

Short communication

Probing gold nanoparticles for the desensitization to β -lactoglobulin from binding mechanism, structure and IgE binding changes

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ABSTRACT

Because of the adsorption of proteins, gold nanoparticles (AuNPs) create potential biological risks in biomedicine, leading to the formation of the protein corona. This adsorption is mainly due to the formation of gold–sulfur (Au–S) covalent bonds between the AuNPs and the -SH groups, causing bioactivity denaturation and biological problems; however, it could also lead to some biological benefits. We explored AuNPs as a potential material for desensitization to allergens, such as β -lactoglobulin (β LG). To address the desensitization of AuNPs, we investigated the binding mechanism and the specific relationship of the time evolution of Au–S bond, secondary structure, and allergy changes. The formation of Au–S bond takes approximately 9 h, consistent with the complete changes time in secondary structure and immunoglobulin E (IgE) combining capacity of the β LG, decreasing allergic reactions. These results indicate that AuNPs have the potential to minimize allergic reactions in the future.

1. Introduction

Milk allergy can induce the most common and severe immediate hypersensitivity reactions in terms of persistence and severity (Canani et al., 2016; Wood et al., 2016). β -lactoglobulin (β LG) is considered as the main allergenic protein contributed to milk allergy (Zhang et al., 2019), which can be mediated by allergen-specific immunoglobulin E (IgE) antibodies and cause mast cell receptors to crosslink, inducing a signal transduction cascade and ending in the degranulation and releasing of a variety of mediators (Kurpiewska et al., 2019). Structurally, for stabilizing the spatial structure of β LG, two disulfide bridges between Cys66–Cys160 and Cys106–Cys119 work as the main force (Falahati et al. 2012). Breaking the disulfide bonds changes the structure and chemical properties of β LG, which could decrease the IgE binding and the allergic reactions caused by β LG (Liao et al., 2018), such as heating and hydrolyzing. However, these methods have some application limits in some cases (Kubota et al., 2016; Sari et al., 2015; Corzo-Martínez et al., 2017). Thus, developing a better desensitization method is important in food safety.

Gold nanoparticles (AuNPs) with high surface energy, physicochemical properties, and adequate biosafety in relation to human health have garnered significantly developments in the fields of biotechnology and biomedicine as an imaging and photothermal treatment (Zhang et al., 2017), in cancer therapy areas as targeted drug delivery (Silvia et al., 2010). Furthermore, it makes useful platforms for the treatment of some intractable problems in the fields of food, for example, they are commonly designed as sensitive colorimetric probes for the rapid detection of melamine, tetracycline, and tobramycin in raw milk (Ma et al., 2018; Ammar et al., 2016). However, when exposed to a biological matrix, such as blood or milk, AuNPs' surfaces can be coated by proteins, forming a protein corona (Cedervall et al., 2007), which consists of a hard corona with strong binding forces and a soft corona with weak forces to the AuNPs' surface (Wei et al., 2017; Zhang et al., 2018). It is well known that the protein corona may change the conformation of adsorbed proteins and then alter their normal function and activity, resulting in adverse effects in some applications (Wang et al., 2015; Visalakshan et al., 2019). Here, we proposed on turning this normally disadvantageous effect into an advantageous effect by

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exploring AuNPs as a novel strategy for allergic desensitization, such as desensitization of β LG.

Binding and denaturation are preconditions for AuNPs working as a desensitizer. The formation of protein corona through the adsorption of proteins on NPs surface is a dynamic process, which depends on many factors (Zhang et al., 2018), such as the size, charge, and chemical composition of the NPs and the nature of the adsorbed protein (Zhang et al., 2017). A protein corona can be formed by fast electrostatic forces (e.g., ionic interactions and hydrogen bonds), as well as by the slow formation of gold–sulfur (Au–S) covalent bonds (Wang et al., 2015). However, because of the dynamic formation and complexity of proteins, the structure and bioactivity changes induced by the protein corona, especially the specific relationship of Au–S bonds, secondary structure, and bioactivity changes are still unclear.

In our present work, the binding parameters, namely, molar ratio, binding affinity (K_a), and time evolution of the Au–S bonds, were determined to explore the binding mechanism. We investigated the specific relationships among Au–S bonds, the changes in the secondary structure of β LG, and the changes in β LG combining capacity with IgE to gain a better understanding of the desensitization mechanism of AuNPs adsorbed β LG. The results might lead to important applications of AuNPs in the field of desensitization, as well as in the fields of biotechnology and biomedicine.

2. Materials and methods

2.1. Materials

Bovine β LG from bovine milk, goat anti-human IgE-horse radish peroxidase (HRP), and tetrachloroaurate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) were purchased from Sigma Aldrich (St. Louis, MO, USA). Human serum samples from milk-allergic patients were generously provided by Shandong Children's Hospital (Jinan, China). Other reagents (analytical grade) were bought from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China).

2.2. Synthesis of AuNPs

AuNPs (~40 nm) were synthesized by the sodium citrate-based reduction method (Frens, 1973). In detail, the HAuCl_4 solution (100 mL, $10^{-2}\%$, w/v) was heated to boiling, and then sodium citrate (1 mL, 1%, w/v) was added vigorously. The solution color changed from clear to purple to bright red sequentially, after which keep boiling and stirring for 20 min. The resulting suspension was then cooled at room temperature under stirring and stored at 4 °C before usage. The concentration was calculated using the ultraviolet–visible method (Haiss et al., 2007).

2.3. Transmission electron microscopy (TEM)

AuNP- β LG conjugates were prepared by incubating AuNPs (5 mL, 5×10^{-4} μM) with β LG (1 mL, 50 μM) for 1 h (20 °C), and then we removed the unbound proteins by centrifugation (10 000 rpm for 5 min). A 50- μL phosphate buffer solution (PBS; 10 mM, pH 7.2) was added to re-suspend the sediment. To obtain coronas, we repeated the centrifuging and re-suspending steps three times. Then, to prepare samples for field emission TEM (Tecnai G2 G20 S-Twin, FEI, USA) observation, we dripped the re-suspended solution (5 μL) on aluminum stubs covered with a carbon substrate and then dried at room temperature.

2.4. Dynamic light scattering (DLS)

AuNP- β LG conjugates were prepared by incubating at the molar ratios (β LG:AuNPs) of 100:1, 500:1, 1000:1, 2000:1, 3000:1, 4000:1, 6000:1, 10000:1, and 30000:1, following the same preparation protocol

for TEM. The hydrodynamic diameters of the β LG, AuNPs, and AuNP- β LG conjugates were determined by DLS (Nicom 380; PSS Inc., Santa Barbara, CA, USA) with an average of three separate measurements. The collection time was set to 120 s for temperature equilibrium and 5 s during kinetic series acquisition.

2.5. Isothermal titration calorimetry (ITC)

ITC experiments were conducted using a MicroCal ITC-200 system (GE Healthcare, Milwaukee, WI, USA) in 10-mM PBS (pH 7.2) at 20 °C. A 30- μM β LG was titrated into the cell, which contained 200- μL AuNPs solutions (5×10^{-4} μM). The first injection was 0.5 μL , and the subsequent injection was 2 μL , consisting of 20 injections. To eliminate system heats, we also performed a blank titration.

2.6. Surface-enhanced Raman spectroscopy (SERS)

The SERS experiments were performed on a Jobin Yvon XploRA (Villeneuve d'Ascq, France). The conjugates were obtained by incubating β LG with AuNPs for 1, 4, 7, 9, and 11 h. The spectra were measured at 532 nm (10 mW) excitation, and Rayleigh scattering light was removed by an edge filter. The collection time was set to 30 s to record the signals from 200 to 800 cm^{-1} . All SERS spectra were the results of a single 1-s accumulation.

2.7. Fourier transform infrared spectroscopy (FTIR)

The AuNPs incubated with β LG for 1, 4, 7, 9, and 11 h, following the protocols as for TEM. FTIR spectra were obtained by a Bomem MB series FTR Spectrometer (Quebec, Canada) with a dTGS detector and constantly purged with dry air at 20 °C (Kong & Yu, 2007; Yang, Yang, Kong, Dong, & Yu, 2015). A 128-scan interferogram was collected in single-beam mode with a 4- cm^{-1} resolution. The relative secondary structural components were calculated according to the curve-fitting analysis of the amide I band from 1600 to 1700 cm^{-1} .

2.8. Allergic experiments

The IgE combining capacities of AuNPs- β LG conjugates were evaluated by an indirect enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA with minor modifications (Neiser et al., 2016). Briefly, microtiter plates polystyrene MaxiSorp 96 U-well (NUNC, Roskilde, Denmark) were coated with AuNPs- β LG conjugates (120 μL /well, 5 μg /mL) in PBS (10 mM, pH 7.2) for overnight. The 100- μL patient's serum (1:30 in PBS/Tween solution, PBST) was added in the wells, incubated 2 h at 37 °C after washing three times by PBST, and then blocked the non-specific free-binding sites by skimmed milk. Afterward, 100- μL goat anti-human IgE-HRP (1:200 in PBST) was added and incubated. Then, 100- μL tetramethylbenzidine was immediately added to each well after washing. The absorbance was measured at 450 nm using a Bio-Rad Microplate Reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA). For the experiments of inhibition ELISA, we followed the same protocols as for the indirect ELISA. The inhibition rate was evaluated as follows: % inhibition = $(1 - B/B_0) \times 100$, where B and B_0 are the absorbance of the wells at 450 nm with and without AuNPs- β LG conjugates, respectively.

3. Results and discussion

3.1. AuNPs- β LG conjugates and the molar ratio

TEM and DLS are used to characterize the protein corona from the image and size (Tenzer et al., 2013). Fig. 1 shows the TEM images and DLS spectrums of β LG, AuNPs, and AuNP- β LG conjugates. It can be deemed that the surface of AuNPs was covered by layers of β LG to form a protein corona (Fig. 1B). DLS measurements show that the

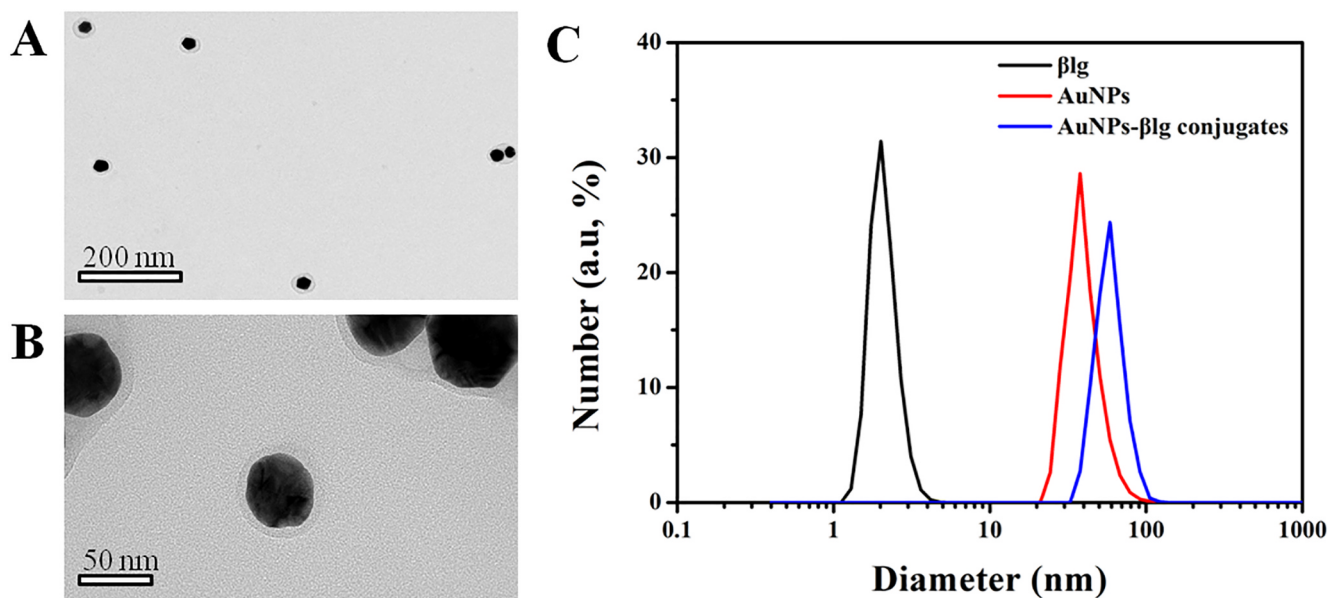


Fig. 1. A, B) The TEM images of AuNPs-βLG conjugates; C) typical DLS size-distribution for βLG, AuNPs, and AuNPs-βLG conjugates.

hydrodynamic diameters of the AuNPs increased dramatically from 36.7 ± 4.2 nm for AuNPs to 56.7 ± 5.1 nm for AuNP-βLG conjugates (Fig. 1C), which is attributed to the adsorption of βLG and not to NPs aggregation (Fig. 1A). The βLG bound layers onto AuNPs were estimated according to the corona thickness divided by the βLG diameter, and the theoretical binding number of βLG on a single AuNP was estimated by the minimal cross-sectional area (Lindman et al., 2007). Briefly, the surface area of the particles with half βLG diameter was divided by the βLG's cross-sectional area to obtain the binding numbers of βLG in each layer. For instance, the diameter of βLG molecules in the neutral condition is approximately 2.1 ± 0.8 nm (Fig. 1C). Thus, assuming four layers, made of hard and soft coronas, approximately 6.5×10^3 molecules are theoretically adsorbed on the surface.

The hydrodynamic diameter of AuNPs-βLG conjugates for different molar βLG/AuNPs ratios from 100 to 30 000 were also obtained by DLS measurements (Fig. S1). The results show that the diameter of the conjugates first increases with increasing of βLG concentration, and it reached a plateau of approximately 56 nm when the βLG/AuNPs molar is approximately 4000. It indicated that the surface coverage was approximately 60% of the theoretical number obtained above, which were based on assuming close-packing at the interface and spherical shapes of the βLG molecules.

3.2. Detection of K_a and stoichiometry

ITC is a direct method to study the protein-NPs interaction from some binding parameters, such as stoichiometry, K_a , and enthalpy change (ΔH). All the ITC data were analyzed by the published methods (Li et al., 2013).

K_a is one of the important criteria that describes the binding strength between proteins and NPs, and it can also give a general idea of whether one protein could be replaced by another one (Vilanova et al., 2016). The thermogram for βLG titrated into AuNPs solution was exemplified in Fig. 2. The negative injection signals implied a strongly exothermic process ($\Delta H < 0$). When the molar ratio reached approximately 7200, all of the AuNPs were coated and reached an equilibrium, which suggested that approximately 3600 βLG molecules adsorbed on a single AuNPs molecule (Fig. 2A). This value of approximately 3600 is in excellent agreement with the results obtained by DLS (Fig. S1).

The thermodynamic parameters of stoichiometry, K_a , ΔH , and ΔS

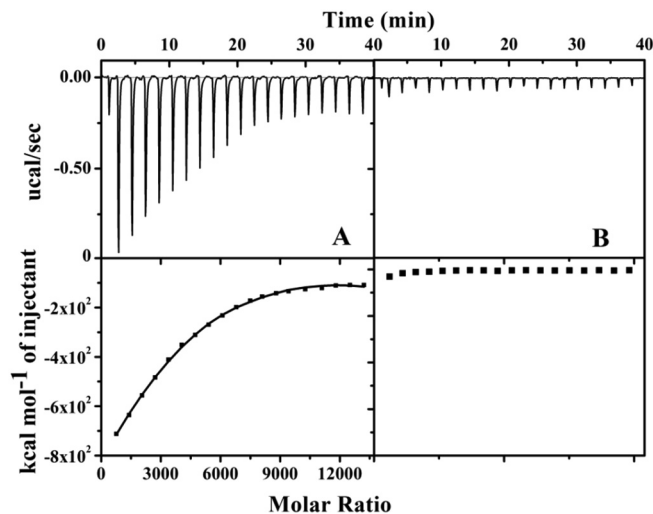


Fig. 2. A) ITC data from the βLG (30 μM) titrated into the AuNPs (5×10^{-4} μM) at 20 °C; B) 30-μM βLG titrated into PBS solution for comparison.

can be derived using nonlinear least-squares fitting (Table 1). The results indicate that βLG bound on AuNPs is an entropy-driven process (i.e., $\Delta S < 0$). K_a was measured as being $2.9 \pm 0.7 \times 10^6$ M⁻¹, which is well in agreement with that derived from fluorescence quenching measurement (2.5×10^6 M⁻¹) (Fig. S2 and Table S1). The K_a value suggests a moderate-strength binding (Creutz & Peters, 2017).

3.3. Detection of Au-S covalent bond

SERS has been proven to be an ultra-sensitive and reliable analytical tool for the detection of analytes from the single-molecule level (Reguera et al., 2017). βLG contains two pairs of disulfide bonds and a free cysteine residue, which can be easily attached to AuNPs in the formation of the Au-S covalent bond, proved by Raman spectroscopy at 296 cm⁻¹ (Zhang et al., 2009).

The covalent bond is the main force involved in the attachment of proteins on the surface of NPs, which can induce a change in the secondary structure and bioactivity of adsorbed protein (Fu et al., 2015).

Table 1Stoichiometry parameter, K_a , ΔH , and ΔS determined by ITC.

Temperature	Stoichiometry ^a ($\times 10^3$)	K_a ($\times 10^6$ M ⁻¹)	ΔH ($\times 10^6$ J M ⁻¹)	ΔG ($\times 10^4$ J)	ΔS ($\times 10^4$ J M ⁻¹ deg ⁻¹)
20 °C	3.6 $\pm$ 0.5	2.9 $\pm$ 0.7	-3.2 $\pm$ 1.3	-3.6	-1.1

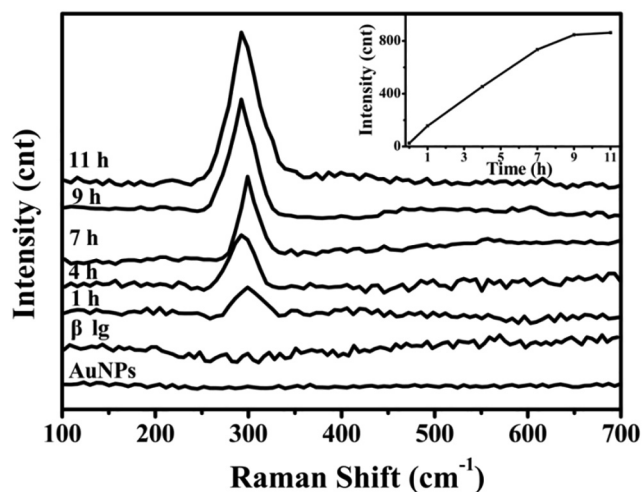
^a The values are derived from the one-site binding model.**Fig. 3.** Raman spectra of AuNPs, β LG, and AuNPs- β LG conjugates at different incubation times. Inset, the intensity of AuNPs- β LG conjugates as a function of incubation time.

Fig. 3 shows the SERS spectrum of AuNPs- β LG conjugates as a function of the incubation time. It can be seen that the conjugates exhibit a Raman peak at 296 cm^{-1} , and β LG and AuNPs alone do not show any peak at this shift. The intensity of Au-S bonds peak increases with time to reach a maximum after approximately 9-h incubation, indicating that formation of Au-S bonds takes approximately 9 h in the AuNPs- β LG conjugates. In β LG molecule, the disulfide bridges and the free thiol group are located at different places, as shown in Fig. S3A. Cys66-Cys160 bridge is inferred to contact with AuNPs surface quickly, whereas Cys106-Cys119 and the free thiol group (Cys121) located at the core region are supposed to require more time to come into contact with the AuNPs.

3.4. Detection of secondary structure

The second-derivative spectrum of the amide I band ($1700\text{--}1600\text{ cm}^{-1}$) has been used to characterize the secondary structure of proteins and polypeptides without the limitation of size or the physical state (Yang et al., 2015). To explore the specific relationship of Au-S bonds and the changes in secondary structures of β LG, we performed FTIR. Fig. 4 shows the fitted inverted second-derivative amide I curve of β LG in different time coronas, and Table S2 summarizes the resulting secondary structural components. The β -sheet (1628 and 1637 cm^{-1}) are the main secondary structural component of β LG (Fig. 4A). The results show that the significant changes in the secondary structure composition of β LG occurred over time after adsorbed AuNPs. The intensity of bands assigned to β -sheet and 3_{10} -helix (1663 cm^{-1}) decreased, and the bands assigned to α -helix (1658 cm^{-1}), β -turn (1667 and 1675 cm^{-1}), and random coil (1648 cm^{-1}) increased. After 9 h, the spectrum and structural composition remain unchanged (Fig. 4E), which is well consistent with the time needed to form the Au-S bonds. This result indicates that Au-S bonds are the main effect inducing the changes in secondary structure changes upon β LG adsorption on AuNPs.

3.5. Inhibition of IgE combining capacity

β LG is the main whey protein that is most frequently and intensively recognized by human IgE, which mediates type I hypersensitivity reactions, including both systemic and localized anaphylaxis. The combining capacity of IgE is the main assessment criteria for the allergic reaction, and it can be determined by indirect ELISA and inhibition ELISA (Neiser et al., 2016).

The formation of protein corona is a continuous dynamic exchange process where the proteins with higher K_a displace those with a lower one on the surface of NPs (Cedervall et al., 2007). The titration of IgE-HRP into AuNPs was also carried out to determine the binding parameters (Fig. S4 and Table S3). The results show that the K_a is $1.1 \pm 0.4 \times 10^6\text{ M}^{-1}$, which is lower than that of AuNPs- β LG ($2.9 \pm 0.7 \times 10^6\text{ M}^{-1}$), indicating that the adsorption of β LG on AuNPs would not be hindered by the presence of IgE (Zhang et al., 2018). We also conjugated β LG with FITC to obtain the β LG-FITC (β LG*) at pH 8 (Zhang et al., 2019), followed by incubation with AuNPs to form AuNPs- β LG* conjugates and then added IgE-HRP to monitor the β LG* fluorescence intensity changes over time (Fig. S5). The results show that the intensities were not changed, which confirmed that the absorbed β LG* would not be replaced by IgE during indirect ELISA and inhibition ELISA experiments (Zhang et al., 2018).

Fig. 5 shows the IgE binding capacity of β LG at a different time of AuNPs- β LG conjugates. The results show that the IgE combining inhibition increased with the increase of time and reached a maximum at approximately 9 h, which is consistent with the time required for the complete formation of Au-S bonds and the complete change in the secondary structure of β LG. This time point introduced the specific relationship of Au-S bonds, in which secondary structure changes and IgE combining capacity decrease when β LG adsorbed on AuNPs.

Three main IgE allergenic epitopes located at 41–60, 102–124, and 149–162, and most of these epitopes are assigned β -sheet (Hartmann et al., 2007). The three-dimensional model (Fig. S3B) indicates that these three epitopes are stabilized by a ring formed by disulfide and hydrogen bonds. The reaction of β LG and AuNPs needs to break the disulfide bonds of β LG, which would destroy the structure of these epitopes, diminish their IgE binding capacity, and decrease the stimulus of anaphylaxis. Furthermore, the FTIR confirmed a decrease in β -sheet contents in β LG molecules after conjugation with AuNPs, indicating the conformation change of the three main IgE allergenic epitopes.

4. Conclusion

In summary, the binding mechanism is the precondition to study the bioapplication of NPs. We assessed the binding parameters, namely, molar ratio, K_a , and time evolution of Au-S bond and general secondary structure changes to explore the desensitization mechanism. The results showed that approximately 3600 β LG molecules are adsorbed on a single AuNPs, and the K_a is $2.9 \pm 0.7 \times 10^6\text{ M}^{-1}$, indicating a moderate-strength interaction. The formation of Au-S bonds takes approximately 9 h, which is the time needed for complete changes in the secondary structure and IgE combining capacity. We found that the structure and conformation of allergenic epitopes in the β LG were destroyed by the formation of Au-S bond, which would diminish allergic reaction of β LG.

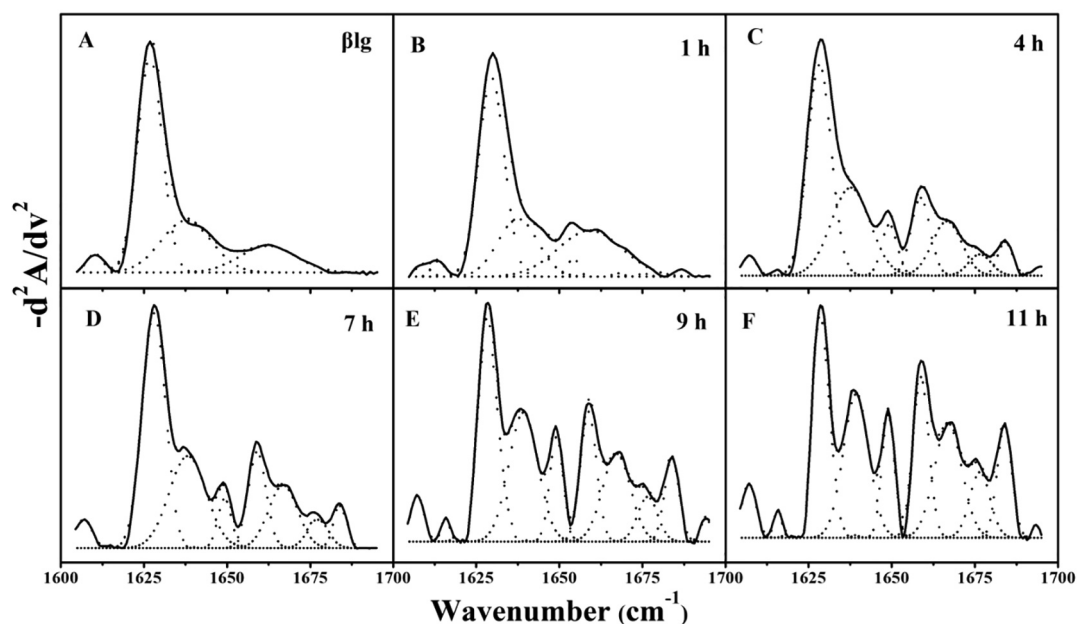


Fig. 4. Curve-fitted inverted second-derivative amide I (1600–1700 cm^{-1}) spectrum of β lg adsorbed on AuNPs at different incubation times: A) β lg alone, B) 1 h, C) 4 h, D) 7 h, E) 9 h, and F) 11 h.

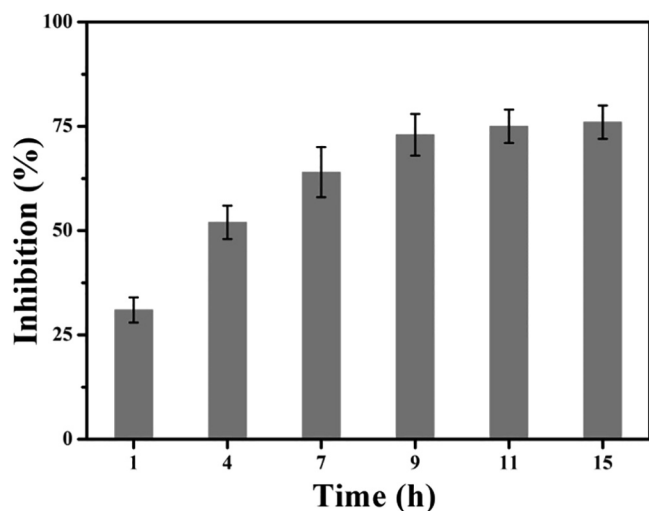


Fig. 5. The changes of IgE binding capacities of AuNPs- β lg conjugates at different incubation times.

CRediT authorship contribution statement

Xiaoning Zhang: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Meifeng Li:** Formal analysis, Software, Validation. **Yuanping Lv:** Funding acquisition, Writing - review & editing. **Xiaoling Sun:** Data curation, Methodology. **Yao Han:** Data curation, Methodology. **Bing Liu:** Funding acquisition, Resources, Visualization. **Xiangzhong Zhao:** Resources, Project administration. **Xiaowen Huang:** Conceptualization, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128329>.

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Supporting information

Probing gold nanoparticles for the desensitization to β -lactoglobulin from binding mechanism, structure and IgE binding changes

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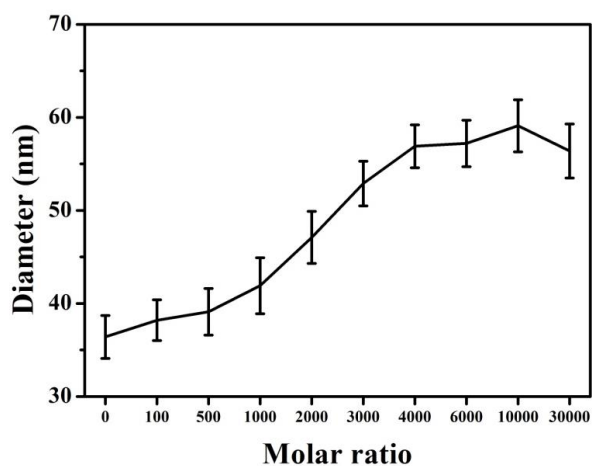


Fig. S1. Diameter as a function of AuNPs- β lg conjugates made with molar ratio of β lg/AuNPs. Bars corresponds to standard deviations.

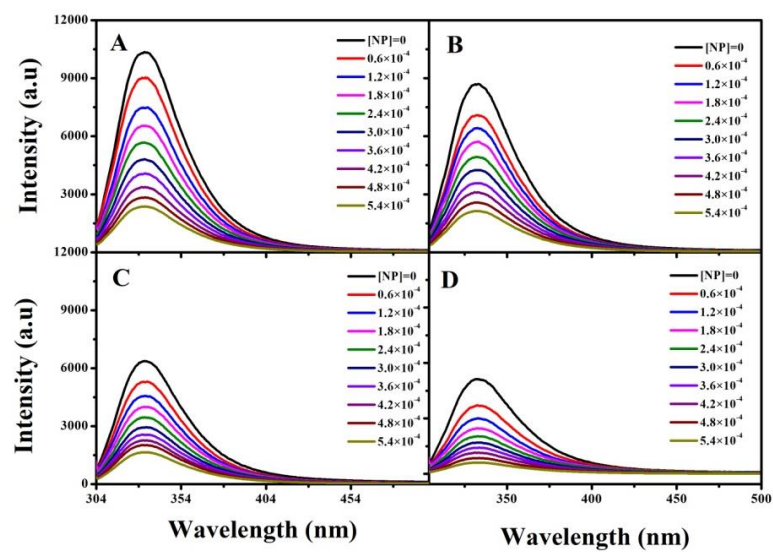


Fig. S2. Fluorescence quenching spectra of β lg (0.01 mM) by 40 nm AuNPs (0 to 5.4×10^{-4} μ M) at A) 0°C , B) 20°C , C) 40°C , and D) 60°C .

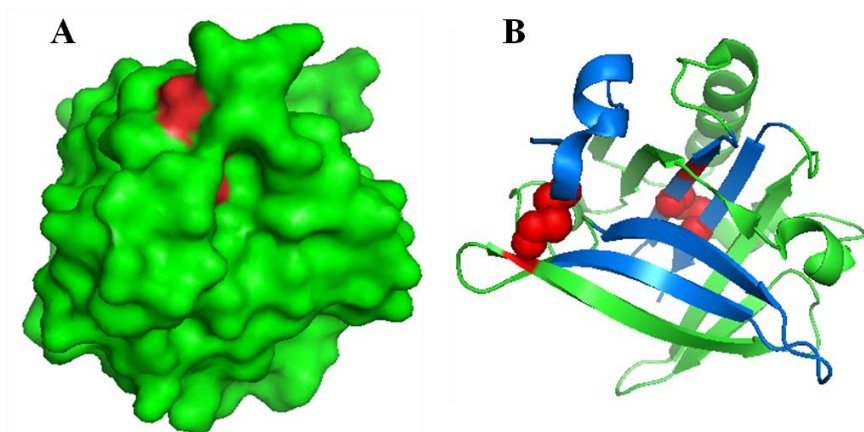


Fig. S3 A) The location of disulfide bridges and cysteine residues in the and marked in red. The Cys66-Cys160 is located on the surface, while, Cys106-Cys119 and free thiol group (Cys121) are located in the core region of the protein. B) The location of disulfide bridges and cysteine residues and marked in red, the IgE allergenic epitopes located at AA 41-60, 102-124, 149-162 marked in blue.

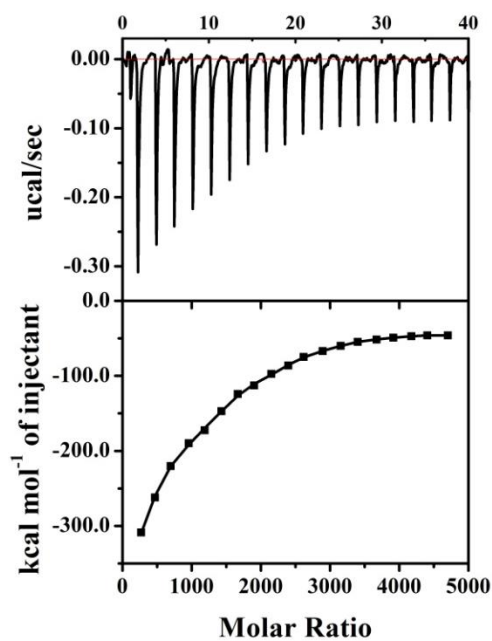


Fig. S4. ITC data. The titration of IgE-HRP (30 μ M) was titrated into the AuNPs (5×10^{-4} μ M) at 20 °C. (Upper) Raw data. (Lower) Integrated heats in each injection versus molar ratio of protein to NPs together with a fit using a one site binding model.

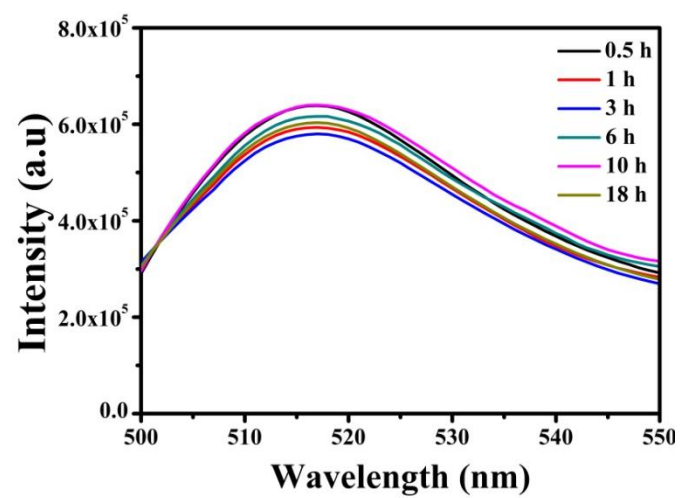


Fig. S5. The fluorescence intensity of βlg^* on the surface of AuNPs-protein conjugates after added IgE-HRP at different incubation time.

Table S1. The K_a and n of β lg adsorbed on AuNPs at different temperature determined by fluorescence quenching

Temperature	$K_a (\times 10^6 \text{ M}^{-1})$	n^a	R^2^b
0 °C	3.1	1.03	0.993
20 °C	2.5	0.97	0.989
40 °C	2.2	1.10	0.994
60 °C	1.8	0.95	0.992

^a n is the number of β lg binding site.

^b R^2 is the correlation coefficient from the least squares fit.

Table S2. Secondary structural component of β lg adsorbed to AuNPs- β lg conjugates as different incubation time as detected by FTIR.

Time	3_{10} -helix ^a	α -helix ^b	β -sheet ^c	β -turn ^d	Side chain ^e	Random coil ^f
0	15%	0	81%	0	4%	0
1 h	8%	8%	65%	12%	4%	3%
4 h	0	15%	55%	22%	6%	2%
7 h	0	21%	39%	30%	5%	5%
9 h	0	24%	32%	35%	3%	6%
11 h	0	22%	35%	33%	4%	6%

^a 3_{10} -helix is 1663 cm^{-1} , ^b α -helix is 1658 cm^{-1} , ^c β -sheet is 1628 and 1637 cm^{-1} , ^d β -turn is 1667 and 1675 cm^{-1} , ^eSide chain is 1617 cm^{-1} , ^fRandom coil is 1648 cm^{-1} in the FTIR spectrum.

Table S3. The binding parameters of IgE-HRP titrated into AuNPs determined by ITC

	Stoichiometry ($\times 10^3$)	K_a ($\times 10^6 \text{ M}^{-1}$)	ΔH ($\times 10^6 \text{ J M}^{-1}$)	ΔS ($\times 10^3 \text{ J M}^{-1} \text{ deg}^{-1}$)
20°C	1.9	1.1 ± 0.4	1.7 ± 0.8	5.6