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Fast and sensitive detection of protein concentration in mild environments

Chen Wang [a,b], Yue Zhou [a], Ke-Xin Zhang [a], Xing-Guo Nie [a,b], Xing-Hua Xia [a,*]

[a] State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China
[b] Department of Physical Chemistry, School of Science, China Pharmaceutical University, Nanjing 211198, China

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ABSTRACT

Determination of protein concentration in mild environments is of great significance in the clinic diagnose and bioassay. Herein, a simple, fast and sensitive method for protein quantitative determination in neutral solution (pH 7.0) is developed. This assay is based on competition adsorption of the sample protein and fluorescently labeled dog serum albumin (FITC-DSA) onto gold nanoparticles (AuNPs). As the competitor FITC-DSA molecules are added into the mixture solution of sample protein conjugated AuNPs, they will compete for active sites of AuNPs, resulting in decrease in fluorescence intensity due to the quenching effect of AuNPs via fluorescence resonance energy transfer (FRET). Thus, quantitative determination of sample protein concentration can be achieved. Under the optimum conditions, the decrease in fluorescence intensity of the solution is related to the concentration of sample protein and a low detection limit of 0.01 μg/mL BSA can be achieved in 5 min. For the validation of our strategy in practical applications, the total protein content in human serum was determined using the as-proposed method. The result is in well agreement with that of measured by other conventional methods, suggesting a simple, accurate, and mild approach for protein detection in bioassay.

1. Introduction

Proteins play fundamental roles in growth and repair of biological cells, metabolism, origin and evolution of life, virtually participating in every process in organisms. In nature, many proteins often express at low abundance. Therefore, the recognition and quantification of low-abundance proteins are crucial in medical diagnosis, prevention, and treatment and others [1]. Till today, various methods have been developed for the determination of protein concentration such as colorimetric and fluorescence method. Colorimetric methods including biuret assay [2], Lowry [3], BCA (bicinchoninic acid) [4], and Bradford [5] are usually available and easy to be performed. However, they are easily interfered by contaminants, and the precision and sensitivity are often relatively low. In contrast, fluorescence technique has been proved to be highly sensitive. Several fluorescence-based methods have been developed for protein quantification including CBQCA (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) [6], OPA (o-phthaldialdehyde) [7], and NanoOrange [8]. In these methods, the procedures are relatively laborious and time-consuming, which usually require a heating step and long incubation time. It is desirable to develop rapid, sensitive, and simple analytical approaches for detection of trace amount of proteins.

Fluorescence resonance energy transfer (FRET) occurs efficiently when the distance of a donor molecule to an acceptor molecule is in the range of 1–10 nm. This concept has been extensively adopted in protein analysis [9–11]. Gold nanoparticles (AuNPs) are usually used as excellent quenchers in a FRET system instead of traditional organic quenchers due to the high fluorescence quenching efficiency, stable optical property, and ease of labeling [12]. The chromophore in close proximity to AuNP experiences strong electronic interaction with the particle surface, resulting in donation of the excited electrons to gold nanoparticle, and therefore a nearly complete quenching of the fluorescence occurs. On the other hand, as a kind of biocompatible nanomaterials, AuNPs can serve as perfect adsorbents in protein adsorption due to the reproducible size and variable surface properties. Thus, gold nanoparticles have been often used to improve the efficiency and sensitivity of proteins quantification. For example, Zhu et al. presented a sensitive protein detection method based on click-chemistry using AuNPs. A low detection limit of 0.2 μg/mL protein was achieved in less than 10 min [13]. Pihlasalo et al. developed a series of highly sensitive methods for protein quantification based on competition adsorption of sample and labeled protein onto the same particles [14–20]. In these methods, time-resolved luminescence resonance energy transfer (TR-LRET) [14], or FRET [15–19] techniques were used and thus low detection limit of 500 pg (7.0 μg/mL) for bovine serum albumin (BSA) was achieved [15], which is lower than that of the most sensitive commercial methods (20 ng for the NanoOrange method [8], 10 ng for CBQCA method [6], and 100 ng

* Corresponding author. Tel./fax: +86 25 83597436.
E-mail address: xhxia@nju.edu.cn (X.-H. Xia).

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for the widely used Bradford method [5]). These methods are of great significance in pushing forward the progress of protein quantitative analysis. However, an acid environment (pH 3.0–5.0) has to be used in all these assays. It is well-known that a wide range of pH values exist in biological systems (*e.g.*, pH 1.5–3.5 in the human stomach; pH 4.7 in lysosomes and pH 7.4 in human blood). Among these, blood detection is most often required in clinical diagnosis. Therefore, protein analysis in neutral pH (7.0–7.4) environments is of great significance.

Herein, a simple protein quantitation method in neutral environments using the FRET technique is developed. First, sample protein is physically adsorbed to AuNPs by simply mixing with AuNPs in PBS buffer (10 mM, pH 7.0). Then, dye-labelled protein as competitor is added into the above mixture. The competition adsorption of competitor protein with sample protein on AuNPs occurs (Scheme 1). As soon as the labelled protein adsorbs on the AuNPs, its fluorescence will be effectively quenched by AuNPs due to the FRET, thus, the total fluorescence intensity of the system will decrease after the competition reaction. High concentration of sample protein leaves less surface active sites of AuNPs for competitor, while low concentration of sample protein leaves more surface active sites of AuNPs for the competitor. Thus, quantitation of sample protein can be made simply by measuring the fluorescence change. As demonstration, bovine serum albumin (BSA) and fluorescein isothiocyanate labelled dog serum albumin (FITC-DSA) are respectively used as sample protein and competitor protein. Using the present method, a trace concentration of sample protein as low as 0.1 μg/mL can be successfully monitored in 5 min, with a detection limit down to 0.01 μg/mL. For the validation of the present strategy in practical applications, human serum is used as sample protein for total protein concentration measurement. The result is consistent with the ones obtained using other conventional methods, suggesting the potential application of the as-proposed method in bioassays and clinical diagnosis.

2. Experimental

2.1. Reagent and instruments

Phosphate buffer (PBS, pH 7.0, 10 mM) solution was used as the buffer system. Proteins including fluorescein isothiocyanate labelled dog serum albumin (FITC-DSA), bovine serum albumin (BSA), myoglobin, glucose oxidase (GOx), lysozyme and β-casein were obtained from Sigma and used as received. Human serum was purchased from BioSino Bio-technology and Science Inc. (Beijing, China). Sodium citrate, NaOH, $CuSO_4$ and $HAuCl_4$ were purchased from Nanjing Chemical Reagent Company. All the chemicals and solvents were of analytical purity and used as received. All solutions were kept in a freezer to prevent deterioration. All liquid samples were filtered with a 0.22 μm syringe filter to remove particulates before use. All aqueous solutions were prepared from deionized water (18 MΩ cm, PURELAB Classic, PALL, USA). An UV–vis spectrophotometer (Shimadzu UV-3600) was used to measure absorption spectrum of AuNPs solutions. Fluorescence spectrometry was conducted on a fluorescence spectrophotometer (Cary Eclipse) with excitation at 495 nm and emission at 525 nm wavelengths. The morphology of spherical gold nanoparticles was characterized using high resolution transmission electron microscope (TEM, JEOL-JEM-200CX microscope, Japan) operated at 200 kV.

2.2. Synthesis of AuNPs

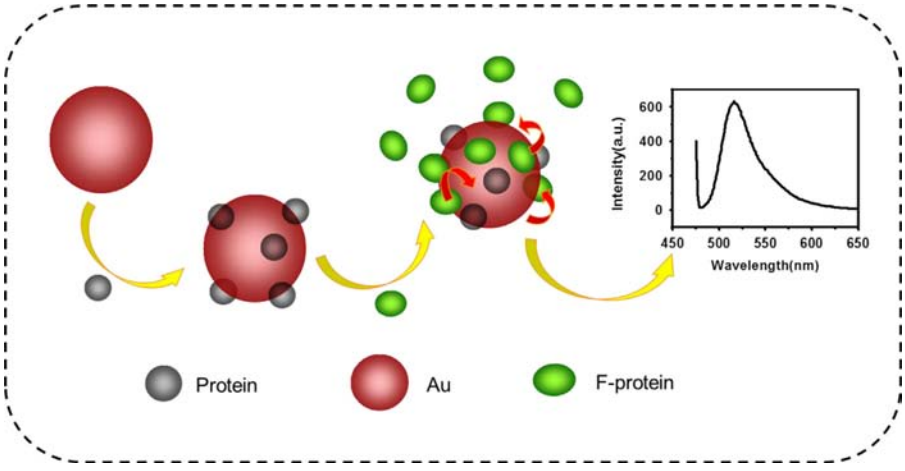
All the glassware used were cleaned in a bath of freshly prepared 3:1 HCl:HNO_3 and rinsed thoroughly with H_2O prior to use. Colloidal gold solutions of *ca.* 13 and 25 nm in diameter were prepared by citrate-reduction of $HAuCl_4$ as previously described [21]. Preparation of "seed colloid" gold solution of *ca.* 2–4 nm-diameter particles was performed by adding 1 mL of 1% aqueous $HAuCl_4{\cdot}3H_2O$ to 100 mL of H_2O with vigorous stirring, followed by the addition of 1 mL of 1% aqueous sodium citrate within 1 min. After an additional reaction for 1 min, 1 mL of 0.075% $NaBH_4$ in 1% sodium citrate was added. The solution was stirred for 5 min and then stored at 4 °C [22,23].

2.3. Fabrication of BSA/AuNPs conjugation

For sample preparation of BSA/AuNPs conjugation, different volume of BSA (10 μg/mL in 10 mM PBS buffer, pH 7.0) solution was prepared and then mixed with 100 μL AuNPs suspension. The reaction mixture was incubated for 5 min at room temperature for conforming the formation of the BSA/AuNPs conjugation [22]. TEM images and UV–vis spectra were used to characterize the changes in size of the AuNPs before and after BSA adsorption.

2.4. Competitive protein assay

Different concentrations of sample protein BSA were mixed with AuNPs solution. Subsequently, FITC-DSA was added into the reaction mixture, and incubated for 5 min at room temperature. Then the fluorescence spectra were measured using a Cary Eclipse fluorescence spectrophotometer with excitation at 495 nm and emission at 525 nm wavelengths.



Scheme 1. Schematic illustration of AuNPs-based FRET for protein detection. Competitor protein (FITC-DSA) competes for surface active sites of AuNPs previously occupied by sample protein (BSA). The remaining FITC-DSA molecules fluorescence labelled in solution can be monitored using a fluorescence spectrophotometer .

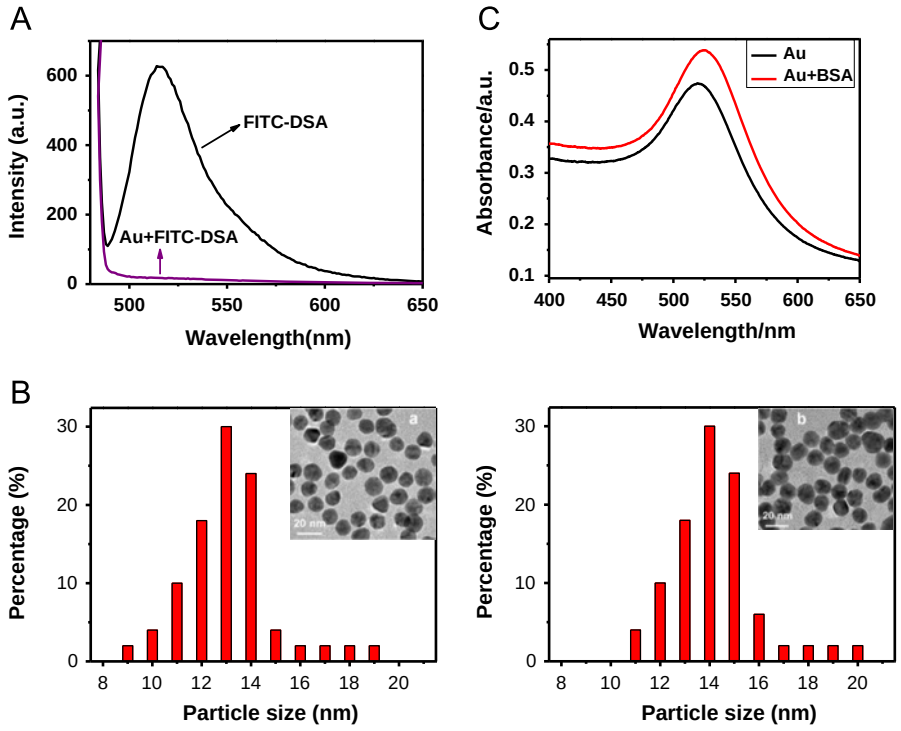


Fig. 1. (A) Fluorescence emission spectra of FITC-DSA before and after AuNPs addition upon excitation at 495 nm; (B) TEM images of the AuNPs before (a) and after (b) BSA adsorption; (C) UV–vis spectra of the AuNPs before and after BSA adsorption. The AuNPs solution has been diluted by 10 folds using PBS buffer before UV–vis spectra measurements.

2.5. Real serum sample assay

Diluted real human serum instead of BSA was used as sample protein to perform the same experiment as described above.

3. Results and discussion

3.1. Synthesis of AuNPs

The size and morphology of AuNPs were investigated using UV–vis absorption and TEM characterizations. As shown in Fig. 1A, B and Fig. S1, the AuNPs are nearly monodispersed with an average size of 13.0 ± 1.5 nm and 25.0 ± 2.5 nm. The surface plasmon band at about 520 nm wavelength was used to estimate the concentration of the AuNPs. Using the present method, the AuNPs concentration of $\sim$17.0 nM was deduced according to the Beer–Lambert's law using an extinction coefficient of $\sim 2.7 \times 10^8\ M^{-1}\ cm^{-1}$ [25].

3.2. Characterization of conjugation of protein to AuNPs

The constructed protein detection sensor relies on the competitive adsorption of sample protein and competitor protein to the surface of AuNPs. The adsorption phenomenon is influenced by multiple interactions, including electrostatic, hydrogen bonding, hydrophobic, and van der Waals forces. The pH value plays important function in the weak interactions. Since the isoelectric points of most proteins are below 7, a low pH (< 7.0) is favorable for protein adsorption through coulombic force for the gold particle surface carries negative charges. However, a wide range of pH values exist in biological system, and a neutral environment (pH 7.0–7.4) is crucial in bioanalysis and clinical diagnosis. In a recent study, the adsorption and conformation of BSA on gold nanoparticles has been investigated in detail [24]. The results indicated that in neutral pH (7.0–7.4), the BSA exists in normal conformation leaving a free thiol (Cys-34) accessible on the external surface of globular BSA, which can be used for efficient protein immobilization onto AuNP surface. In addition, there are a lot of $-NH_2$ groups in protein, which can also react with AuNPs besides physical interaction between protein and nanoparticle. All these interactions help adsorption of protein to AuNPs.

To investigate the adsorption behavior of protein to AuNPs in neutral environments, FITC-DSA is used as the model protein, and the change in fluorescence of the system is monitored. It is found that once the AuNPs solution is added, the fluorescence of FITC-DSA decreases due to FRET (Fig. 1A), indicating successful adsorption of FITC-DSA onto the AuNPs surface. A quenching efficiency of nearly 100% is obtained by comparing the fluorescence intensities before and after addition of AuNPs. The TEM images and UV–vis spectra of AuNPs solution before and after physically adsorption of BSA are shown in Fig. 1. The TEM images (Fig. 1B) shows no obvious difference in the size of AuNPs and BSA/AuNPs (diameter of *ca.* 13.3 and 14.2 nm, respectively). However, the observed 5 nm shift (from 520 nm to 525 nm) in absorption band of AuNPs in UV–vis spectra (Fig. 1C) further confirmed the existence of interactions between AuNPs and proteins.

3.3. Optimizing the ratio of protein to AuNPs

It is well-known that AuNPs have good biocompatibility, high fluorescence quenching efficiency and stable optical property. It has been widely used as excellent quenchers in the FRET system for bioanalysis. However, to eliminate the fluorescence background for achieving higher detection sensitivity, the ratio of protein to AuNPs should be optimized. We use FITC-DSA as the model protein to investigate the adsorption behavior of protein to AuNPs. Different volume of AuNPs was added into 20 μl FITC-DSA solution (80 μg/mL in 10 mM PBS buffer, pH 7.0), and then was diluted by PBS buffer to obtain a final concentration of 4.0 μg/mL FITC-DSA. The concentrations of AuNPs were calculated according

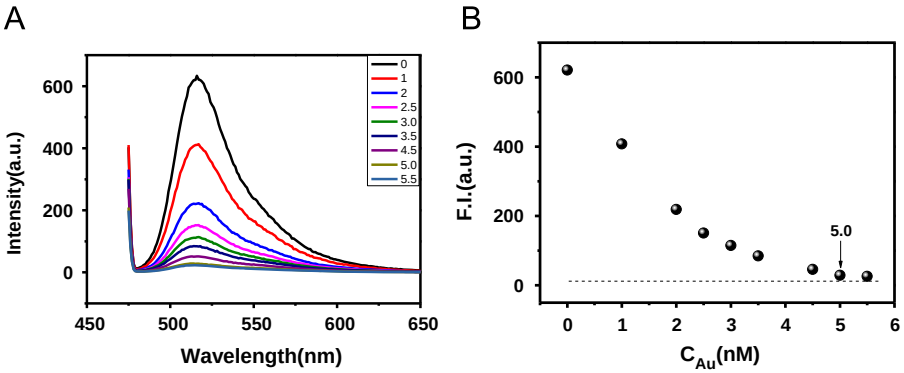


Fig. 2. (A) Fluorescence emission spectra of FITC-DSA in the presence of various concentrations of AuNPs following incubation for 5 min at room temperature upon excitation at 495 nm. The AuNPs concentration increases from 0 to 5.5 nM (from top to bottom); (B) corresponding fluorescence intensity of the solution as a function of AuNPs concentration.

to the volume of added AuNPs. Fig. 2A indicates the fluorescence spectra of nanoprobes with a different ratio of FITC-DSA to AuNPs. The corresponding fluorescence change *versus* AuNPs concentration is shown in Fig. 2B. The optimum ratio of protein to AuNPs (under which the background interference is minimal) is determined as 12.0:1.0 in the solution containing 5.0 nM AuNPs and 4.0 μg/mL FITC-DSA. The surface area of 13 nm AuNP is calculated as 530 nm^2 using the equation of $S=4\pi r^2$ (r is the radius, 6.5 nm). The surface area of BSA is about 25.9 nm^2 based on an equilateral triangular model for globular N-form BSA with 8 nm edges and 3 nm thickness [24,26]. Due to relatively larger size of serum protein compared to small molecule FITC and the similar structures between BSA and DSA, we approximately used the BSA size as that of FITC-DSA. Therefore, about 58.6% ($12\times 25.9/530$) of the maximum monolayer surface coverage is achieved according to adsorption of 12 protein molecules per AuNP. The result is in good accordance with the previous report [24], which is also similar to the results reported by Härmä et al. [17]. The apparent submonolayer coverage could be due to intermolecular electrostatic repulsion and steric hindrance between neighboring proteins absorbed on AuNPs surface.

To investigate the effect of particle size on the protein adsorption and quenching, 25 nm AuNPs solution is also used as absorbent for the FRET assay. The fluorescence emission spectra of FITC-DSA before and after addition of 25 nm AuNPs are shown in Fig. S2. The similar quenching phenomenon occurs for the 25 nm AuNPs. The maximum quenching efficiency with sufficient AuNPs is calculated as 98.9%. However, less amount of gold is required for 13 nm AuNPs if the same sensitivity and signal response in the assay are considered due to the larger specific surface area for smaller nanoparticles. Therefore, AuNPs of 13 nm is used for the following measurements.

3.4. Protein concentration determination

Rapid signal response is an important factor which should be considered in sensitive protein concentration measurement. As previously described, successful adsorption between AuNPs and proteins occurs once the AuNPs are mixed with the protein solution. To make sure the time needed for competitive adsorption of labelled protein to BSA/AuNPs conjugation, the change of fluorescence intensity upon addition of FITC-DSA into the BSA/AuNPs system was investigated. The result is shown in Fig. 3 (the relative fluorescence intensity, I/I_0 is indicated in supporting information, as Fig. S3). The fluorescence intensity changes rapidly within the first 3 min, then levels off. The result indicates that competitive adsorption of labelled protein to the BSA/AuNPs conjugation occurs very

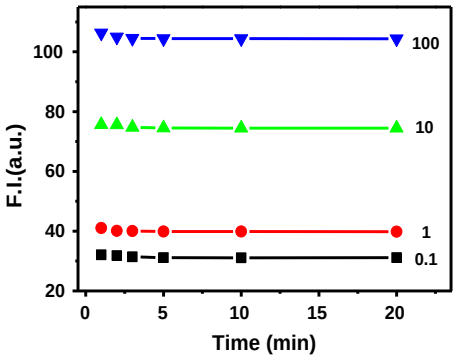


Fig. 3. Fluorescence intensity of the BSA protein assay using 13 nm AuNPs with different sample protein concentrations (0.1–100 μg/mL) as a function of time after adding FITC-DSA (4.0 μg/mL).

fast. In the following work, the fluorescence spectra were all recorded after 5 min mixing.

For protein concentration determination, the reaction mixture was prepared using the optimal conditions (12.0:1.0 for protein to AuNPs) by keeping the final concentration of AuNPs and FITC-DSA respectively 5.0 nM and 4.0 μg/mL (~60.0 nM). The fluorescence intensities of the reaction mixture at varied BSA concentrations are shown in Fig. 4. The more sample protein BSA in the medium, the less surface active sites of AuNPs leave for the competitive adsorption of FITC-DSA, resulting in more free FITC-DSA molecules in solution, and accordingly higher fluorescence intensity. It is found that trace amount of BSA as low as 0.1 μg/mL can be successfully detected in 5 min using the proposed assay. The similar detection sensitivity for BSA can be achieved using 25 nm AuNPs, as indicated in Figs. S4 and S5. These results are comparable to the reported ones previously [12], but slightly larger than other reports [13–15]. This could be due to the change of solution pH (from acid to neutral), which would influence the adsorption of protein to nanoparticles.

To test the general applicability of our approach, we investigated the response of the assay to four different proteins of BSA (MW 66,000 g/mol, isoelectric point (pI) 4.6), glucose oxidase (GOx, MW 156,000 g/mol, pI 4.2), β-casein (MW 24,000 g/mol, pI 4.6), myoglobin (MW 16,000 g/mol, pI 7.07). A series of concentrations of each protein ranging from 0.001 to 1000 μg/mL were measured using the proposed method. The total protein concentration of four proteins mixture was also measured. The results are shown in Fig. 5. The reproducibility experiments were conducted by repeatedly measuring five parallel reaction mixtures under

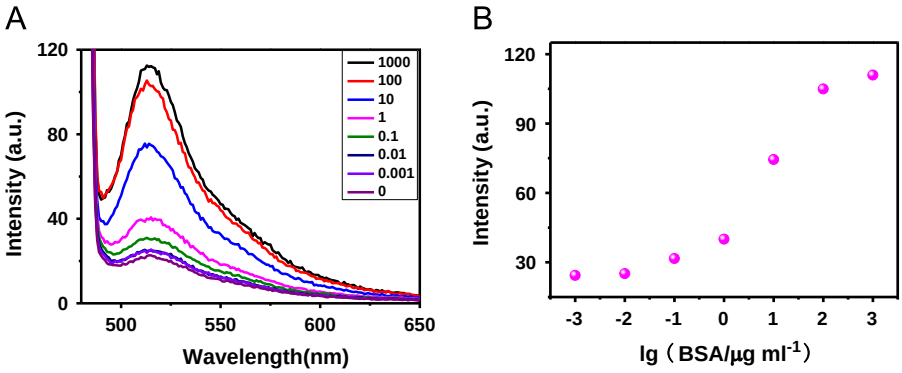


Fig. 4. Fluorescence spectra of 4 μg/mL FITC-DSA solution in presence of varied concentrations of BSA and AuNPs mixture upon excitation at 495 nm at pH 7.0 in 10 mM PBS. The BSA concentration increases from 0 to 1000 μg/mL (from bottom to top); (B) corresponding fluorescence intensity of the mixture as a function of logarithm of BSA concentration (lgC_{BSA}).

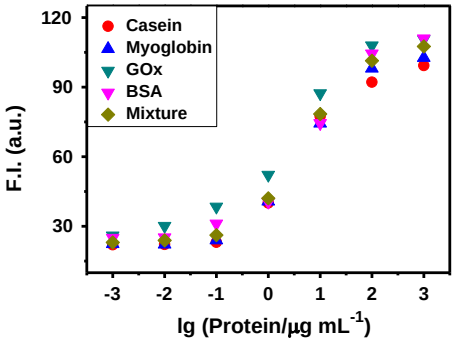


Fig. 5. Fluorescence intensity of the reaction solution as a function of the logarithm of proteins concentration ($lgC_{protein}$) for different proteins.

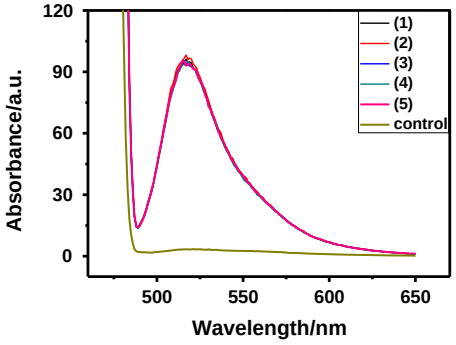


Fig. 6. Fluorescence spectra of 4 μg/mL FITC-DSA solution in the presence of 2000-fold diluted human serum and AuNPs mixture upon excitation at 495 nm at pH 7.0 in 10 mM PBS.

Table 1
Detection of total protein in serum using three different methods.

Sample numbers	Methods	Total protein concentration (mg/mL)	RSD (%) ($n=5$)
1	As-proposed strategy	67.2	3.2
	Biuret assay	65.1	4.2
	UV–Vis	76.3	3.2
2	As-proposed strategy	68.6	3.8
	Biuret assay	65.2	4.2
	UV–Vis	75.1	3.5

the same conditions. The quite low relative standard deviation (RSD) value (ranging from 2.1% to 5.2%) indicates a relatively good reproducibility. As expected, these four proteins almost present the same trend for concentration detection, and the calibration curves of the each protein nearly overlap, indicating the similar adsorption properties of these proteins. However, it is worthy to note that GOx has a 10 folds lower detection limit (0.01 μg/mL) as compared to other three proteins. This is because GOx has a larger molecular weight and thus a bigger volume, which occupies more surface active sites of the AuNPs than the smaller ones. Therefore, even 10 fold lower concentration of GOx can be sensitively monitored using our method.

3.5. Real serum sample assay

For the validation of our strategy in practical applications, five parallel diluted human serum samples were analyzed quantitatively using the proposed method. The result is shown in Fig. 6. It is clear that very similar results of five parallel experiments were achieved, indicating the potential application of the present method. Using the calibration curves in Fig. 4B, the total protein concentration in the human serum sample is calculated as 67.2 mg/mL.

For comparison, two other methods including the conventional biuret assay and the UV–vis method were also used for total protein concentration determination in serum sample. The results are listed in Table 1 and Figs. S6 and S7. All the data were obtained by averaging five parallel measurements. It can be seen that the results from three different methods are similar, suggesting that our strategy shows potential application in clinic analysis and bioassay.

4. Conclusion

In summary, we have proposed a simple, fast, and sensitive method for quantitative determination of trace amount protein in neutral environments. This approach relies on competitive adsorption of sample protein and competitor protein on AuNPs surface. A super low detection limit down to 0.01 μg/mL of BSA can be achieved within 5 min, which is more sensitive than commercial protein quantification methods reported. Using the present method, the total protein content in human serum can be determined, and the measured value is compared to the ones determined using other methods. Results show that the proposed method is accurate and precise, providing an easy and sensitive approach for protein concentration determination in clinic diagnose and bioanalysis.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.046.

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