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Trypsin-Stabilized Fluorescent Gold Nanocluster for Sensitive and Selective Hg^{2+} Detection

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We report on trypsin-stabilized fluorescent gold nanoclusters (Au NCs) for the sensitive and selective detection of Hg^{2+} ions. The Au NCs have an average size of 1 nm and show a red emission at 645 nm. The photostable properties of the trypsin-stabilized Au NCs were examined, and their photochemical stability was found to be similar to that of CdSe quantum dots. The fluorescence was particularly quenched by Hg^{2+}, and therefore the Au NCs can be used as fluorescent sensors for sensitive and selective Hg^{2+} detection to a detection limit of 50 ± 10 nM and the quantitative detection of Hg^{2+} in wide and low concentration range of 50 – 600 nM.

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Introduction

Ultra-small gold nanoparticles (Au NPs) from subnanometer scale to approximately 2 nm (also called gold nanoclusters [Au NCs]) have recently attracted much attention in the fields of physics, chemistry, material science, and biosciences because of the relative ease of their synthesis by chemical reduction of their salts in solution. This is done using stabilizing agents such as thiol and phosphine compounds.[1-9] The specific characteristics of Au NCs are attributed to the ultra-small size of the NPs around 1 nm. In contrast to Au NPs with sizes of more than 3 nm, the Au NCs show no localized surface plasmon resonance (LSPR) band in the optical absorbance spectra. They display various interesting molecular-like properties such as discrete electronic states and size-dependent fluorescence, and have a variety of applications in the field of catalysis, chemical sensing, electronic devices, optics, and biomedics.[10-25]

Recently, there has been increasing interest in the development of biological synthesis for Au NPs because of the need for an environmentally acceptable solvent system and eco-friendly reducing and capping agents.[26-28] Xie *et al*. first demonstrated the synthesis of highly fluorescent protein-stabilized Au NCs using a bovine-serum alubumin (BSA)-templated method.[22] The as-prepared Au NCs consisted of 25 gold atoms (Au_{25}) with a red emission at 640 nm. It has been suggested that the surface of Au NCs are stabilized with Au^+, which were further utilized for luminescence sensing of Hg^{2+} ions through fluorescence quenching by Hg^{2+}-Au^+ interaction.[29] Although it is not yet clear how the protein-stabilized Au NCs are formed in the solution synthesis, it has been suggested that rich tyrosine (Tyr) and cystein (Cys) residues (34 Cys and 21 Tyr) in BSA are important to produce the protein-stabilized Au NCs. This is because Cys residues, similar to thiol-protected Au NCs, are able to stabilize Au NCs, and Tyr residues can reduce Au(III) ions in alkaline pH above the pK_a of Tyr (~10).[22]

On the basis of these previous studies, proteins with rich Cys and Tyr sequences are considered to be strong candidates for the synthesis of protein-stabilized Au NCs. Thus, there are many natural proteins that are highly likely to produce such Au NCs, and the use of various proteins for protein-stabilized Au NCs may lead to the production of nanomaterials with highly specific or multiple functions, or protein-mediated self-assembly. However, so far only a few protein systems (*i.e*., BSA and lysozyme) are available for protein-stabilized fluorescent Au NCs.[22,29-31]

In the present paper, we report on the synthesis of trypsin-stabilized fluorescent Au NCs with a red emission by mixing trypsin and $HAuCl_4$ at pH 12. Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins. Trypsin is also a strong candidate for the synthesis of protein-stabilized Au NCs, since trypsin includes rich amino acid residues with 7 Cys and 10 Tyr. The photostable properties of the trypsin-stabilized Au NCs and the effectiveness of fluorescent-based heavy metal ion sensing were examined. We found that the fluorescence was particularly quenched by Hg^{2+}, and therefore, the Au NCs can be used as sensors for sensitive and selective Hg^{2+} detection to a detection limit of 50 ± 10 nM. The quantitative detection of Hg^{2+} was possible over the wide and low concentration range of 50 – 600 nM.

Experimental

Reagents

$HAuCl_4{\cdot}4H_2O$ (99.9%) as a source of gold atoms was obtained from Wako Chemical Co., from where we also purchased trypsin. Standard heavy metal solutions ($CaCO_3$ (1002 mg/l), $Cd(NO_3)_2$ (1001 mg/l), $Co(NO_3)_2$ (1001 mg/l), $HgCl_2$ (1000 mg/l), $Mg(NO_3)_2$ (1001 mg/l), $Ni(NO_3)_2$ (1001 mg/l), $Pb(NO_3)_2$ (997 mg/l), $Zn(NO_3)_2$ (1005 mg/l)) were obtained from Wako Chemical Co. The ultra-pure water used throughout

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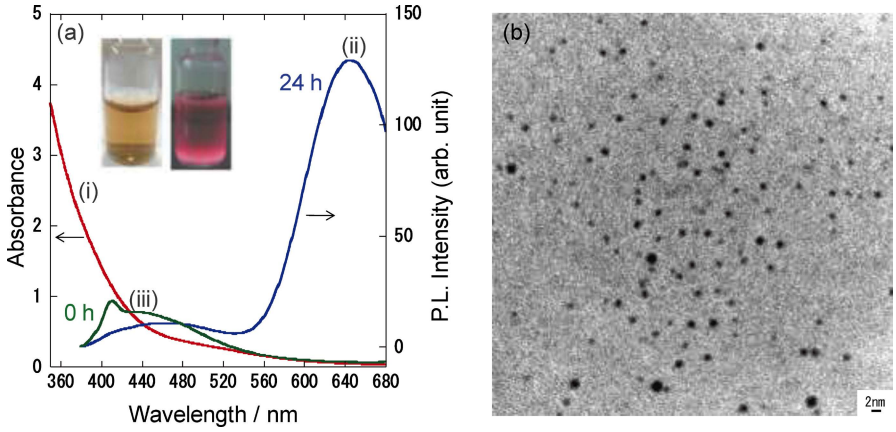

Fig. 1 (a) (i) UV-visible spectrum of aqueous solution of trypsin-stabilized Au NCs at pH 12 (a red line). Photoluminescence spectra of an aqueous solution of trypsin-stabilized Au NCs at λ_{ex} = 360 nm (ii) after 24 h of the reaction at 37°C (a blue line) and (iii) before the reaction (0 h) (a green line). Photographs of trypsin-stabilized Au NCs in solutions under a room light (left side) and UV light of 365 nm (right side) are shown. (b) TEM image of trypsin-stabilized Au NCs.

all experiments was purified with an Advantec RFD 250 NB system. All other chemicals used in this study were of reagent grade from Wako Chemical Co., and were used without further purification.

Synthesis of trypsin-stabilized Au NCs: Trypsin-stabilized Au NCs with a red emission were prepared according to the procedure by Xie *et al.*[22] During a typical synthesis, the trypsin-stabilized Au NCs were prepared by mixing an aqueous trypsin solution (0.5 mL, 20 mg/mL) and an aqueous $HAuCl_4$ solution (0.5 mL, 5 mM) under vigorous stirring. After 1 min, an aqueous NaOH solution (0.15 mL, 1 M) was added to this solution to adjust the pH to 12, and the mixture was incubated at 37°C for 24 h.

Apparatus

Optical properties. UV-visible absorption spectra were measured using a JASCO V-670 spectrometer. Fluorescence excitation and emission spectra were obtained on a JASCO FP-6300 fluorometer. The photochemical stability (*i.e.*, photobleaching) of Au clusters was examined using UV light (SLUV-6, AS ONE Co., Japan) of 1.3 mW/cm^2 at a distance of 5 cm from the light.

Circular dichroism (CD) spectroscopy. The CD spectra of aqueous solutions (pH 12) of trypsin and trypsin-stabilized Au NCs were obtained using a JASCO J-820 spectropolarimeter.

X-ray photoelectron spectroscopy (XPS). X-ray photoelectron spectra were recorded with a Quantera SXM spectrometer (Physical Electronics, Inc.) using the monochromatic Al K_α line (1486.7 eV). The base pressure was approximately 2×10^{-8} torr. The binding energies were referenced to C 1 s at 284.7 eV from hydrocarbon to compensate for the charging effect.

Transmission electron microscopy (TEM). A drop of NCs dispersed in water was placed on a carbon-coated Cu grid, and TEM images were recorded with a JEOL JEM-2010F at an acceleration voltage of 200 kV.

Results and Discussion

Trypsin-stabilized fluorescence Au NCs with a red emission

The UV-visible absorption spectrum of the as-prepared trypsin-stabilized Au NCs solution shows a continuous absorption increase from around 700 nm (Fig. 1a (i)). It is known that Au NPs with sizes of more than 3 nm show the LSPR absorption band (~520 nm) in the UV-visible spectrum, while Au NCs with sizes less of than 2 nm exhibit no LSPR band. The fact that the LSPR band was not observed for as-prepared trypsin-stabilized Au NCs suggests that the Au NCs have core diameters of less than 2 nm. Such tiny particle formation of the trypsin-stabilized Au NCs was confirmed by TEM observations which show their diameter to be approximately 1 – 2 nm (Fig. 1b). This indicates that $AuCl_4^-$ ions are reduced to Au_0 atoms and grow to form Au NCs by using trypsin as a reducing and stabilizing agent; however, there is no further growth into large Au NPs.

After just mixing $HAuCl_4$ and trypsin (*i.e.*, the reaction time of 0 h), the yellow solution showed no red fluorescence (Fig. 1a (iii)). After reacting for 0.5 h, trypsin-stabilized Au NCs showed a red fluorescence under UV light (365 nm), characteristic of Au_{25} NCs. The fluorescence intensities gradually increases with the reaction times (not shown). After the reaction for 24 h, the red emission peak of the trypsin-stabilized Au NCs was observed, as shown in the photograph in Fig. 1a. The fluorescence peak appeared at an emission of wavelength of λ_{em} = 645 nm with an excitation of wavelength of λ_{ex} = 360 nm (Fig. 1a (ii)), which is consistent with red emission at λ_{em} = 640 nm from the BSA-stabilized Au_{25} NCs_{22} and that at λ_{em} = 657 nm from the lysozyme-stabilized Au_{25} NCs.[29] Therefore, the trypsin-stabilized Au NCs are presumed to consist of 25 gold atoms (Au_{25}).

It is known that the binding energy of the metal cluster increases with a decrease in the cluster size in the XPS spectra.[13] Bulk Au metal has a Au ($4f_{7/2}$) binding energy of 84 eV, while the Au binding energy of Au clusters could be 1.0 – 2.0 eV higher. Tanaka and coworkers found from detailed peak shape analysis that the Au ($4f_{7/2}$) peaks can be deconvoluted into two components associated with the inner and surface atoms of gold.[32] The Au ($4f_{7/2}$) peak positions for the inner Au atoms were found to monotonically shift from 84.0 to 84.3 eV with a reduction of the core size. The Au ($4f_{7/2}$) peak positions of the surface Au components were at higher energy (84.3 – 84.7 eV) than those of the corresponding inner components. It has also been reported that the Au ($4f_{7/2}$) peak component from Au–S

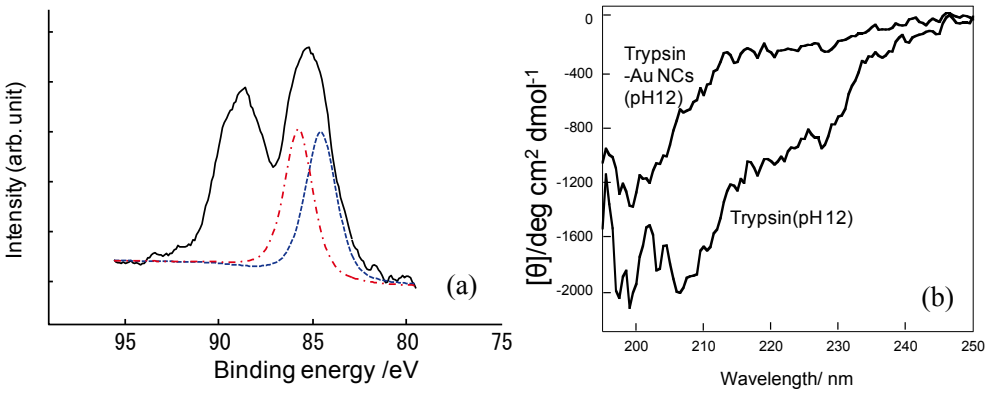

Fig. 2 (a) XPS spectra of the binding energy of Au $4f_{2/7}$ peak in trypsin-stabilized Au NCs. The binding energy of Au $4f_{2/7}$ could be deconvoluted into two distinct components at 84.0 eV (a dot blue line) and 85.5 eV (a dot red line). (b) CD spectrum of the aqueous solution of trypsin-stabilized Au NCs at pH 12 and that of pure trypsin at pH 12.

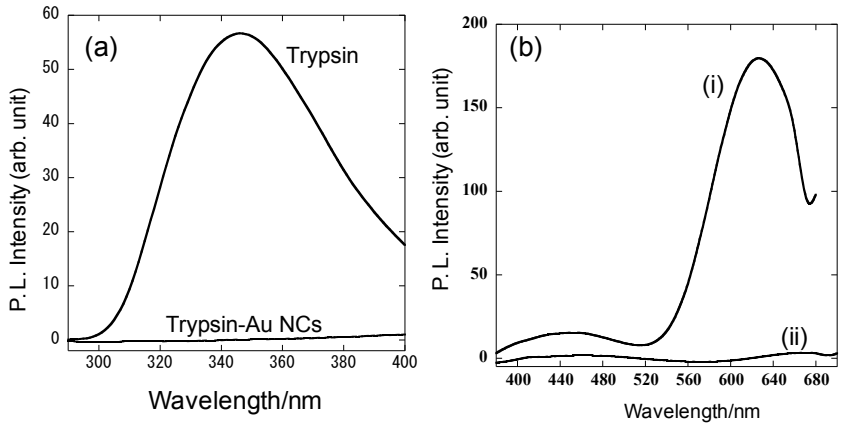

Fig. 3 (a) Photoluminescence spectra from aqueous solutions of trypsin and trypsin-stabilized Au NCs with $\lambda_{ex} = 280$ nm at pH 12. (b) Photoluminescence spectra of aqueous solution of trypsin-stabilized Au NCs at $\lambda_{ex} = 360$ nm obtained from the preparation (i) in the absence and (ii) in the presence of the 8 M urea at pH 12.

bonds in thiolate-stabilized Au NPs is positively shifted relative to that from the Au–Au bonds.[33] In the present case, thus, the binding energy of the Au ($4f_{7/2}$) peak for trypsin-stabilized Au NCs could be deconvoluted into two distinct components at 84.0 and 85.5 eV (Fig. 2a). The binding energy at 84.0 eV can be assigned to Au (0) atoms. Two contributions may be considered for the shifts in binding energy by 1.5 eV toward the higher energy of trypsin-stabilized Au NCs (relative to the Au (0)). One contribution is that the higher binding energy (85.5 eV) of trypsin-stabilized Au $4f_{2/7}$ can be attributed to the surface Au components with higher energy.[32] Another contribution is that the higher binding energy originated from Au–S bonds in trypsin-stabilized Au NCs.[33]

To check whether or not any conformational changes occurred in the trypsin of Au NCs, CD spectroscopy was performed for trypsin-stabilized Au NCs and pure trypsin. The CD spectrum is useful for studying the secondary structures of proteins. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of the CD spectrum. The CD spectrum shows negative bands around 208 and 227 nm for proteins with rich alpha-helix structures, while it shows a negative band around 215 nm for proteins with rich beta-sheet structures. For the random coil structure of a protein, a small positive band at 212 nm and a large negative one at around 195 nm are observed in the CD spectrum. The CD spectra of the trypsin-stabilized Au NCs and pure trypsin at pH 12 both showed a large negative band at around 195 nm from the random coil structure (Fig. 2b), indicating a large conformational change for the trypsin-stabilized Au NCs as well as pure trypsin at pH 12. This is in contrast to the case of BSA-stabilized Au NCs, where the encapsulation of Au NCs in BSA has little effect on the structure of the BSA scaffolds.[22]

The addition of NaOH was essential to produce the trypsin-stabilized Au NCs, and when NaOH was not added, or under acidic conditions of pH 2 with HCl addition, there was no formation of Au NCs (not shown). This is probably because the reduction ability of tyrosine in trypsin is activated with the alkaline pH.[22,27] Recently, Tan *et al*. have also investigated the amino-acid sequence in peptides for the synthesis of peptide-stabilized Au NPs.[34] It was found that tyrosine and tryptophan showed fairly gold reduction ability of $AuCl_4^-$ when present in the peptide sequence. In addition, $AuCl_4^-$ ions have been reported to lead to the formation of Au hydroxy species such as $[AuCl_x(OH)_{4-x}]^-$ ($x = 0 - 4$) with alkaline pH.[35]

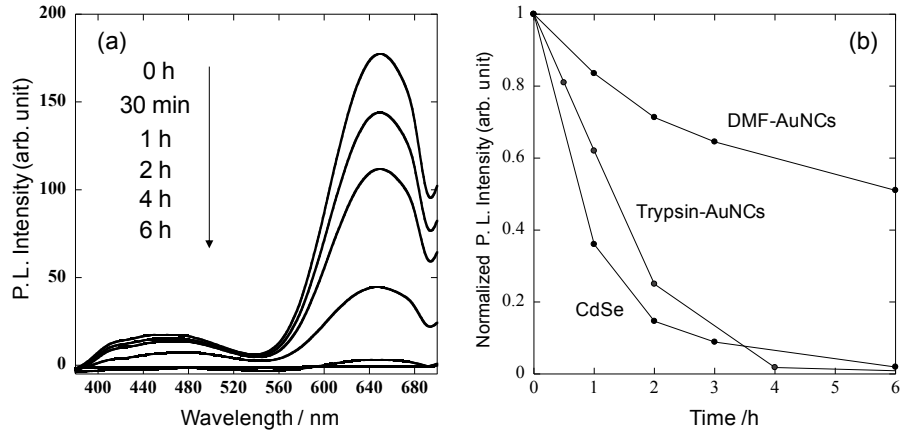

Fig. 4 (a) Photoluminescence spectra of trypsin-stabilized Au NCs at λ_{ex} = 360 nm in water at pH 8 as a function of the UV irradiation time (356 nm, 2 mW/cm^2) for up to 6 h. (b) Normalized photoluminescence intensities of trypsin-stabilized Au NCs at λ_{ex} = 360 nm as a function of the UV irradiation time (356 nm, 1.3 mW/cm^2) for up to 6 h. For comparison, results on DMF-protected Au NCs and CdSe in toluene,[25] exposed to the same conditions, are also shown.

The formation of such hydroxylated species under the alkaline pH condition may lower the reduction potential of the Au ions,[36] leading to an easier reduction of Au ions by tyrosine in trypsin. The involvement of tyrosine residues in the reduction of the Au ions was further confirmed by the observation of fluorescence from tyrosine before and after the reduction of Au ions by trypsin. The fluorescence of tyrosine is sensitive to oxidation, and the loss of fluorescence indicates the oxidation of tyrosine in protein.[37] As shown in Fig. 3a, the fluorescence of tyrosine was observed in trypsin aqueous solution at pH 12; however, most of the fluorescence of tyrosine residues in trypsin was lost in the trypsin-stabilized Au NCs, suggesting the involvement of tyrosine residues in the reduction of Au ions. It should be noted that some types of protein structures in trypsin should contribute to initiate the reduction of $AuCl_4^-$ and the template scaffold in the formation of trypsin-stabilized Au NCs, since we could not obtain the fluorescent trypsin-stabilized Au NCs in the presence of the denaturant of 8 M urea, which unfolded the protein (Fig. 3b).

Photostability of trypsin-stabilized Au NCs

Photochemical instability (*i.e.*, decrease in fluorescence intensity against light irradiation) irreversibly destroys photoluminescence molecules stimulated by radiation within the excitation spectrum. This eliminates potentially useful photoluminescence properties. It has been reported that Au NCs are highly photostable when compared with organic fluorophores, such as fluorescein and rhodamin 6G, although the Au NCs were not as photostable as polymer-coated CdSe/ZnS quantum dots (QDs).[38] Recently, we also reported that dimethylformamide (DMF)-protected Au NCs that include at least Au_8 and Au_{13} are much more photostable compared to CdSe QDs.[25] In this study, the photostable properties of trypsin-stabilized Au NCs in water at pH 8 were examined. Figure 4a shows photoluminescence spectra of trypsin-stabilized Au NCs as a function of the UV light continuous irradiation time (356 nm, 1.3 mW/cm^2). For comparison, the results for DMF-protected Au NCs and CdSe in toluene,[25] exposed to the same light conditions, are also shown in the figure. The fluorescence intensities decreased to approximately 60% of their maximum after 1 h and disappeared after 4 h (Fig. 4b). The photostability of trypsin-stabilized Au NCs is high, similar to that of CdSe QDs. The high photostability of trypsin-stabilized Au NCs has an advantage concerning the analytical application of Au NCs for fluorescent sensing of metal ions. On the other hand, the photostability of trypsin-stabilized Au NCs is no better than those of DMF-protected Au NCs. The lower photostability of the trypsin-stabilized Au NCs compared to that of DMF-protected Au NCs may originate from the low stability of trypsin against UV light.

Analytical application of trypsin-stabilized Au NCs for Hg^{2+} ion sensing

It has been reported that BSA- or lysozyme-stabilized fluorescent Au NCs can be used as sensitive and selective sensors of metal ions, such as Hg^{2+} ions, through fluorescence quenching by the Hg^{2+}-Au^+ interaction.[29-31] Here, the effect of metal ion addition on the fluorescence of trypsin-stabilized Au NCs was investigated in the presence of 10 μM of Cu^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Na^+, Pb^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, or Cd^{2+} at pH 8. Figure 5a shows the relative fluorescence (I/I_0) in the presence of 10 μM of the above metal ion. Only the addition of the Hg^{2+} ion resulted in the dominant quenching of the fluorescence of the Au NCs, although there was a slight quenching of fluorescence in the presence of other metal ions. Thus, the fluorescence quenching of trypsin-stabilized Au NCs can be used for Hg^{2+} ion sensing, similar to BSA- or lysozyme-stabilized fluorescent Au NCs. The fluorescence intensity decreased linearly over the Hg^{2+} concentration range of 50 – 600 nM (Figs. 5b and 5c), but the quantitative decrease in the fluorescence intensity was not observed in the low concentration range of 0.5 – 10 nM. The limit of detection (LOD) for the Hg^{2+} ions was estimated to be 50 ± 10 nM from the minimum concentration in the linear region of fluorescence decreases. The LOD value for Hg^{2+} ion is higher than those of BSA-stabilized Au NCs (0.5 nM) and lysozyme-stabilized Au NCs (10 nM).[29] The advantage of trypsin-stabilized Au NCs is the wide and low quantitative detection range of 50 – 600 nM, compared to those of BSA-stabilized Au NCs (the fluorescence decreases in the linear region of 1 – 20 nM)[29] and Lyz-stabilized Au NCs (the fluorescence decreases in the linear region of 100 – 2000 nM).[30] If it is assumed that the LOD value for the Hg^{2+} ion sensing is defined as the minimum concentration in the linier region of fluorescence decreases, the LOD values of

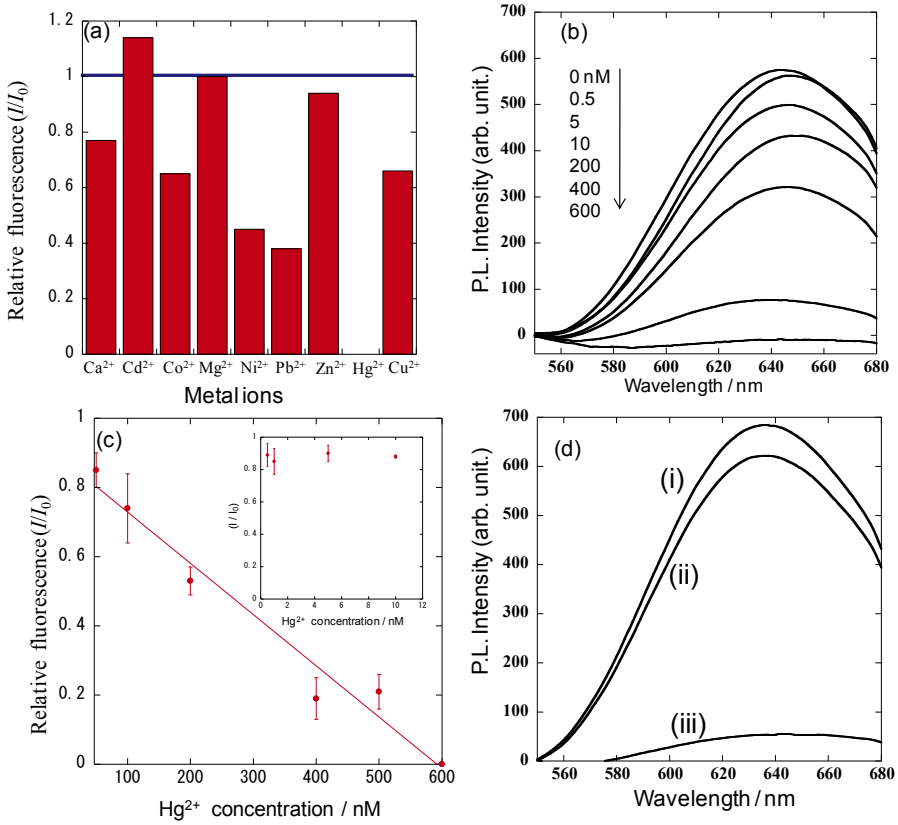

Fig. 5 (a) Relative fluorescence (I/I_0) of trypsin-stabilized Au NCs at λ_{ex} = 360 nm in the presence of 10 μM of Cu^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, or Cd^{2+} at pH 8. I_0 and I: the photoluminescence intensities of trypsin-stabilized Au NCs in the absence and presence of the above ions. (b) Photoluminescence intensities of trypsin-stabilized Au NCs at λ_{ex} = 360 nm with increasing concentration of Hg^{2+} ions at pH 8 (concentrations shown in the figure). (c) I/I_0 of trypsin-stabilized Au NCs at λ_{ex} = 360 nm as a function of Hg^{2+} ion (inset shows the concentration range 0.5 – 10 nM). (d) Photoluminescence intensities of trypsin-stabilized Au NCs at λ_{ex} = 360 nm (i) in water at pH 8; (ii) in coexistence with the following metal ions at pH 8: Cu^{2+}, Ni^{2+},Ca^{2+}, Mg^{2+}, Na^{+}, Pb^{2+}, Zn^{2+}, Co^{2+}, and Cd^{2+} (each metal ion concentration, 500 nM); (iii) in coexistence with the following metal ions at pH 8: Cu^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, Pb^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, and Hg^{2+} (each metal ion concentration, 500 nM).

protein-stabilized Au NCs are as follows: 1 nM for BSA-stabilized Au NCs, 50 nM for trypsin-stabilized Au NCs, and 100 nM for lysozyme-stabilized Au NCs. To further demonstrate the potential practical application of detecting Hg^{2+} ions in real samples using trypsin-stabilized Au NCs, 600 nM of Hg^{2+} was spiked in non filtered aqueous solutions from tap, mineral, and river water (the Yodo River in Osaka, Japan). The fluorescence of trypsin-stabilized Au NCs completely disappeared in the presence of Hg^{2+} ions (600 nM), corresponding to the values of $I/I_0 \sim 0$ for tap water, mineral water, and river water. The naked-eye detection of Hg^{2+} ions is possible for the Hg^{2+} concentrations of more than 600 nM.

We also examined the possibility of quantitatively detecting Hg^{2+} ions (500 nM) in aqueous solution using trypsin-stabilized Au NCs, in coexistence with interference metal ions: Cu^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, Pb^{2+}, Zn^{2+}, Co^{2+}, and Cd^{2+} (each metal ion concentration was 500 nM). The addition of the above-mentioned metal ions into aqueous solutions of trypsin-stabilized Au NCs (5 μM) resulted in a slight decrease in the fluorescence intensity (Fig. 5d (ii)). The further addition of the Hg^{2+} ion of 500 nM into this solution led to a dramatic decrease in the fluorescence intensity (I/I_0 = 0.08) (Fig. 5d (iii)). From the fluorescence intensity in the presence of Hg^{2+}, we estimated the concentration of Hg^{2+} ions to be 540 nM, using the calibration curve of Fig. 5c. This is almost consistent with the concentration (500 nM) of previously spiked Hg^{2+}. These features contribute to the selectivity in the quantitative detection of Hg^{2+} ions, even in the presence of interference ions.

The above results indicate that the fluorescence of trypsin-stabilized Au NCs was particularly quenched by Hg^{2+}, and therefore the Au NCs can be used as sensors for sensitive and selective Hg^{2+} detection. The specific characteristics of Au NCs reflect the specific interaction between trypsin-stabilized Au NCs and Hg^{2+}. Trypsin has rich amino acids with thiol-groups (6 cysteine and 3 methionine). Hg^{2+} ions can strongly bind to the thiol-compounds due to the fact that the thiol-group has a stronger binding of Hg^{2+} to thiol-sites than do other metal ions.[39] The stability constants between heavy metal ions and the chelating ligand, like L-cysteine, are log K(Hg) = 10.1, log K(Pb) = 4.1, log K(Cd) = 3.2, respectively.[40] It is also known that in aqueous solutions, Hg^{2+} spontaneously reacts with Au to form a Au amalgam.[36,39,41] One possible explanation for the specific interaction between trypsin-stabilized Au NCs and Hg^{2+} is as follows: the Hg^{2+} ions deposit on the surface of the trypsin-stabilized Au NCs because of the strong binding of Hg^{2+} to thiol-sites, which results in the accumulation

of trypsin on the Au surfaces. Moreover, by direct interaction with the Au surfaces, Hg^{2+} spontaneously reacts with Au to form a Au amalgam, resulting in fluorescence quenching of the trypsin-stabilized Au NCs. Based on these results, we consider that trypsin-stabilized Au NCs can selectively detect Hg^{2+} with a relatively high limit of detection *via* the specific interaction between trypsin-stabilized Au NCs and Hg^{2+}.

Conclusion

Trypsin-stabilized fluorescent Au NCs with a red emission of 640 nm in basic aqueous solution were synthesized using trypsin as a reducing and stabilizing agent. The trypsin-stabilized Au NCs are approximately 1 nm in size. The CD spectroscopy of trypsin-stabilized Au NCs showed a large conformational change by the encapsulation of Au NCs in trypsin, which contrasted with the case of BSA-Au NCs, which had little effect on the structure of the BSA scaffolds. The photostability of trypsin-stabilized Au NCs was similar to that of CdSe QDs, while it was no better than that of DMF-protected Au NCs. The fluorescence was particularly quenched by Hg^{2+}, and therefore the Au NCs can be used as sensors for sensitive and selective Hg^{2+} detection to a detection limit of 50 ± 10 nM and the quantitative detection of Hg^{2+} in the wide and low concentration range of 50 – 600 nM.

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