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Graphical Abstract

Synthesis of ovalbumin-stabilized AuNCs highly fluorescent gold nanoclusters and their application as Hg^{2+} sensor

H. Shi, M. Y. Ou, J. P. Cao and G. F. Chen
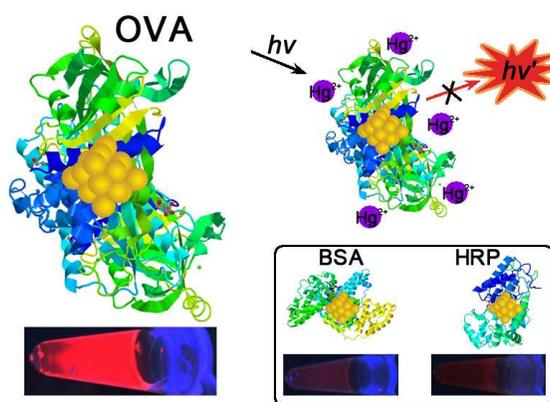
Highly fluorescent Au nanoclusters were synthesized by using ovalbumin (OVA) as the template. Application of the nanoclusters for the detection of mercury ions was also achieved.

Journal Name

ARTICLE

Synthesis of ovalbumin-stabilized highly fluorescent gold nanoclusters and their application as Hg^{2+} sensor

H. Shi, M. Y. Ou, J. P. Cao and G. F. Chen

Biomolecules-functionalized fluorescent gold nanoclusters (AuNCs) have attracted much attention because of their good biocompatibility and considerable environmental/cost advantages. Recently, some proteins rich in tyrosine and cysteine have been proven to work as templates for the direct synthesis of AuNCs under alkaline. However, the low quantum yield (QY) of AuNCs is still a restraining factor, which constrains its wide applications. In this work, highly fluorescent AuNCs have been synthesized in basic aqueous solution by using ovalbumin (OVA) as reducing and stabilizing agent. The QY of ovalbumin-stabilized AuNCs (OVA@AuNCs) was the twice of the reported BSA-stabilized AuNCs (BSA@AuNCs) under same conditions. Moreover, the good pH stability and time stability of the OVA@AuNCs were examined. These properties will be helpful for AuNCs-based sensing and imaging. Further research revealed that the fluorescence of OVA@AuNCs could be quenched by Hg^{2+}, and it can be used as a sensor for sensitive Hg^{2+} detection with a detection limit of 10 nM.

Introduction

Metal nanoclusters, composed of a few to tens of atoms, are a new type of luminescent nano-materials that have attracted a deal of interest.[1-3] Compared with nanoparticles, a distinct feature is that they have some characteristic properties such as photoluminescence, photostability, and stokes shift. AuNCs with sizes smaller than 3 nm are a specific type of nano-materials. Unlike the well-known large gold nanoparticles, AuNCs do not exhibit surface plasma resonance (SPR) absorption in the visible region. Instead of this, AuNCs can be excited fluorescence under UV light. With advantages of long lifetime, discrete electronic states, size-dependent fluorescence, AuNCs have attracted much attention in bio-imaging and bioanalysis.[4-7]

The preparation of AuNCs from Au^{3+} in the presence of small thiol compounds such as 2-phenylethanethiol (PhCH$_2$CH$_2$SH) has been reported over the past decade.[8] However, the low quantum yield (usually less than 1%), poor water dispersibility, and instability limited their bioapplications. In the past decade, two major categories for the preparation of stable, water dispersible AuNCs have been reported. The first category is an etching process using polymers,[9] and another category is a chemical reduction in the presence of thiol ligands.[10, 11] For chemical reduction, chemicals, polymers, and biomolecules that act as capping agents are required for the preparation of stable and good fluorescent AuNCs. Biomolecules such as peptides and proteins can be used as structure-defined scaffolds to induce the nucleation and growth of AuNCs. In recent years, an interesting method has been developed to synthesize AuNCs by using proteins as sole reduction agents.[12-15]

It was suggested that rich tyrosine (Tyr) and cysteine (Cys) residues in proteins are critical to produce the protein-stabilized AuNCs. This is because Tyr residues can reduce Au(III) ions in alkaline pH and Cys residues, similar to thiol-protected AuNCs, are able to stabilize AuNCs. The synthesis of proteins-stabilized AuNCs is a simple, one-pot synthetic route and these proteins are some common commercially available. Moreover, the synthesized proteins-stabilized AuNCs have good biocompatibility, stability, and considerable environmental/cost advantages. However, the QY of synthesized proteins-AuNCs is still low in comparison with Ag nanoclusters (QY: 95% in ethanol),[16] which will limit the wide application of proteins-stabilized AuNCs in bio-imaging and bioanalysis. So it is still necessary to develop the new synthetic method of proteins-stabilized AuNCs to enhance the QY.

Inspired by previous works, we wonder whether other proteins, which contain rich Cys and Tyr residues, can also be applied to synthesize fluorescent AuNCs. After several attempts, we found that AuNCs could be synthesized by using OVA acted as a reducing and stabilizing agent. OVA is an N-linked glycoprotein derived from chicken egg white that is made up of 385 amino acids. It is a strong candidate for the synthesis of protein-stabilized AuNCs, since OVA contains rich amino acid residues with 6 Cys and 10 Tyr. The preparation of OVA@AuNCs was easy mixing OVA and chloroauric acid (HAuCl$_4$) under basic conditions. The pH stability, time stability of OVA@AuNCs and the effectiveness of fluorescent-based heavy metal ion sensing were examined. Moveover, the synthesized OVA@AuNCs had high QY (~12%), which was the twice of the reported BSA-stabilized AuNCs (BSA@AuNCs) (QY: ~6%). In this work, we also demonstrated that the highly fluorescent AuNCs could be designed as Hg^{2+} sensor with sensitivity.

Experimental section

Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai 200444, PR China.

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Chemicals and materials

$HAuCl_4$, OVA, sodium hydroxide (NaOH), horseradish peroxidase (HRP), manganese chloride, potassium chloride, lead chloride, zinc chloride, cobalt chloride, nickel chloride, ferric chloride, ferrous chloride, cadmium chloride, copper chloride, and silver nitrate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Bovine serum albumin (BSA), hydrochloric acid, barium chloride, magnesium chloride, calcium chloride, and mercuric acetate were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Water used throughout all experiments was purified by a Milli-Q system (Branstead, USA) to a specific resistance of > 18 $M\Omega \cdot cm$.

Synthesis of ovalbumin-stabilized fluorescent gold nanoclusters

The OVA@AuNCs sample was prepared according to the following procedure: First, 100 μL of 250 μM OVA aqueous solution and 100 μL of 1.5 mM $HAuCl_4$ aqueous solution were mixed under vigorous stirring at room temperature. After mixing, NaOH was added into the mixed solution to adjust the pH to 12.65, and the mixture was incubated at 37 °C on a constant temperature shaking table under 250 rmp for 10.5 h. After reaction, the mixture was transferred into a 30 KD ultrafiltration device and centrifuged under 12000 rmp for 20 min to remove unreacted reagents and terminate the reaction. At last, OVA@AuNCs was redissolved with ultrapure water and stored at 4 °C. BSA@AuNCs and HRP@AuNCs were synthesized with the same method.

Characterization of the ovalbumin-stabilized fluorescent gold nanoclusters

The fluorescence spectra of the synthesized OVA@AuNCs were obtained with a Hitachi F-7000 Fluorescence Spectrophotometer. The excitation spectra were obtained by fixing the emission wavelength of 650 nm, whereas the emission spectra were obtained by fixing the excitation wavelength of 490 nm. Data points were recorded with an interval of 1 nm. Transmission electron microscopy (TEM) was performed on JEOL JEM-2100 high resolution transmission electron microscopy at 200 KV.

Hg^{2+} detection using the ovalbumin-stabilized fluorescent gold nanoclusters

Different concentrations of mercuric acetate (aqueous solution or spiked in lake water, which was collected from Meilan Lake of Shanghai) and prepared OVA@AuNCs were mixed with equivalent volume and the mixture was incubated at ambient temperature for 5 min. After that, 50 μL mixtures were used to record the fluorescent spectra.

To examine the selectivity of Hg^{2+} detection using the OVA@AuNCs, other ions (i.e. Ca^{2+}, Na^+, K^+, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ag^+, Cd^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Co^+, Ni^{2+}, and Pb^{2+}) were used instead of Hg^{2+}.

Results and discussion

In order to find potential candidates for the preparation of protein-stabilized AuNCs, some common proteins were tried to synthesize

the AuNCs following the procedure of Xie *et al.*[12] The related datas were not shown here. After serval attempts, we found that OVA could be used as reducing and stabilizing agent to synthesize AuNCs. In fact, it was not unexpected that Au(III) ions could be reduced by OVA since it contains 10 Tyr residues and possibly other residues with reduction capability. Next, the synthetic conditions of OVA@AuNCs, reaction time and pH, were optimized in our work.

Effect of reaction time on the synthesis of ovalbumin-stabilized fluorescent gold nanoclusters

To obtain the stable and high QY of OVA@AuNCs, the synthetic time of OVA@AuNCs was considered in the method. It was observed from Fig. 1 that the fluorescence intensity of OVA@AuNCs
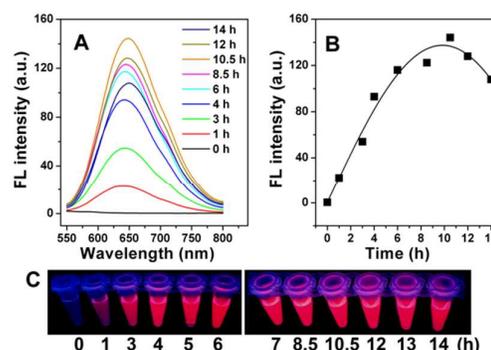

Fig. 1 Effect of reaction time on the synthesis of OVA@AuNCs at pH 12.65, the excitation wavelength was 490 nm. (A) The fluorescence responses of OVA@AuNCs at different synthetic time and the synthetic time were varied from 0 h to 14 h. (B) The evolution of the fluorescence intensity of OVA@AuNCs over the synthetic time. (C) The fluorescence of OVA@AuNCs at various synthetic time under UV light.

increased gradually over time until 10.5 h it reached maximum. It indicated that Au^{3+} in $HAuCl_4$ was reduced to Au^0 by tyrosine residues in OVA and AuNCs was formed in situ. However, the results showed that the fluorescence intensity of OVA@AuNCs started to decrease after 10.5 h. It was thought that the continuous growth of AuNCs destroyed the spatial structure of OVA so that the microenvironment for the formation of AuNCs was changed. Therefore, in order to maintain the high fluorescence of the synthesized OVA@AuNCs, ultrafiltration experiment was performed to remove redundant NaOH and $HAuCl_4$ and terminate the reaction after incubation for 10.5 h.

Effect of pH on the synthesis of ovalbumin-stabilized fluorescent gold nanoclusters

The gold cluster of 0 valence could be produced at high pH where Au^+ can be reduced to Au^0 by the Tyr residue. So the addition of NaOH was critical to form the OVA@AuNCs. Fig. 2 showed that the fluorescence intensity of the OVA@AuNCs increased gradually with the increasing of pH value from 9 to 12.65. However, when NaOH was not added, or under acidic conditions with HCl addition, the fluorescence of AuNCs disappeared and the characteristic peak of the OVA@AuNCs at 650 nm could not also be detected at the same time, so it indicated that there was no formation of AuNCs. As a
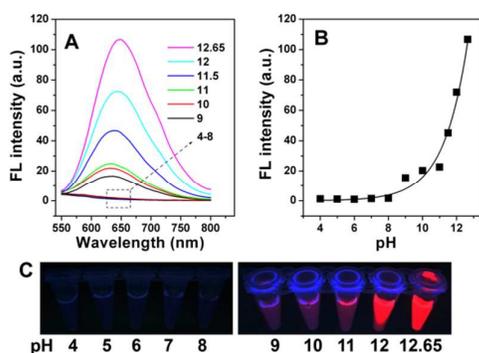
Fig. 2 Effect of pH on the synthesis of OVA@AuNCs. (A) The fluorescence responses of OVA@AuNCs at different pH and the pH value were varied from 4 to 12.65. (B) The evolution of the fluorescence intensity of OVA@AuNCs over pH value. (C) The fluorescence of OVA@AuNCs at different pH under UV light.

Fig. 4 (A) Fluorescence spectra of synthesized BSA@AuNCs. (B) Fluorescence spectra of synthesized HRP@AuNCs. (C) Fluorescence spectra of synthesized OVA@AuNCs. (D) The photograph of different proteins-stabilized AuNCs in solution under the visible light on the left and the fluorescence of different proteins-stabilized AuNCs in solution under UV light on the right.

consequence, the OVA@AuNCs was synthesized only in alkaline solution at pH value above 9. But considering that the spatial structure of OVA may be destroyed in strong alkali environment, pH value above 12.65 was not considered in our experiment. Therefore, pH 12.65 was chosen as the optimal synthesis condition of OVA@AuNCs.

Preparation and characterization of the ovalbumin-stabilized fluorescent gold nanoclusters

Based on the above results, the fluorescent OVA@AuNCs was synthesized by mixing OVA and $HAuCl_4$ at pH 12.65 and a further incubation at 37 °C for 10.5 h. The formation of the OVA@AuNCs was first confirmed by high resolution transmission electron microscope (HRTEM). As shown in Fig. 3, typical spherical particles
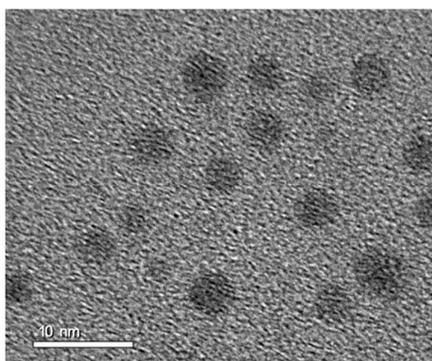

Fig. 3 TEM image of as-prepared OVA@AuNCs.

with an average diameter of about 3.8 nm can be observed. As for the production of fluorescence, it has been reported that both BSA and HRP could be used as reducing and stabilizing agents.[12, 14] So the OVA@AuNCs was compared with BSA@AuNCs and HRP@AuNCs, the results were showed in the Fig. 4. As shown in Fig. 4, the maximum emission peak of OVA@AuNCs could be observed at 650 nm and the maximum excitation peak was 490 nm (Fig. 4C). The same results can be seen in BSA@AuNCs and HRP@AuNCs systems (Fig. 4A and 4B). However, the fluorescence emission peak intensity of OVA@AuNCs was higher than the fluorescence
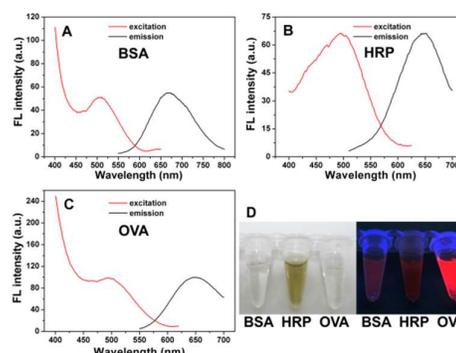
emission peak intensity of BSA@AuNCs and HRP@AuNCs at 650 nm. It could be visually observed that the high fluorescence of OVA@AuNCs from the photograph under UV light (Fig. 4D).

The excellent fluorescence properties of the synthesized OVA@AuNCs attracted our attention. Hence, the photoluminescence quantum yield (QY) of OVA@AuNCs was calculated in our work. Rhodamin B was chosen as standard substance to measure the quantum yield with reference to the report.[17] The calculated QY of OVA@AuNCs was about 12%, and the QY of BSA@AuNCs and HRP@AuNCs are 5.5% and 6.7%, respectively. The QY of BSA@AuNCs was consistent with the reported literature.[9] The high QY of OVA@AuNCs will be helpful for the application in bio-imaging and bioanalysis.

The stability of synthesized ovalbumin-stabilized fluorescent gold nanoclusters

The stability is often seen as an important indicator of the practicability of nano-materials. So the stability of synthesized OVA@AuNCs was taken into consideration in our experiments. Firstly, the impacts of the storage time on the stability of synthesized OVA@AuNCs were investigated. The synthesized OVA@AuNCs were redissolved with ultrapure water and stored at 4 °C. The results in Fig. 5 illustrated that the synthesized OVA@AuNCs could be stored at 4 °C for at least 15 days. In addition, we tested the effect of pH on the stability of synthesized OVA@AuNCs. As shown in Fig. 6, although the pH value was varied from 2 to 12.65, the fluorescence intensity of synthesized OVA@AuNCs was changed little in 15 days. So the results demonstrated that the synthesized OVA@AuNCs could be used in samples with different acidity. In conclusion, the synthesized OVA@AuNCs is stable, which is in favour of the utilization and exploitation in further.

Hg^{2+} detection using the as-prepared ovalbumin-stabilized fluorescent gold nanoclusters as fluorescent probes

Hg^{2+} pollution is highly toxic, endocrine system, brain, and kidney by interaction with thiol groups in protein and aminophospholipids.[18,19]
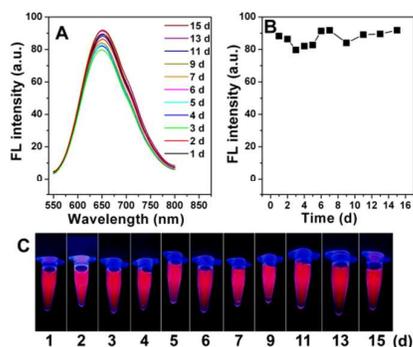
Fig. 5 Effect of stored time on the stability of synthesized OVA@AuNCs. The synthesized OVA@AuNCs were stored at 4 ºC and the storage time was varied from 1 day to 15 days. (A) The fluorescence responses of OVA@AuNCs at different storage time. (B) The evolution of the fluorescence intensity of OVA@AuNCs over storage time. (C) The fluorescence of OVA@AuNCs at different storage time under UV light.

Some methods have been de veloped for the detection of Hg^{2+}.[20, 23] However, new strategies with considerable sensitivity and usability are still badly needed.
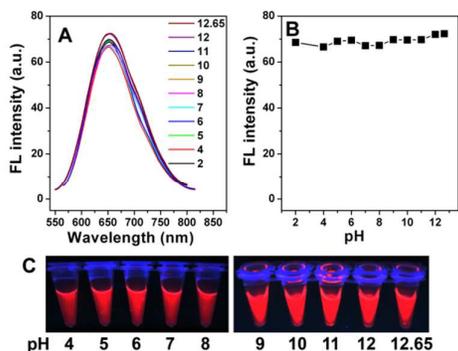

Fig. 6 Effect of pH on the stability of synthesized OVA@AuNCs. The synthesized OVA@AuNCs were redissolved in ultrapure water with special pH and the solution was incubated at 4 °C for 30 min. The fluorescence intensity was recorded after incubation. (A) The fluorescence responses of OVA@AuNCs, which were redissolved in solution with different pH. (B) The evolution of the fluorescence intensity of OVA@AuNCs over different pH value. (C) The fluorescence of OVA@AuNCs at different pH under UV light.

Firstly, in next set of experiments, we measured the modulation in the photoluminescence intensity of OVA@AuNCs in the presence of various metal ions. The metal ions tested including Hg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Ag^+, Cu^{2+}, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, and Mn^{2+}. As shown in Fig. 7A, 2 mM of Na^+, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Ba^{2+} could hardly change the fluorescence of the OVA@AuNCs in solution. On the contrary, we found that the fluorescence emission intensity of OVA@AuNCs was obviously enhanced by Zn^{2+} and Cd^{2+} in Fig. 7B. It was reported that the chelation between fluorescent molecule and metal ions enhanced the fluorescence.[24] However, the enhancement was gradually weakened when the concentration of Cd^{2+} was more than 500 μM,
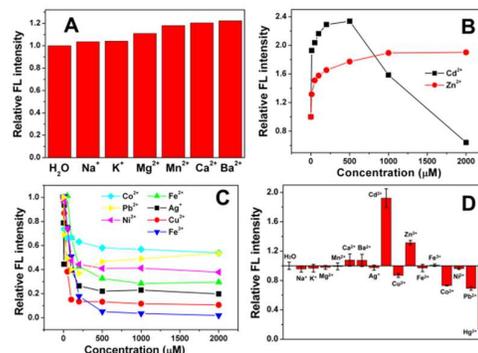

Fig. 7 (A-C) Effect of different metal ions on the fluorescence of synthesized OVA@AuNCs. (D) Relative fluorescence (I/I_0) of OVA@AuNCs at λ_{ex} = 490 nm in the presence of 10 μM of Na^+, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, Ag^+, Cd^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Pb^{2+}, Hg^{2+} at pH 12.65. I_0 and I: the photoluminescence intensities of OVA@AuNCs in the absence and presence of the above ions.

and even the florescence of OVA@AuNCs could be quenched by Cd^{2+} in the end. Unlike Cd^{2+}, the fluorescence enhancement of OVA@AuNCs by Zn^{2+} reached equilibrium when the concentration was as high as 500 μM. Further research revealed that several metal ions (Co^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Ag^+, and Cu^{2+}) could quench the fluorescence of synthesized OVA@AuNCs. But the results indicated that the fluorescence of OVA@AuNCs cannot be quenched completely by these metal ions except Fe^{3+}. Moreover, only high concentration of Fe^{3+} could quench completely the fluorescence of prepared OVA@AuNCs (Fig. 7C). Compared with other metal ions, a strong decrease of the fluorescence was observed in the presence of 10 μM Hg^{2+} (Fig. 7D), which could be explained the specific and strong interactions between Hg^{2+} and Au^+.[25-27] The mechanism can be explained using a photo-induced electron transfer process.[28, 29]

Finally, we designed a method for the detection of Hg^{2+} in aqueous solution based on the induced fluorescence quenching of OVA@AuNCs. It was found that there was a linear correlation between the fluorescence intensity and the concentrations of Hg^{2+} over the ranges of 0-10 μM, the curve-fitting equation was y = -0.0094x + 106, R^2 > 0.99 (Fig. 8, black curve), and the corresponding
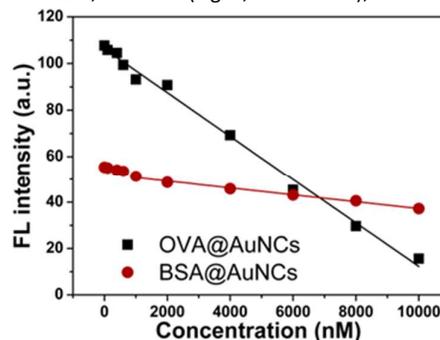

Fig. 8 Quenching efficiency plotted with respect to the Hg^{2+} concentration. The concentration of Hg^{2+} was 0-10 μM.

limit of detection (LOD) was 10 nM. In comparison, the commonly used BSA@AuNCs were not as sensitive as the OVA@AuNCs. The sensitivity was 1.5 a.u./μM (calculated from the slope of the red

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linear curve in Fig. 8), ca. 6 times lower than that of OVA@AuNCs. Detection of Hg^{2+} in real samples using OVA@AuNCs was also conducted. As shown in Table 1, lake waters which had been spiked

Table 1 Detection of Hg^{2+} in lake water

Sample number	Added (μM)	Found (μM)	Recovery (%)	RSD (%) (n=3)
1	0.1	0.94	94%	6.6
2	1	1.06	106%	4.3
3	10	8.97	90%	6.9

with different concentrations of Hg^{2+} were adopted. Results showed that acceptable recoveries and relative standard deviation (RSD) could be obtained, suggesting the potential application of our methodology for environmental monitoring.

Possible reaction mechanism

We have developed the synthesis of ovalbumin-stabilized highly fluorescent gold nanoclusters. Here we tried to explain the produced mechanism of high fluorescence of OVA@AuNCs. Recent studies have demonstrated that Tyr or Tyr residues in peptides can reduce Au(III) ions through their phenolic groups, and the reduction capability of Tyr can be greatly improved by adjusting the reaction pH above the pKa (~10).[30] Therefore, it was expected that Au(III) ions could be reduced by OVA since it contains 10 Tyr residues and possibly other residues with reduction capability. BSA contains 21 Tyr residues, but the QY of OVA@AuNCs was higher than the QY of BSA@AuNCs in our experiments. It was thought that Au(III) ions were reduced by other residues with reduction capability, like tryptophan, because tryptophan could also reduce Au(III) ions above the pKa in alkaline pH in previous report.[31]

Conclusion

In our work, OVA-stabilized fluorescent AuNCs with a red emission of 650 nm in aqueous solution were synthesized using OVA as a reducing and stabilizing agent. The OVA@AuNCs had higher QY than BSA@AuNCs and HRP@AuNCs. Moreover, the synthesized OVA@AuNCs were stable in different pH values of solution and can be stored at 4 °C for at least 15 days. Finally, the fluorescence intensity of as-prepared OVA@AuNCs was sensitive to Hg^{2+} and decreased as the concentration of Hg^{2+} increased. The calibration graphs were linear over the range of 0-10 μM, and the corresponding LOD was 10 nM.

Acknowledgements

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