Herein, we demonstrate a novel, facile, and suitable strategy for imaging GSH based on mercaptopyrimidine-directed gold nanoclusters (Au NCs). GSH can specifically induce the fluorescence enhancement of Au NCs by attacking the surface of the NCs to form dual ligand stabilized Au NCs at the millimolar level. Very importantly, the Au NCs can selectively image cancer cells.

Glutathione (GSH) is an important antioxidant widely distributed in cells, which plays pivotal roles in maintaining cell redox equilibrium. As the most abundant endogenous biothiol and free radical scavenger in biological systems, GSH can widely participate in detoxification, immune regulation, signal transduction, and other physiological processes. In particular, abnormal GSH levels are key risk indicators for aging and cancerization. Due to the fact that the GSH levels in cancer cells are significantly higher than those in normal cells, it may represent an opportunity to evaluate different cell statuses and cancer occurrence, which shows great promise for early diagnosis of cancer. Thus, the development of a simple and reliable method to evaluate intracellular GSH levels has important significance for cancer and other clinical diagnoses. To date, a number of analytical strategies have been proposed for the determination of GSH, including high-performance liquid chromatography, surface enhanced Raman scattering, colorimetric methods, electrochemical methods, photoelectrochemical methods, and electrochemiluminescence. However, these proposed analytical methods have some limitations in clinical applications, such as the requirement of expensive instruments, and being labor intensive and time-consuming. In addition, these methods usually have relatively lower limit of detection (LOD) values and the detection ranges are mostly concentrated at the micromolar and nanomolar levels. Thus, they may not be suitable for the detection of intracellular GSH because it is usually present at millimolar levels (1–15 mM). Therefore, it is still necessary to explore a suitable approach that enables intracellular GSH evaluation in a simple way.

Recently, fluorescence assays for intracellular composition detection have been of great interest, including organic fluorophores, quantum dots, upconversion nanoparticles, and noble metal nanoclusters. Among them, noble metal nanoclusters (NCs), especially Au NCs, were proposed as alternatives for biosensing because of their excellent biocompatibility, photostability, and tunable emission ranges. Several reports have also demonstrated that Au NCs are suitable for the detection of intracellular thiols. For example, Tian and coworkers showed that a bovine serum albumin (BSA)-capped Au NC-based sensor has been designed for GSH sensing in living cells and blood samples. Recently our group also found that surface “unsaturated” GSH-coated Au NCs can be used for the imaging of intracellular thiols. However, these probes still cannot distinguish sensitively GSH from other thiols such as cysteine (Cys) and N-acetyl cysteine (NAC).

Herein, we have demonstrated a simple and efficient method for intracellular GSH imaging using thiolated Au NCs as fluorescent probes. We first synthesized a kind of thiolated Au NC with weak fluorescence by using 4,6-diamino-2-mercaptopurin (DAMP) as both a stabilizer and a reducing agent. The fluorescence of the DAMP-capped Au NCs (AuDAMP) was found to be significantly and selectively enhanced after further introduction of GSH. The mass spectroscopic results indicated that the AuDAMP samples are mainly composed of Au13 species, and the additional GSH can efficiently attach to the surface of the AuDAMP by forming dual ligand (DAMP and GSH) stabilized Au NCs. The formation of dual ligands might contribute to the fluorescence enhancement. The fluorescence enhancement is specific to GSH, with limited response to other common thiol species such as Cys and NAC. More importantly, AuDAMP can selectively distinguish cancer cells from normal cells due to the fact that the GSH levels in cancer cells are significantly higher than those in normal cells.

Ultrasmall AuDAMP can be produced in a one-pot synthesis by simply mixing gold precursors with DAMP at 70 °C under
stirring (Scheme 1). DAMP, an analogue of 2-mercaptopyrimidine in bacterial tRNA, is known as a stabilizer for synthesizing large size Au nanoparticles in the presence of NaBH₄, a strong reducing agent. Here we demonstrate that DAMP can act as both a reducing agent and a stabilizer to prepare ultrasmall AuDAMP. Compared to the synthesis of large-sized Au nanoparticles by reduction of NaBH₄, the reaction presented here is mild and slow, which allows the generation of ultrasmall clusters. The as-synthesized AuDAMP is mustard under visible light (Fig. 1a inset 1), which shows a dark red fluorescence under UV illumination (Fig. 1a inset 2). The fluorescence peaks are centered at 420 nm (excitation) and 620 nm (emission), respectively (Fig. 1a). The fluorescence emission peak wavelength is well-defined and does not change upon excitation from 400 to 500 nm (Fig. S1, ESI†), demonstrating that the fluorescence originates from Au NCs rather than light scattering.†

The UV-vis absorption spectrum of the Au NC solutions is shown in Fig. S2, ESI†. The absorption curve decays roughly exponentially toward the visible region and there is no characteristic absorption peak (i.e., surface plasmonic band) in the UV region, corresponding to the successful formation of NCs. Indeed, there are many well-dispersed ultrasmall spherical clusters (Fig. 1b), with an average diameter of 1.8 ± 0.7 nm (Fig. 1c). Furthermore, the zeta-potential of Au NCs is as high as +37.6 mV (Fig. S3, ESI†), indicating the excellent colloidal stability of AuDAMP in aqueous solution. Indeed, the Au NC aqueous solutions were stable for months by storing at room temperature, without observable precipitation and flocculation. As a typical feature, the fluorescence emission intensity showed negligible changes over the entire retention period (Fig. S4, ESI†). Using Rhodamine B (quantum yield = 95% in ethanol) as the contrast, the quantum yield of AuDAMP is measured to be approximately 0.92% in aqueous solution, which is comparable to that of penicillamine-reduced Au NCs and is significantly higher than that of thiol-stabilized Au NCs reduced by NaBH₄. Similar to fluorescent Au(i) clusters, AuDAMP has a very large Stokes shift up to 200 nm. Generally, these fluorescent Au NCs are intermediates between fluorescent Au(i) complexes and non-fluorescent large-size Au NPs, and the fluorescence is associated with the composition of the Au(i) moiety in the cluster. To verify whether this insight also applies to AuDAMP, we inspected the valence states of Au in AuDAMP. As illustrated in Fig. 1d, the X-ray photoelectron spectroscopy (XPS) spectrum shows the binding energy of Au4f₅/₂ and Au4f₇/₂ at 87.6 eV and 83.8 eV, respectively, indicating that both Au(0) and Au(i) exist in AuDAMP. To further validate the key role of Au(i) in the emitter, we also investigated the spectroscopic changes of AuDAMP fluorescence upon addition of borohydride (Fig. S5, ESI†). As expected, a sharp reduction in fluorescence intensity was observed due to the further reduction of Au(i). These observations strongly support the hypothesis that the Au(i) part that existed in the Au NCs is responsible for the fluorescence.

The optimization of the synthetic conditions (i.e., DAMP-to-Au molar ratios, pH, reaction temperatures and heating time) to improve the fluorescence properties of AuDAMP was performed sequentially. The molar ratio of DAMP to Au during the reaction affected the synthesis of AuDAMP greatly. At a constant gold precursor concentration of 1.0 mM, the fluorescence intensity of AuDAMP increases drastically before the DAMP-to-Au molar ratio reaches 1.5 (Fig. S6a, ESI†). In contrast, the fluorescence intensity of AuDAMP severely decreases when the DAMP-to-Au molar ratio exceeds 2.0, implying that superfluous DAMP may recede the fluorescence from AuDAMP. Also, the reaction times distinctly influence the fluorescence intensity of AuDAMP (Fig. S6b, ESI†). As for the choice of pH, the reaction can only be carried out under non-alkaline conditions (Fig. S6c, ESI†), which is different from other reports. Naturally, this synthetic reaction strongly depends on temperature (Fig. S6d, ESI†). It shows a fast reaction kinetics when the reaction temperature is between 70 °C and 85 °C.

Fig. 1 Characterization of AuDAMP. (a) Fluorescence excitation and emission spectra of Au NCs centered at 420 nm (blue line) and 620 nm (red line), respectively. The inset shows the photographs of AuDAMP under visible light (1) and 365 nm UV light (2). (b) Transmission electron microscopy (TEM) image, (c) size distribution histogram, and (d) XPS spectrum (Au4f zone) of AuDAMP.
We found that the simple Au NCs can selectively detect GSH over other main biomolecules including glutamic acid (Glu), glycine (Gly), lysine (Lys), Cys, NAC, methionine (Met), glutamine (Gln), phenylalanine (Phe), arginine (Arg), serine (Ser), and glucose and cell culture medium (DMEM) in phosphate buffer saline (PBS). As illustrated in Fig. 3a, the fluorescence intensity of AuDAMP displays a significant enhancement (≈7-fold) upon addition of GSH, whereas other common molecules do not cause obvious fluorescence enhancement compared to GSH. In particular, the main interfering agents during the detection of intracellular GSH, Cys and NAC, do not show a significant response (Fig. 3a). This clearly demonstrates that AuDAMP can selectively distinguish GSH from Cys and NAC. To further verify the selectivity of the fluorescent sensor toward GSH, competitive experiments were carried out by adding the same concentration of GSH to an assay system containing the potential interfering substances mentioned above (Fig. S7, ESI†). The results showed that common potential interfering substances hardly affect the AuDAMP in the GSH assay.

The effect of thiols on the fluorescence of Au NCs has been investigated recently. Generally, charge transfer from the ligands to the metal center, chemical etching, and the steric hindrance effect are the main mechanisms for fluorescence enhancement of Au NCs. Obviously, the etching mechanism is unrealistic in our case as this process generally requires a long reaction time (days to weeks), while the fluorescence enhancement of AuDAMP by GSH can be achieved rapidly. The charge transfer mechanism from the ligands to the metal center has been widely employed to clarify the fluorescence enhancement of Au NCs stabilized by thiol ligands such as GSH. When thiols are added, the charge transfer might occur between the Au NC core and thiols through the formation of a strong Au–S bond. GSH contains more electron-rich groups (e.g. amino group and carboxyl group) than Cys and NAC, which might also contribute to the fluorescence enhancement. The fluorescence enhancement of noble metal nanoclusters ascribed to the large steric hindrance by GSH has also been presented. These proposed mechanisms seemingly show some evidence resembling our results. However, due to the lack of direct support, the GSH-induced fluorescence enhancement requires further study.

The fluorescence spectra of AuDAMP after adding different concentrations of GSH are shown in Fig. 3b. As the concentration of GSH increases, the fluorescence intensity of AuDAMP increases. The change in fluorescence intensity ratio (FL/FL0) at 620 nm as a function of GSH concentration ([GSH]) is shown in Fig. 3c. The fluorescence intensity enhancement is linearly correlated with the GSH concentration range of 2–25 mM, with a LOD of 1.21 mM, suggesting that this NC may be suitable for the detection of intracellular GSH.

Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was then employed to investigate the NC species of AuDAMP and after the addition of GSH. It is worth noting that we can only infer that the newly formed Au species will cause fluorescence enhancement because separating the fluorescent species effectively is very difficult at the current stage. The result shows that Au13 are the major species in AuDAMP, including the highly abundant species [Au13(DAMP)5]4− (m/z 818.95, peak 1) and [Au13(DAMP)4]4− (m/z 852.93, peak 2), and other species such as Au9 (m/z 1014.89, peak 3), Au12 (m/z 1210.82, peak 4), and Au17 (m/z 1048.84, peak 5) are also present (top panel, Fig. 3d and Table S1, ESI†). Compared with AuDAMP alone, several new Au species appeared after the addition of GSH (bottom panel, Fig. 3d and Table S1, ESI†), i.e., [Au8(DAMP)6(GSH)]3− (m/z 761.07, peak 6); [Au14(DAMP)6(GSH)]4− (m/z 930.70, peak 7); [Au13(DAMP)15(GSH)]3− (m/z 971.09, peak 8); and [Au8(DAMP)12(GSH)]3− (m/z 1135.18, peak 9). These results clearly show that GSH can successfully attack AuDAMP and form dual ligand (DAMP and GSH) stabilized Au NCs. The formation of dual ligands might contribute to the fluorescence enhancement.

Considering that AuDAMP exhibits an excellent fluorescence response to GSH at the millimolar concentration level, we have...
investigated their possible role as candidate probe for the evaluation of intracellular GSH, as well as its capability to selectively image cancer cells. For a concentration of AuDAMP of 100 \(\mu g\) mL\(^{-1}\), significant fluorescence in cancerous A549 and HepG2 cells was observed after 3 h of incubation (Fig. 4), while relatively dark fluorescence was observed for normal AT II and L02 cells (Fig. S8, ESI†). When a medium containing GSH (10 mM) is further added to the AuDAMP treated cells, the fluorescence intensity of the cells, particularly the normal cells (i.e. L02 and AT II), is further increased after 2 hours of incubation (Fig. S9, ESI†). For cells pretreated with buthionine sulfoximine (BSO), a known GSH synthase inhibitor unresponsive to AuDAMP (Fig. S10, ESI†), the fluorescence of A549 and HepG2 was significantly inhibited after further treatment with AuDAMP (Fig. 4), corresponding to the repression of intracellular GSH levels. As a control, almost no changes were observed for AT II and L02 cells after treatment with BSO (Fig. S8, ESI†). The extraction of related fluorescence grayscale values inside cells may offer some semi-quantitative results (Fig. 5). We have also further confirmed this result by using commercial kits to measure the intracellular GSH levels (Table S2, ESI†). These results unarguably confirmed that the observed bright fluorescence in cancerous cells is triggered by highly fluorescent Au nanocomposites generated from the reaction between intracellular GSH and internalized AuDAMP. This strongly supports the observation that the prepared AuDAMP can be used as a potential probe for intracellular GSH imaging and cancer diagnostic studies. Compared with other fluorescent probes for GSH imaging, the preparation of AuDAMP is facile and safe, without sundry synthesis and separation procedures and without the introduction of other hazardous materials (such as Hg).\(^{35}\) So it is believed that the versatile Au NCs will be a promising candidate probe for future personalized biomedical applications.

In summary, we have demonstrated the rapid GSH-induced fluorescent enhancement of AuDAMP by formation of dual ligand stabilized Au NCs. The fluorescence enhancement is specific to GSH, with a limited response to other common thiol species especially Cys and NAC. Based on this nature, a biocompatible fluorescent probe is proposed for selectively distinguishing cancer cells from normal cells due to the fact that GSH levels in cancer cells are significantly higher than those in normal cells. These results showed that the biocompatible fluorescent probe can be used as a candidate to image the intracellular GSH, which shows great prospects to reveal the cell redox equilibrium and the cell state.

Conflicts of interest
There are no conflicts to declare.

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Notes and references


