SERS characteristic peak intensity at 1170 cm^{-1} was observed with a gradual increase with the increasing concentrations of α -LA (from 0 to 1200 ng/mL), and the calibration curve of the established method indicated a linear relationship between the SERS characteristic peak intensity at 1170 cm^{-1} and the α -LA concentrations in a wide range of 0.5–600 ng/mL ($R^2 = 0.994$). Moreover, the limit of detection (LOD) of the established nanozyme-based SERS immunoassay for α -LA was calculated as 0.01 ng/mL ($3\sigma/S$, where σ represented the standard deviation of the blank signal). The established nanozyme-based SERS immunoassay was in comparison to the current methods for the α -LA assay in the

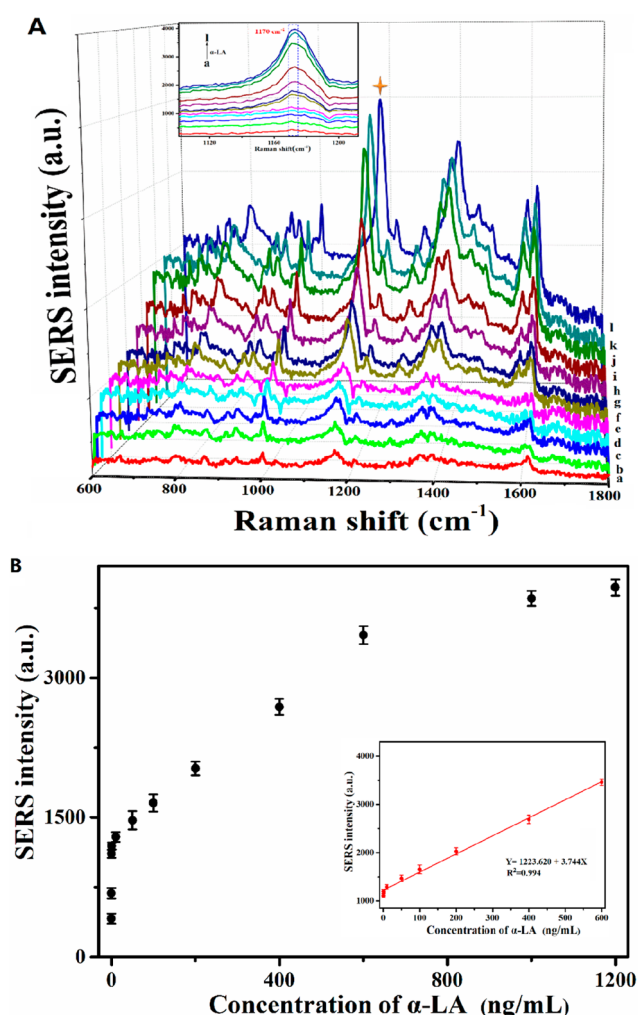


Figure 5. (A) Quantitative determination of α -LA at different concentrations (α -LA concentrations from a to k were 0.05, 0.5, 1, 10, 50, 100, 200, 400, 600, 1000, and 1200 ng/mL) were performed by recording the SERS spectra of MG generated in the nanozyme-based SERS immunoassay. The inset in panel A was the enlarged range from 1100 to 1220 cm^{-1} . (B) SERS characteristic peak intensity at 1170 cm^{-1} in the nanozyme-based SERS immunoassay of α -LA with various concentrations. The inset in panel B showed the correlation analysis between the α -LA concentration and the SERS characteristic peak intensity at 1170 cm^{-1} . The error bars represented the standard deviation in the repeated tests.

fields of LOD and linear detection range. As Table 1 showed, the LOD and linear detection range of the proposed nanozyme-based SERS immunoassay showed superior detection performances. Although traditional ELISAs based on the colorimetric signal readout has achieved a wide application in the quantification of multiple analytes, their detection accuracy and reliability may be easily influenced by the potential interferences from the sample background color. Traditional ELISAs based on the fluorescence signal readout are difficult to realize portability. In comparison to colorimetric and fluorescent methods, SERS exhibits distinctive advantages, such as a high signal enhancement, single-molecule-level sensitivity, and unique molecular fingerprint spectrum. Benefit from the combination of SERS with the nanozyme-catalyzed Raman signal “turn-on”, the established nanozyme-enhanced SERS immunoassay could not only efficiently eliminate the background interferences but also achieve a high detection

Table 1. Comparison of the Developed Methods and the Current Methods for the Detection of α -LA

detection method	signal readout	LOD (ng/mL)	linear range (ng/mL)	reference
FLISA ^a	fluorescence	0.1	0.1–1000	12
amperometric immunosensors	electrochemical	0.011	0.037–5	43
nano-ELISA with a smartphone	colorimetric	0.056	0.12–3.46	46
amperometric immunoassay	electrochemical	0.07	0.75–630	47
ELISA	UV–vis	0.128	NM ^b	48
nanozyme-based SERS immunoassay	SERS	0.01	0.5–600	this study

^a“FLISA” represent “fluorescence-linked immunosorbent assay”.

^b“NM” means “not mentioned”.

sensitivity. Moreover, the detection portability was also realized using a portable Raman spectrometer. In general, the proposed method showed great potential in the detection of α -LA.

The specificity of the developed nanozyme-based SERS immunoassay for α -LA was studied by measuring the cross-reactivity with different non-target allergenic proteins (including α -CN, β -CN, κ -CN, β -LG, egg protein, peanut protein, soybean protein, and wheat protein) at the concentrations of 0.01, 0.4, and 1 μ g/mL. As shown in Figure 6A, the egg protein, peanut protein, soybean protein, and wheat protein showed no cross-reactivity with the detection and capture antibodies. However, caseins (i.e., α -CN, β -CN, and κ -CN) and β -LG exhibited slight cross-reactivity with the detection and capture antibodies, which may be attributed to the residue of α -LA in caseins and β -LG. Additionally, the negative control tests were carried out by adding the Tris–HCl buffer solutions and BSA, and no obvious SERS spectrum intensities were observed in both tests, indicating a negligible non-specific adsorption of the developed nanozyme-based SERS immunoassay.

Stability acts as a key indicator of the practical usage in the analytical methods. To achieve the stability assessment of the nanozyme-based SERS immunoassay, Ab2-modified CeO₂ (CeO₂@Ab2) stored for different times at 4 °C was applied for the nanozyme-based SERS immunoassay of α -LA. As shown in Figure 6B, the variation coefficients of the established nanozyme-based SERS immunoassay for detecting 1 μ g/mL α -LA were recorded to be still less than 30%, even after being stored for 35 days, indicating good detection stability. Generally, the above results demonstrated that the developed nanozyme-based SERS immunoassay with excellent detection performances was very promising for the determination of α -LA in food samples.

Detection of Allergenic Proteins in Food Samples.

Encouraged by its superior analytical ability, the potential usage of the established nanozyme-based SERS immunoassay in the detection of allergenic proteins in milk samples was further verified using the raw cow milk, UHT cow milk, peanut milk drink, and walnut milk drink. The recovery test was achieved by adding 1 and 5 mg/mL α -LA in raw cow milk, UHT cow milk, peanut milk drink, and walnut milk drink, respectively. The recovery was calculated on the basis of the following formula:

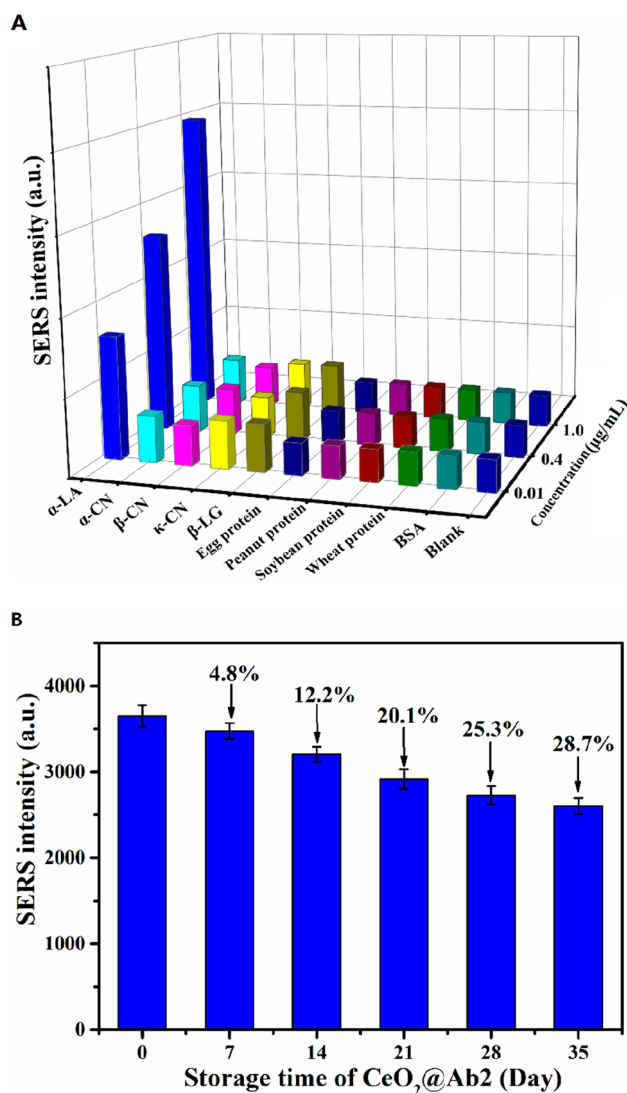


Figure 6. (A) Cross-reactivity of the developed nanozyme-based SERS immunoassay for α -LA with α -CN, β -CN, κ -CN, β -LG, egg proteins, peanut proteins, soybean proteins, wheat proteins, and BSA. (B) SERS characteristic peak intensity at 1170 cm^{-1} in the nanozyme-based SERS immunoassay for the determination of α -LA using CeO₂@Ab2 stored for different times at 4 °C. The values on the top of the histogram indicated the variation coefficients of the nanozyme-based SERS immunoassay for 1 mg/mL α -LA in comparison to freshly prepared CeO₂@Ab2.

$$R = \frac{k_2 - k_1}{k_3} \times 100\%$$

where R , k_1 , k_2 , and k_3 mean the recovery, the concentration of samples, the concentration of spiked samples, and the concentration of the spiked α -LA standard, respectively.

As Table S1 of the Supporting Information showed, the α -LA content in the raw cow milk was determined to be 1.16 mg/mL with the established nanozyme-based SERS immunoassay, and the results were almost consistent with the reported literature that showed that the content of α -LA in the mature bovine milk was about 1.2 g/L,⁴⁵ while a lower α -LA content in the UHT cow milk, peanut milk drink, and walnut milk drink was discovered, which may be attributed to the heat denaturation-induced molecular unfolding of α -LA during processing.⁴³ Therefore, the detection of α -LA could also be

applied in the evaluation of the milk heat treatment. In addition, the average recoveries of α -LA from the spiked raw milk, UHT milk, peanut milk drink, and walnut milk drink samples were 99.51, 98.70, 100.40, and 98.70%, respectively. The relative standard deviation (RSD) was in the range of 0.93–3.24%. According to the above results, the nanozyme-based SERS immunoassay was further confirmed to have great potential for detecting trace-level α -LA in food samples with good recovery.

In conclusion, we established a novel nanozyme-enhanced SERS immunoassay platform based on the nanozyme-catalyzed SERS signal amplification for the rapid, reliable, and ultra-sensitive detection of allergy proteins in food samples. In the proposed method, CeO_2 nanozyme with high peroxidase-like activity was used to catalyze the oxidation of Raman-inactive reporter LMG into active MG with H_2O_2 for enhancing the SERS signal amplification. As a benefit from the nanozyme-catalyzed SERS signal amplification and the formed Raman “hot spot” by MG-induced AuNP aggregation, the developed nanozyme-enhanced SERS immunoassay showed plenty of advantages in the detection of allergy proteins, such as high sensitivity, good selectivity, and improved accuracy. Moreover, the established method was successfully applied in the detection of allergy proteins and achieved a fast, portable, and highly sensitive determination of α -LA with a low LOD of 0.01 ng/mL, which was also carried out well in milk samples with satisfactory recoveries and high reliability. Given the good detection performances of the established nanozyme-enhanced SERS immunoassay, this study will pave a novel way for the detection of other trace analytes.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c04309>.

Schematic illustration of the synthesis of CeO_2 nanozymes (Figure S1), oxidation of TMB catalyzed by CeO_2 nanozymes with the existence of H_2O_2 (Figure S2), recyclability of CeO_2 nanozymes in the catalytic reaction after recycling and reusing (Figure S3), UV–vis spectra of LMG, LMG + H_2O_2 , and LMG + H_2O_2 + CeO_2 at 620 nm (Figure S4), Raman intensity corresponding to MG when nanozymes were modified with Ab2 or no Ab2 in immunoassays (Figure S5), optimization of the CeO_2 @Ab2 concentration on the nanozyme-based SERS immunoassay in the presence and absence of antigens (Figure S6), optimization of the blocking agent amount on the nanozyme-based SERS immunoassay in the presence and absence of antigens (Figure S7), optimization of the incubation time on the nanozyme-based SERS immunoassay in the presence and absence of antigens (Figure S8), and results of the nanozyme-based SERS immunoassay for detection of α -LA in food samples (Table S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Guoliang Li – School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi 710021, People's Republic of China; orcid.org/0000-0001-7951-9750; Email: 61254368@163.com

Authors

Xianlong Zhang – School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi 710021, People's Republic of China

Jianghua Liu – School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi 710021, People's Republic of China

Zhuoqun Su – School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, Shaanxi 710021, People's Republic of China

Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.jafc.1c04309>

Notes

The authors declare no competing financial interest.

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Supporting Information

Bio-inspired Nanoenzyme Synthesis and its Application in Portable Immunoassay for Food Allergy Protein

Xianlong Zhang^a, Guoliang Li^{a*}, Jianghua Liu^a and Zhuoqun Su^a

^a School of Food and Biological Engineering, Shaanxi University of Science and
Technology, Xi'an 710021, China

***CORRESPONDING AUTHOR:**

E-mail: 61254368@163.com (Guoliang Li)

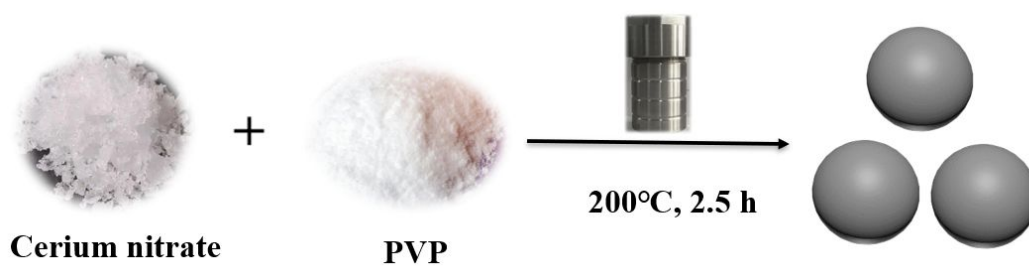


Figure S1. (A) Schematic illustration of the synthesis of CeO_2 nanozymes.

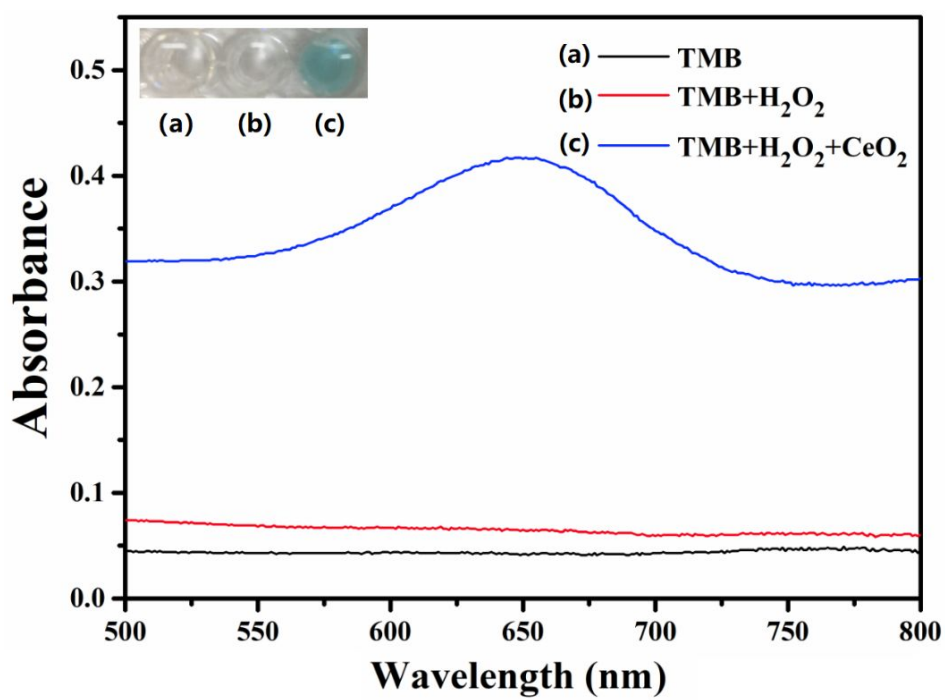


Figure S2. The oxidation of TMB catalyzed by CeO_2 nanozymes with existence of H_2O_2 . UV-vis spectra of the TMB (a), TMB+ H_2O_2 (b), and TMB+ H_2O_2 + CeO_2 nanozyme (c) at 656 nm.

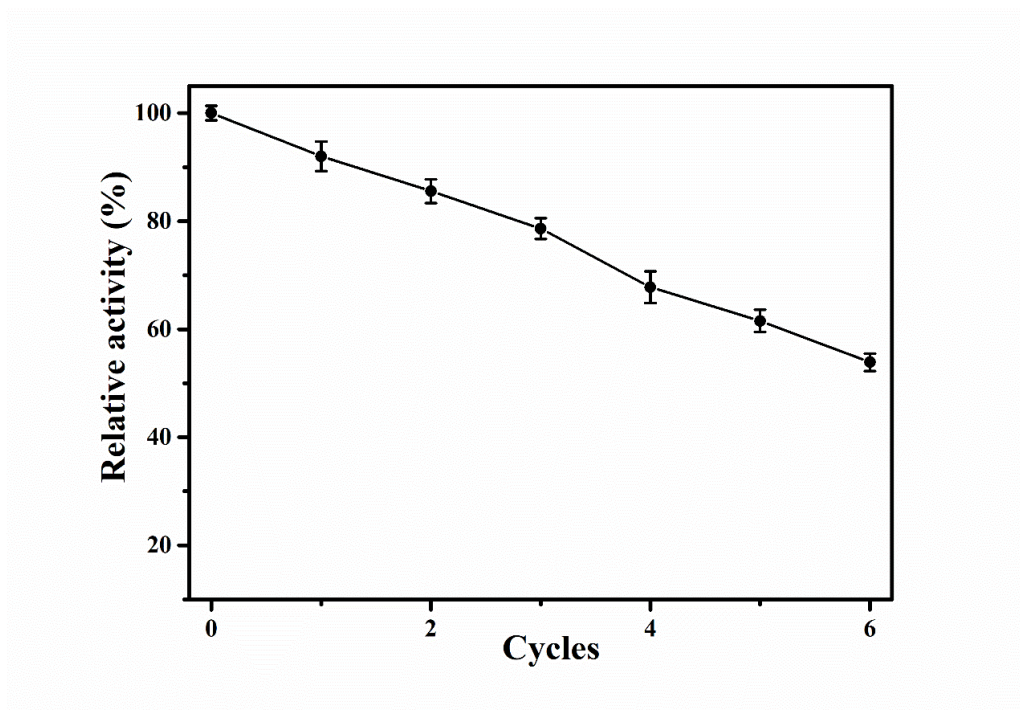


Figure S3. Recyclability of CeO₂ nanozymes in the catalytic reaction after recycling and reusing.

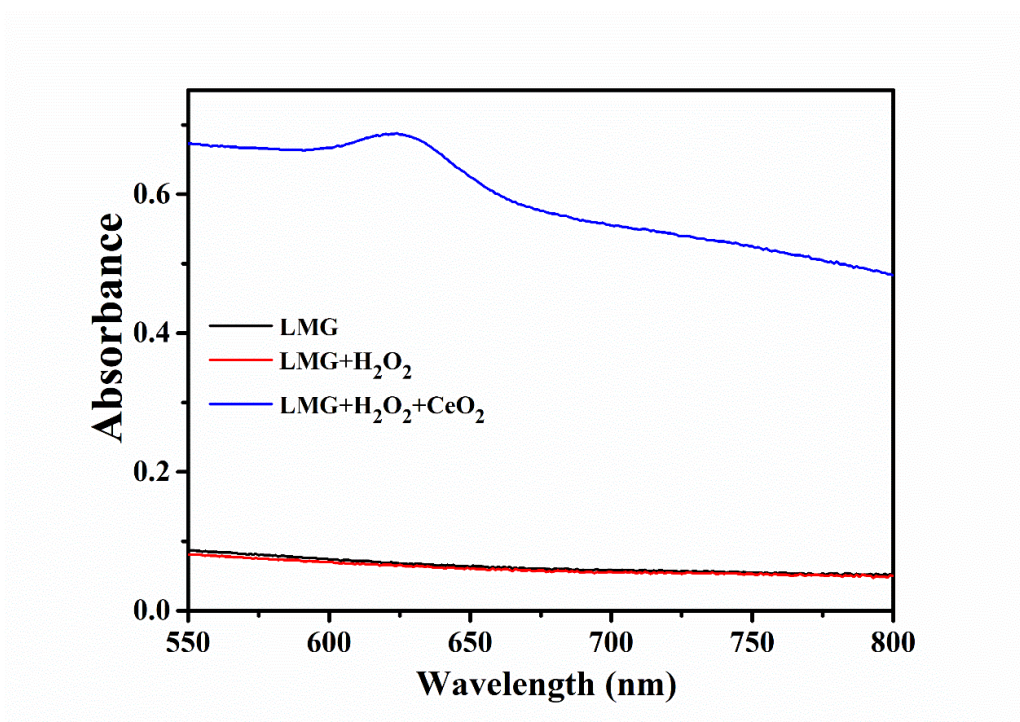


Figure S4. The UV-vis spectra of the LMG, LMG+H₂O₂, and LMG+H₂O₂+CeO₂ nanozyme at 620 nm.

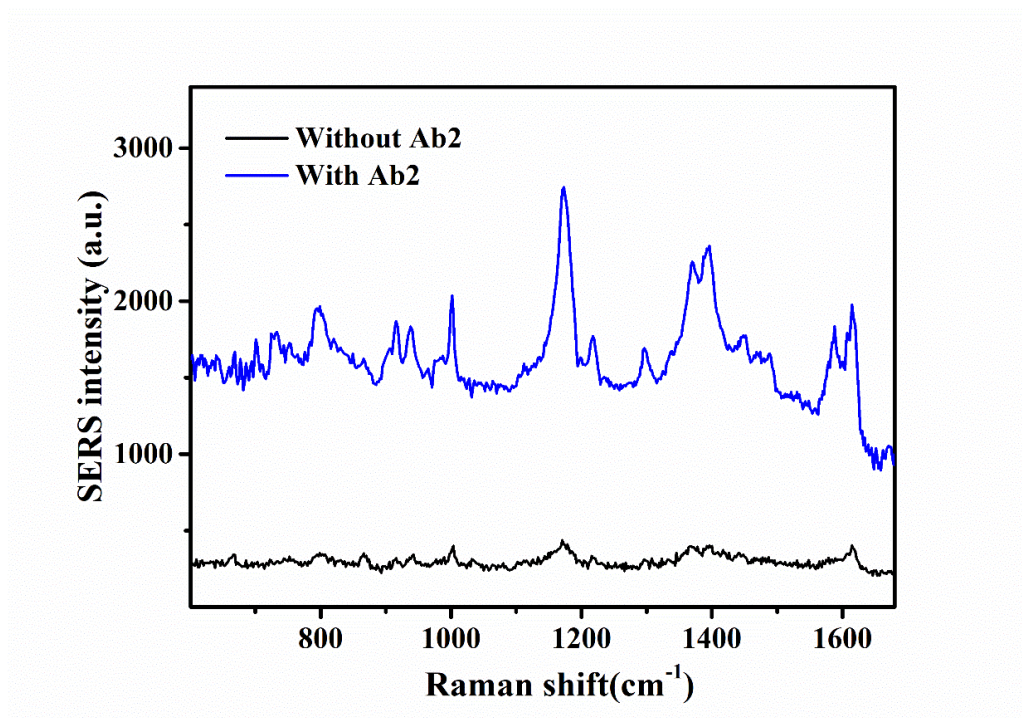


Figure S5. The Raman intensity corresponding to the MG when nanozymes were modified with Ab2 or no Ab2 in immunoassays.

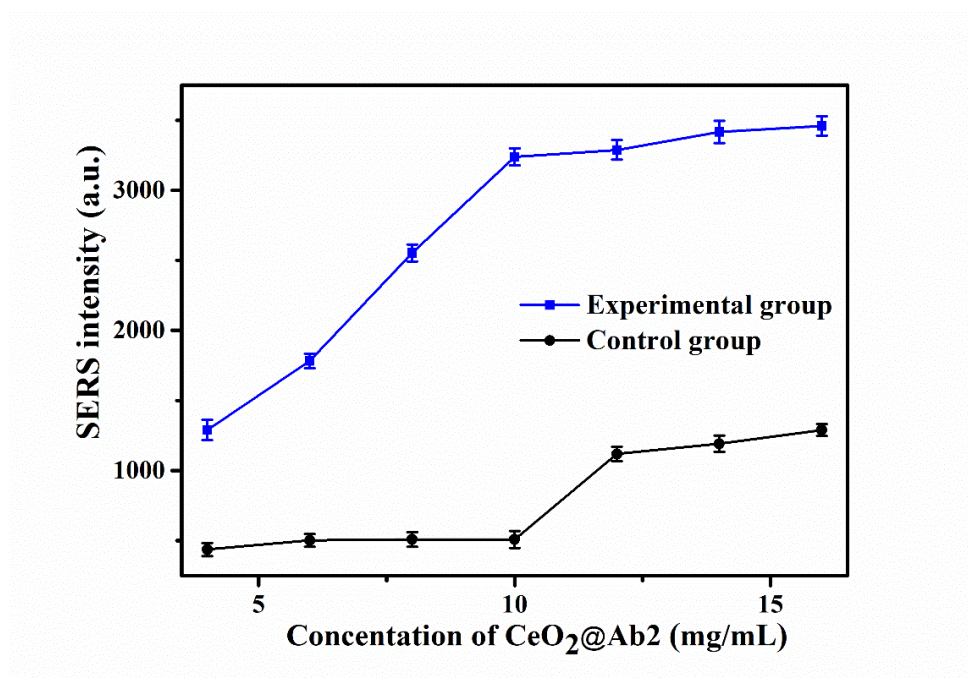


Figure S6. The optimization of CeO₂@Ab2 concentration on the nanozyme-based SERS immunoassay in the presence (the blue represents the experimental group) and absence (the black represents the control group) of antigens.

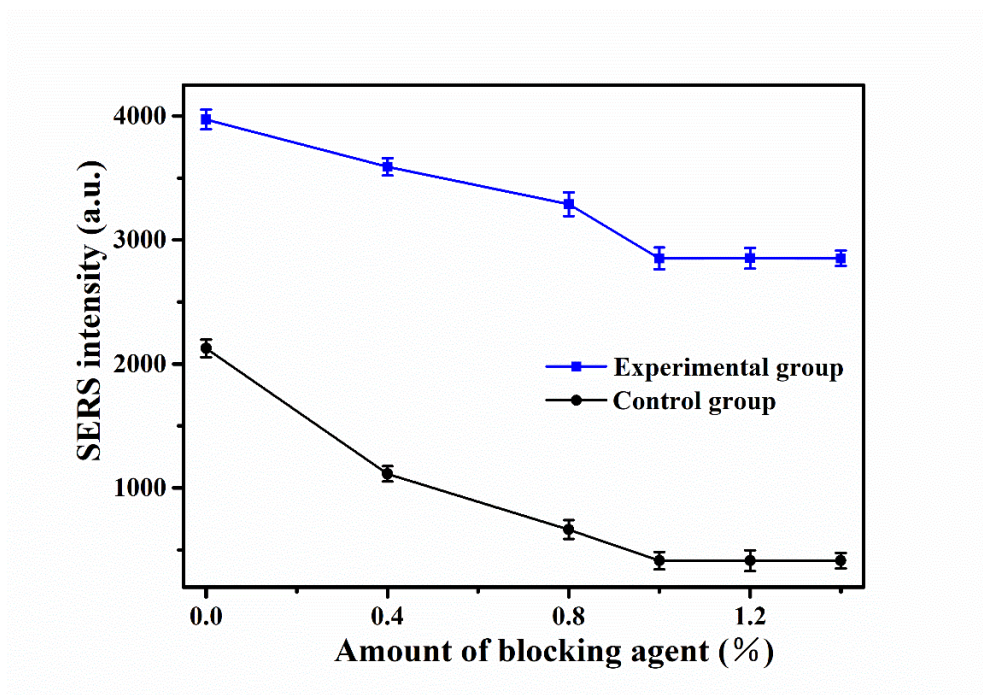


Figure S7. The optimization of blocking agent amount on the nanozyme-based SERS immunoassay in the presence (the blue represents the experimental group) and absence (the black represents the control group) of antigens.

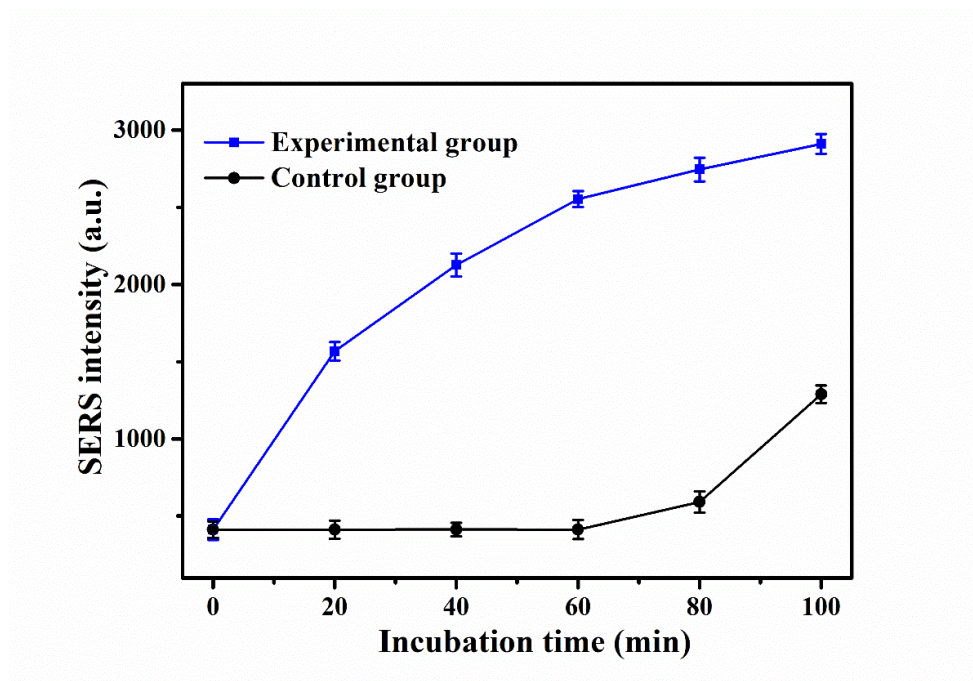


Figure S8. The optimization of incubation time on the nanozyme-based SERS immunoassay in the presence (the blue represents the experimental group) and absence (the black represents the control group) of antigens.

Table S1. Results of nanozyme-based SERS immunoassay for detection of α -LA in food samples

Food samples	Spiked concentration (mg/mL) ^a	Dilution factor	Detected concentration (mg/mL) ^a	Recovery (%)	RSD (%)
Raw cow milk	0	100,000	1.16		1.82
	1	100,000	2.15	99.00	3.24
	5	100,000	6.17	100.02	2.46
UHT cow milk	0	100,000	0.52		2.15
	1	100,000	1.50	98.00	0.93
	5	100,000	5.49	99.40	1.37
Peanut milk drinks	0	100,000	0.14		2.56
	1	100,000	1.15	101.00	2.43
	5	100,000	5.13	99.8	2.32
Walnut milk drinks	0	100,000	0.26		1.88
	1	100,000	1.23	97.00	1.92
	5	100,000	5.28	100.40	2.67

Note: ^aThe “mg/mL” is the unit of raw cow milk, UHT cow milk, peanut milk drinks, and walnut milk drinks.