Unexpected Thiols Triggering Photoluminescent Enhancement of Cytidine Stabilized Au Nanoclusters for Sensitive Assays of Glutathione Reductase and Its Inhibitors Screening

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ABSTRACT: The photoluminescence (PL) of nonthiolate ligand capped Au nanoclusters (NCs) is usually quenched by thiols due to the tight adsorption of thiols to the Au surface and formation of larger non-PL species. However, we here report an unexpected PL enhancement of cytidine stabilized Au (AuCyt) NCs triggered by thiols, such as reduced glutathione (GSH) at sub-μM level, while such phenomena have not been observed for Au NCs capped with similar adenosine/cytidine nucleotides. The mass spectrometric results indicate that this enhancement may be caused by the formation of smaller, but highly fluorescent, Au species etched by thiols. This enables the sensitive detection of GSH from 20 nM to 3 μM, with an ultralow detection limit of 2.0 nM. Moreover, the glutathione reductase (GR) activity can be determined by the initial rate of GSH production, i.e., the maximum PL increasing rate, with a linear range of 0.34–17.0 U/L (1 U means reduction of 1.0 μmol of oxidized glutathione per min at pH 7.6 at 25 °C) and a limit of detection of 0.34 U/L. This method allows the accurate assays of GR in clinical serum samples as well as the rapid screening of GR inhibitors, indicating its promising biomedical applications.

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Metal nanoclusters (NCs) are a family of attractive nanoscale materials containing tens to hundreds of metal atoms. As the intermediate between single metal atom and common metal nanoparticles made of thousands of atoms, NCs show distinguished optical and electric properties due to their transition states from continuous to discrete energy levels. A large variety of NCs have been reported with versatile roles in environmental monitoring, catalysis, and biomedical imaging fields. Among these NCs, Au NCs own excellent biocompatibility, which allows the wide applications in various biochemical assays. In the past decades, dendrimers, DNA or RNA scaffolds, proteins, and peptides (especially glutathione) were applied as templates to prepare Au NCs emitted from blue to near-infrared regions. In virtue of flexible sequence design and high stability, DNA molecules are extremely desirable in the controllable assembly of Au NCs. Kennedy et al. observed that even simple DNA sequences, such as 12-mer or 30-mer polycytosine can act as templates for Au NCs. Recently, we proposed a method to synthesize single cytidine/cytidine monophosphate stabilized Au NCs with multicolor emissions. In this work, we will further discuss the detection of glutathione reductase (GR), an important enzyme involved in metabolic processes by using these NCs. GR is an important enzyme in charge of maintaining the reducing environment inside mammalian cells. The ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG), an important index for evaluating redox homeostasis in cytoplasm, is controlled through GR mediated conversion of GSSG to GSH. Therefore, the rapid and accurate detection of GR activity in biological samples is of great importance. The fluorescent technique is a known convenient and sensitive method for GR assays. Several nanoprobes have been attempted for this purpose. For example, Pavlov and co-workers developed a concept for GR detection by using GSH mediated stabilization of in situ produced CdS QDs. Since this method depends on the surface modification of CdS QDs, it is relatively time-consuming and shows a high limit of detection. On the other hand, although many reports focused on the photoluminescence (PL) inhibition of NCs by GSH, the GR activity assays are seldom reported for these GSH induced PL quenching models due to the limited sensitivity.

Here, we report a simple and efficient strategy to detect GR activity by using cytidine stabilized Au (AuCyt) NCs emitted at 505 nm (Scheme 1). We demonstrate that thiols such as GSH can rapidly and sensitively trigger an ∼3-fold PL enhancement of AuCyt at sub-μM level, while all other tested Au NCs capped...
with similar nucleotides do not have similar features. The possible mechanisms have been attributed to the formation of smaller, but highly fluorescent, Au species etched by thiols. We also validate that it can be extended to the determination of GR activity through controlling the production of GSH and triggering PL enhancement. This strategy allows the assays of GR in clinical serum samples as well as the screening of GR inhibitors, indicating very promising biomedical applications.

MATERIALS AND METHODS

Reagents and Instruments. Cyt, adenosine monophosphate (AMP) disodium, adenosine diphosphate (ADP) sodium, adenosine triphosphate (ATP) disodium, GSH, GSSG, β-nicotinamide adenine dinucleotide phosphate reduced (NADPH, coenzyme II) tetrasodium, β-nicotinamide adenine dinucleotide phosphate (NAPD+) sodium salt hydrate, glutathione reductase (GR, E.C. 1.8.1.7, from bakery yeast), cysteine (Cys), N-acetyl cysteine (NAC), and cysteamine hydrochloride (CyA) are products from Sigma-Aldrich (St. Louis, MO, USA). HAuCl4 hydrochloride (CyA) are products from Sigma-Aldrich (St. Louis, MO, USA). HAuCl4, Cyt, and citrate buffer are purchased from Sinoreagent Co. Ltd. (Shanghai, China). Citrate stock buffer (0.5 M, pH 6.0) was prepared by mixture of phosphate disodium (0.1 M, PB, 0.1 M, pH 6.0) was prepared by a mixture of phosphate trisodium (0.5 M) and citric acid, which may tightly adsorb to the Au surface via the Au-S bond. Such interaction is more intensive than Au-N coordination bonds. We further synthesized cytidine and cytidine monophosphate (CMP) stabilized AuNCs emitting blue/green light, and yellow emissions by changing pH and metal–ligand molar ratios. These nonthiolate ligand capped NCs are formed through weak interactions, such as Au–N coordination bonds among A/C bases and Au cores. As is well-known, biothiols, such as GSH, may tightly adsorb to the Au surface via the Au–S bond. Such interaction is more intensive than Au–N coordination bonds. Here, we will discuss the substitution reactions by thiols and nucleoside/nucleotide capped AuNCs in detail.

The PL responses of AuCyt NCs to GSH were first studied. Both PL excitation and emission intensity from AuCyt NCs of 0.125 mg/mL were greatly enhanced in the presence of GSH even at sub-μM levels in PB (Figures 1A and S1), i.e., from 20 nM to 3 μM (Figure 1B). The PL change can reach equilibrium in 35 s after addition of 1 μM GSH (Figure S1D), indicating its fast responses to GSH. This case was unexpected since many works reported PL quench of metal NCs induced by thiols or sulfide.8,15–28 To the best of our knowledge, few works reported thiol induced NCs PL enhancement at sub-μM level. We recently reported thiol induced PL enhancement of low luminescent GSH capped AuNCs at mM level, which was not very sensitive.8,29 PL of histidine capped AuNCs could also be enhanced by GSH of at least 50 μM, about 1–2 magnitude order higher than that in this work.30 GSH could enhance the PL of DNA-wrapped Ag NCs by a maximum value of ~40%,31 while here ~3-fold PL enhancement was observed. Correspondingly, the typical UV–vis peak absorbance of AuCyt NCs at 380 nm also had an observable increase (~5%) at GSH of 0.5 μM (Figure S1C). The peak was then stable even at GSH concentration up to 4 μM.
The collapse of AuAXP NCs induced by GSH was also verified by UV–vis spectra (Figure S2B,D,F). All AuAXP NCs showed a typical absorption peak at ~290 nm, which could be attributed to the Au–N (from AXP) metal–ligand interactions. This peak decreased at GSH concentrations higher than 2 μM, accompanied by the slow increase in the absorbance at ~260 nm, implying the exposure or release of adenine moiety (with a typical adsorption peak at ~260 nm) from AuAXP NCs caused by GSH attack. The spectral changes gave an isosbestic point at ~270 nm, suggesting that only two species, i.e., AuAXP NCs and some nonfluorescent species, contributed to the absorption around the isosbestic point. Therefore, GSH competed with AXP ligands and occupied their coordination sites on the Au NCs surface, inducing an etch of AuAXP NCs as well as a PL quench.

Note that, in the above experiments, the feeding molar ratio of AuCl₄⁻ and ligands during synthesis was 1:1 for Au/Cyt and 1:2 for Au/AXP. Thus, the possible higher AXP coverage of ligands on the Au NCs surface may resist the GSH adsorption and inhibit the potential PL enhancement. To address this issue, AuCyt NCs (Au/Cyt ratio of 1:2) and AuAXP NCs (Au/AXP ratio of 1:1) were prepared for comparison. The PL excitation and emission intensity for AuCyt (ratio 1:2) NCs increased significantly in the presence of GSH (Figure S3A), while no such PL enhancement was observed for AuAXP (ratio 1:1) (Figure S3B–D). Hence, the feeding molar ratios were irrelevant to PL enhancement, and we consider that the GSH induced PL enhancement may be a feature of Cyt stabilized Au NCs. To further investigate the issue, we also use other kinds of Cyt stabilized Au NCs, AuCyt-490 and Ag doped AuCyt (AuAgCyt-560) NCs (Figure S3E,F). No PL enhancement was found for either NC. AuAgCyt-560 was even more sensitive to GSH than AuCyt NCs. AXP ligands and occupied their coordination sites on the Au NCs surface, inducing an etch of AuAXP NCs as well as a PL quench.

Subsequently, the optimal concentrations of AuCyt for thiols detection were probed. Although the PL emission had a constant peak wavelength at 505 nm, the PL excitation wavelengths shifted significantly with the concentrations of AuCyt (Figure S1A). For AuCyt of 1.0, 0.5, 0.25, and 0.125 mg/mL, there were two excitation bands located at 440 nm/340 nm, 430 nm/340 nm, 420 nm/340 nm, and 410 nm/350 nm, respectively. Interestingly, the two bands gradually became close to each other with dilution and were combined to a single peak at around 380 nm at concentrations lower than 0.0625 mg/mL. Because the PL enhancement ratios by GSH were generally similar from 0.125 to 1.0 mg/mL AuCyt (Figure S1B), a lower response sensitivity, i.e., the slope of PL plot against GSH concentration, was obtained when using higher concentrations of AuCyt. For AuCyt of 0.0625 mg/mL, however, the maximum PL intensity was decreased to 80% of that at AuCyt of 0.125 mg/mL (cyan curve, Figure S1B). Therefore, a concentration of 0.125 mg/mL was applied in the following research.

To determine whether other nucleotide capped Au NCs own similar features, we investigated the PL responses of AuAXP NCs of 0.1 mg/mL to GSH of various concentrations (Figure S2). The adenosine coated Au NCs were not taken into account due to their aggregation in aqueous solution. The PL sensitivity to GSH was different among these AuAXP NCs. The PL of AuAMP and AuADP NCs was generally unchanged in the presence of GSH lower than 1 μM and only shows an observable decrease at GSH concentrations higher than 5 μM (Figure S2A,C), while for AuATP NCs the PL drops were even significant at 0.2 μM (Figure S2E). The concentration of GSH that cause 50% PL drops was ca. 75, 50, and 20 μM for AuAMP, AuADP, and AuATP NCs, respectively. No changes in PL peak wavelengths occurred, indicating that nonfluorescent species were generated in the presence of GSH. These results indicate that GSH has higher Au binding capacity than AXP and AuAMP NCs are most resistant to GSH attack.

Possible Mechanism for PL Enhancement. One may consider that the unusual PL enhancement may be attributed to the aggregated-induced emission (AIE) well-known for Au and Cu NCs. 27–34 The significant increased luminescence was proved to depend on the aggregation of high content of Au(I)–thiolate or Cu complexes on the NCs surface after treatment of 75–95% (v/v) ethanol. No shift in wavelength occurred during the aggregation, which was also shown in this work. To testify such possibility, the morphological changes upon addition of GSH were investigated by TEM. As shown in Figure 2A, the mean size of AuCyt NCs is 2.0 ± 0.3 nm. After addition of GSH, only dots less than 1 nm are present (arrows indicated,
This demonstrates that GSH may also swiftly etch AuCyt NCs, instead of inducing their aggregation. The MALDI-TOF MS information can shed light on the rationale behind the unexpected PL enhancement. Considering that all species should have a molecular weight larger than 3000 Da after ultrafiltration treatment, the MS peaks at \( m/z \) (peak number, abundance, \%) of 901 (peak 3, 100), 917 (peak 4, 33), 1075 (peak 5, 14), 1122 (peak 6, 20), and 1318 (peak 7, 12) corresponded to the species of \([\text{Au}_6(\text{Cyt})_{10}]^4^-\), \([\text{Au}_{10}(\text{Cyt})_7]^4^-\), \([\text{Au}_9(\text{Cyt})_6]^3^-\), \([\text{Au}_6(\text{Cyt})_9]^3^-\), and \([\text{Au}_9(\text{Cyt})_9]^3^-\), respectively (Figure 3A). After addition of GSH, peaks 3 and 5−7 diminished and peak 4 significantly decreased (Figure 3B). A novel peak at \( m/z \) 881 (peak 8) showed clear evidence of \([\text{Au}_2(\text{Cyt})_2]^−\) (Figure 3B). Therefore, it is very likely that the addition of thiols induced the etching of larger Au species such as \( \text{Au}_9 \) to generate even smaller, but highly fluorescent, species including \( \text{Au}_2 \). Although all nucleoside/tide capped Au NCs can be rapidly etched by thiols, the results could be totally different. Here, no AuCyt-thiol intermediates were captured, implying that they are unstable and quickly transfer to the final products, resulting in no trace of thiol attached Au NCs.

Since no shift in PL wavelength was observed, the PL emission may be attributed to the metal–ligand charge transfer (MLCT) effect inside AuCyt NCs. This effect has also been reported for adenosine series coated Au NCs. The MLCT effect may be more effective after the dissociation of larger AuCyt NCs, rendering increasing fluorescence for \( \text{Au}_2 \) over larger Au species.

We also used the XPS technique to inspect the metal valence changes (Figure S4). In both samples, the Au 4f double peaks were located at 83.6 and 87.3 eV, with a gap of 3.7 eV and peak area ratio of 4:1. No S 2p peak was found either before or after treatment of GSH, which was in agreement with the results obtained by MS. The unchanged metal valence indicated that the dissociation of NCs belongs to a redox-free process.

Enhanced PL for Detection of Thiols, GR Activity, and GR Inhibitor. As previously described, the PL enhancement of AuCyt NCs allows the quantitative determination of thiol concentrations at sub-μM level. The PL increases upon addition of GSH by using AuCyt of 0.125 mg/mL (Figure 1B). The PL intensity differences (\( ∆I \)) reach saturation at concentrations of GSH of greater than 2 μM (Figure 4). If thiols are adsorbed to the Au surface before dissociation of NCs, then this process can be interpreted by an ideal Langmuir adsorption; i.e., the PL changes (\( ∆I \)) and the concentration of GSH (\( c \)) can be fitted by the following equation:

\[
\frac{c}{\Delta I} = \frac{1}{\Delta I_m} \times a + \frac{1}{\Delta I_m} \times c
\]

where \( \Delta I_m \) is the maximum \( ∆I \) and \( a \) is the equilibrium constant.
According to this equation, the \( c/\Delta I \) values are plotted against \( c \), which gives a satisfactory linear relationship \( (R = 0.995, n = 12) \), with a slope of 0.00302, i.e., \( \Delta I_{\text{m}} \) values (a.u.) of 331 (Figure 4 inset). Moreover, the equation is also suitable for other common thiols such as Cys, NAC, and CyA with excellent fitted results \( (R = 0.998–0.999, n = 4) \) (Figure S5). The thiols show similar \( \Delta I_{\text{m}} \) values of 306, 366, and 265, respectively. These results support that the fast attack of thiols to AuCyt NCs can be described by an ideal single layer adsorption model. The proposed strategy allows the detection of GSH with a linear range from 0.02 to 3 \( \mu \)M, with an ultralow detection limit of 2.0 nM \((S/N = 3)\). Other amino acids with typical side chains, including aliphatic chain (glycine), aromatic chain (phenylalanine), phenolic hydroxyl chain (tyrosine), acidic chain (glutamate), and basic chain (histidine, homo-arginine), did not interfere with the detection even at concentrations up to 100 \( \mu \)M (Figure S6), confirming that only thiol groups are responsible for the PL enhancement.

On the basis of the principle of thiol triggered sensitive PL enhancement, a facile method for assays of GR can be designed. To verify the feasibility of this method, the possible PL interferences by other participants in this system were evaluated. Thus, the PL responses upon addition of individual components, i.e., GSSG, NADPH, GR, and premixed GSSG/GR or NADPH/GR, were studied (Figure S7). Both GSSG and NADPH showed negligible PL changes at 5 \( \mu \)M, which were chosen in the final detection buffer. Similarly, GR and GR premixed with either GSSG or NADPH gave no PL changes. A significant increase in PL emissions at 505 nm occurred only when GR of certain activity was added to the mixture of AuCyt, GSSG, and NADPH in PB \((0.1 \text{ M}, \text{pH}\ 7.6)\) (Figure 5A). Since NADP\(^+\), the coproduct during GR catalysis, did not interfere with PL at a concentration of 5 \( \mu \)M (Figure S7), the PL increase can be fully attributed to GSH generated by enzymatic catalysis. The GR activity corresponds to the production rate of GSH at the beginning of enzymatic catalysis. At this instant, the PL increase is almost linear with the generated amount of GSH. Thus, the maximum/initial PL increasing rates \( [d(\Delta I)/d\tau]_{\text{max}} \) were linearly relevant with the GR activity (Figure S8), ranging from 0.34 to 17.0 U/L \((R = 0.9991)\), with a detection of limit of 0.34 U/L (1 U means reduction of 1.0 \( \mu \)mol of GSSG per min at pH 7.6 at 25 °C, \( S/N = 3)\). The linear equation can be described as follows:

\[
[d(\Delta I)/d\tau]_{\text{max}} \text{(a.u./s)} = 0.184 + 0.322 \times [\text{GR}](\text{U/L})
\]

The possible interferences of several common proteins/ enzymes were checked (Figure S8). No significant PL changes were observed in the presence of these proteins. Only SOD can induce PL decreased at a high activity \((19 \text{ U/mL})\), which was almost impossible in common biological samples. Moreover, GR was accurately detected in clinical serum samples, with an acceptable recovery of 97–112% (Table S1).

The proposed platform was also attempted for screening GR inhibitors. A typical GR inhibitor, Cu\(^{2+}\), was selected as a model. First, the PL interference of AuCyt by only Cu\(^{2+}\) was investigated. Cu\(^{2+}\) of 40 \( \mu \)M showed no observable PL quench or enhancement effect (Figure S9). The initial GR catalytic rates decreased in the presence of Cu\(^{2+}\) at \( \mu \)M level, indicating the effective inhibition on GR activity (Figure 6). The 50% inhibition rate corresponded to \( \sim 10 \mu \)M for Cu\(^{2+}\) according to the fitted logistic model \((R^2 = 0.983)\); see the Supporting Information). This was generally consistent with the literature value,\(^{35}\) taking into account the different experimental conditions.

![Figure 5](image-url) **Figure 5.** (A) PL dynamic curves of the mixture of AuCyt (0.125 mg/mL), GSSG (5 \( \mu \)M), and NADPH (5 \( \mu \)M) after addition of GR with different activities from 0.34 to 17.0 U/L. (B) The linear relationship between initial PL increasing rates and GR activity.

![Figure 6](image-url) **Figure 6.** Inhibition of GR activity by Cu\(^{2+}\). The reaction solution was PB (pH 7.6) containing AuCyt (0.125 mg/mL), GSSG (5 \( \mu \)M), and NADPH (5 \( \mu \)M) after addition of GR with different activities from 0.34 to 17.0 U/L. PB is premixed with Cu\(^{2+}\) of different concentrations.
procedures and materials origin. Hence, the strategy can be used for screening possible GR inhibitors.

**CONCLUSIONS**

Although thiols usually quench PL of metal NCs by formation of nonfluorescent species, here, we demonstrate that thiols can significantly enhance PL of AuCy7 NCs at sub-μM concentrations. The mechanistic investigation supports that the PL enhancement is attributed to the formation of highly fluorescent smaller Au species etched by thiols. The thiols adsorption before inducing dissociation of NCs can be described by a simple Langmuir's adsorption model. On the basis of this PL enhancement, a sensitive GR detection platform can be constructed, which enables the GR inhibitors screening. The proposed platform offers potential applications in fast and efficient clinical diagnosis.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b00112.

PL changes upon addition of thiols to different nucleotide ligand capped Au NCs, XPS characterization, GR detection interference, GR recovery in serum sample, and GR inhibition dynamics (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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