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Dual ligand co-functionalized fluorescent gold nanoclusters for "turn on" sensing of glutathione in tumor cells

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In this work, we develop a facile one-step strategy for rapidly preparing dual ligand co-functionalized fluorescent gold nanoclusters (Au NCs) and establish a "turn on" approach for rapid and selective sensing of glutathione (GSH) in aqueous solution and living cells. The as-prepared Au NCs exhibited orange red fluorescence (λem=608 nm), a long lifetime (5.62 μs), a large stokes shift (>300 nm), considerable stability and were systematic characterized by using fluorescence spectra, high-resolution transmission electron microscopy (HRTEM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), fluorescence lifetimes, infrared spectra (IR) and ultraviolet absorption spectrum. Based on the fluorescence recovery induced by competitive coordination with Cu2+ between Au NCs and GSH, the present "turn on" approach offers a high sensitivity and excellent selectivity toward GSH detection with a detection limit of 9.7 nM. More importantly, this "turn on" approach could also be successfully applied for visualizing and monitoring change of the intracellular GSH level in Hep G2 cells, providing available potential for diagnostic applications.

1. Introduction

Glutathione (GSH), a thiolated tripeptide, is an important antioxidant and the most abundant low-molecular thiol in the intracellular environment.[1,2] It plays pivotal roles in maintaining biological redox homeostasis in biological systems which are crucial for cell growth.[3] More importantly, the abnormal levels of GSH has been proved to be closely associated with certain diseases, such as Alzheimer's disease, AIDS, cancer and liver damage, etc.[4-6] Consequently, sensitive and quantitative detection of GSH are of sustained attention owing to its clinical significance. Thus far, due to the advantages of high sensitivity, simplicity, and nondestructive properties,[7] various fluorescent probes have been proposed for GSH detecting, including conjugated polymers, organic fluorophores and semiconductor quantum dots, etc.[8-13] However, these materials suffer from either time-consuming synthesis , poor photostability or high biotoxicity, which limit their widespread application for routine detection. [14] Therefore, it is necessary and important to make further efforts to develop simple, low-toxic and efficient candidates for sensitive and selective GSH detection.

As a burgeoning and promising kind of fluorophores, gold nanoclusters (Au NCs) below 2 nm in diameter, have gained much attention in biosensing and bioimaging due to their unique photophysical properties, excellent biocompatibility and ultrasmall size in the past decade. [15-18] Particularly, Au NCs with near-infrared (NIR) emission attract special attention in the field of bioimaging application because NIR emission can enhance the signal-to-noise thanks to its negligible tissue scattering. Prompted by their potential applications, NIR fluorescent Au NCs have been synthesized utilizing various protected ligands, including protein, polymers, alkanethiol or peptide, etc. [19-24] Among these protecting ligands, alkanethiol are extensively used because of the considerable stability and easy functionalization for alkanethiol-stabilized NIR fluorescent Au NCs. With reference to the synthesis of alkanethiol stabilized NIR fluorescent Au NCs, phase-transfer, ligand exchange and etching techniques have been previously reported. [25] However, these methods suffer from complicated procedures or post-treatment. Aiming at these problems, recently, Sun et *al* have first simplified the synthetic procedures and synthesized the NIR fluorescent 11-mercaptoundecanoic acid (MUA) protected AuNCs by using MUA both as a reducing and protecting agent. [26] Nevertheless, it is noteworthy that this synthetic strategy is not entirely satisfactory since it still requires long reaction time (20 hours), which adversely affected on practical applications. Therefore, the development of a facile strategy to solve this problem is still highly valuable, but even more challenging.

In this work, we report a facile one-step strategy for rapidly preparing dual ligand co-functionalized fluorescent Au NCs with intense orange red fluorescence (λ_{em}=608 nm). Three obvious advantages of the as-obtained Au NCs make them particularly attractive: (1) The reaction time can be shortened

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from 20 hours to 2 hours, thanks to the direct donation of delocalized electrons of electron-rich atoms or groups of the ligands to the Au core; (2) The large stokes shift (>300 nm) forecast that they could be a prominent fluorescent probe for biological imaging; (3) They have excellent photostability (pH-, temperature-, salt- and time-stability) and water dispersibility. Subsequently, the as-obtained Au NCs together with Cu^{2+} were used as a "turn on" probe for GSH detection. Due to the strong coordination of Cu^{2+} with the carboxyl group of MUA and Methionine (Met), Au-S charge transfer was blocked, leading to the fluorescence quenching of the as-obtained Au NCs. However, the quenched fluorescence can be restored within seconds when GSH is introduced due to the stronger binding preference of GSH with Cu^{2+}. Based on this, this "turn on" probe offers an excellent selectivity in discriminating GSH from other common biomolecules as well as high sensitivity [limit of detection (LOD): 9.7 nM, at a signal-to-noise ratio (SNR) of 3]. Furthermore, it was also successfully applied for monitoring the GSH level in tumor cells (Hep G2), showing feasible potential in bio-imaging and bio-medical applications.

2. Experimental

2.1 Materials

All reagents were of analytical reagent grade. GSH , Met, MUA, Histidine (His), Serine (Ser), Alanine (Ala), Glutamic (Glu), Isoleucine (Iso), Lysozyme (Lys), Human serum albumin (HSA), Trandferrin (Tf), Glucose, uric acid (UA), dopamine and $HAuCl_4 \cdot 3H_2O$ were purchased from Sigma (St. Louis, MO, USA). $CuSO_4$ and NaOH (sodium hydroxide) were supplied by Beijing Fine Chemical Factory (Beijing, China). All solutions were prepared utilizing ultrapure water from a Millipore Simplicity 185 water purification system (Millipore, USA). Serum sample of healthy people were obtained from the Affiliated Hospital of the Beijing Normal University. This sample was collected with informed consent from the human subject. In addition, this sample collection was approved by Institutional Review Board of Beijing Ditan Hospital.

2.2 Instruments

Fluorescence spectra was recorded using an F-4600 fluorescence spectrometer. High-resolution transmission electron microscopy (HRTEM) was carried out on JEOL JEM-2100 microscope operating at 200 kV. X-ray photoelectron spectroscopy (XPS) was carried out on a thermoelectron instrument (Thermo-VG Scientific ESCALAB 250). All spectra were referenced to the C 1s peak at 285.0 eV. Dynamic light scattering (DLS) experiment was carried out on a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). Fluorescence lifetimes were measured utilizing an OB920 single-photon counting fluorometer (Edinburgh Analytical Instruments). Infrared spectra (IR) were recorded using a FT/IR-410 Fourier transform infrared spectrophotometer (JASCO, Japan).

2.3 Preparation of dual ligand co-functionalized fluorescent Au NCs

All glassware were washed with aqua regia and rinsed with ultrapure water. Typically, a solution of NaOH (1 M, 350 μL) was added to a MUA solution (10 mM, 2 mL), and then 150 μL of 10 mM $HAuCl_4$ and 2.5 mL of 0.03 g/mL Met were added to the above mixtures. The mixture was placed at room temperature without stirring for 2 h. The reaction product was the MUA and Met synergistically protected dual ligand co-functionalized Au NCs. It is noteworthy that the as-prepared AuNCs were not stable and tended to precipitate, because the concentration of Au NCs is too high and the presence of NaOH and other un-reacted chemicals. Therefore, the crude product was filtrated and the presence of components in precipitate were the dual ligand co-functionalized Au NCs. Then, they were re-dispersed in pure water. The filtered clear solution (MUA, Met, salts, etc.) was not fluorescent. Thus, the highly stable purified Au NCs were obtained, and they were stored at room temperature before use.

2.4 Fluorescent "turn on" assay for sensing GSH

The as-purified Au NCs was diluted 20 times using phosphate buffer solution (PBS, pH=7.4), named Au NCs solution (1/20). For the GSH detection, 5 μL of 7.5 mM Cu^{2+} solution was added to 2 mL of the Au NCs solution (1/20) to quench the fluorescence. Afterwards, 10 μL of GSH of different concentrations were added to the above mixtures. The recovered fluorescence intensity was recorded with excitation at 275 nm

2.5 Cell imaging

Hep G2 cells were cultured in 12-well plates with Dulbecco's Modified Eagle Medium (DMEM) media with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator to achieve a suitable density. Then, Au NCs solution (1/20) containing Cu^{2+} (18.75 μM) was added to the culture media, incubating for 2 h. To detect the intracellular GSH, the cultured cells were washed with cold PBS (pH 7.4) three times before imaging. For control experiment, the cultured cells were pretreated with a thiol oxidant-hydrazine hydrate. Then, Au NCs solution (1/20) containing Cu^{2+} (18.75 μM) was added to the pretreated cells in culture media. The pretreated cells were also washed with cold PBS (pH 7.4) three times before imaging. Finally, images were observed on a inverted fluorescence microscope.

3. Results and discussion

3.1 Synthesis and Characterization of dual ligand co-functionalized fluorescent Au NCs

We synthesized the dual ligand co-functionalized fluorescent Au NCs with the improved strategy in allusion to the previous research.[24] In our study, we discover that $HAuCl_4$, MUA, Met and the mixture of each two components (The mixed reaction time is 2 h) show non fluorescence, respectively (Fig.1A-a to f). Attractively, after the addition of Met to the mixture of $HAuCl_4$ and MUA for 2 h, strong orange red fluorescence could be obviously observed (Fig. 1A-g), suggesting the formation of fluorescent Au NCs. The fluorescence spectra showed its maxima emission peak located around 608 nm (Fig. 1B), with a quantum yield (QY) of 7.6 %

(calibrated with Rhodamine B as the reference). Meanwhile, the absence of localized surface plasmon resonance absorption peak around 520 nm further confirmed the formation of the Au NCs (Fig. S1). According to the previous reports, thiolate ligand functionalized Au NCs have an Au^0-Au^I core-shell nanostructure and the fluorescence was probably generated by the aggregation-induced emission (AIE) of Au(I)–thiolate complexes, which involved two steps. The first step was the reduction of Au (III) to Au(I) by the thiol group, immediately formed insoluble aggregates of Au(I)–thiolate complexes. The second step, the insoluble aggregates of Au(I)–thiolate complexes was dissolved to be oligomerization of Au(I)–thiolate complexes after adding NaOH. Then, luminescence would be generated when the complexes slowly aggregated to form the Au^0-Au^I core-shell nanostructure by collision and fusion.[27] In addition, previous reports illustrated that direct donation of delocalized electrons of electron-rich atoms or groups of the ligands to the Au core could accelerate the emerge of fluorescence based on ligand to metal charge transfer.[28,29]Moreover, ligands with electron-rich atoms (O or N, etc.) or functional groups (carboxylic and amino groups, etc.) could effectively strengthen the electron transfer which is beneficial to accelerate the emerge of fluorescence of the dual ligand co-functionalized fluorescent Au NCs. [28,30,31]

To characterize the as-prepared Au NCs, a typical transmission electron microscopy (TEM) image in Fig. 1C reveals that they are spherical with good monodispersity. Moreover, the well-resolved lattice planes of approximately 2.3 Å spacing in the HRTEM image (inset in Fig. 1C) indicates the extraordinary crystalline structure of the Au NCs, which is consistent with the previous report.[32] The average diameter, calculated by measuring more than 100 particles in the TEM image, is about 1.3 ± 0.2 nm, which was in accordance with the DLS data (Fig. 1D). The fluorescence lifetime of the Au NCs was 5.62 μs (Fig. 1E), indicating they should be a good candicate fluorescent probe for long-term cell imaging. Furthermore, the as-prepared Au NCs are relatively stable and the fluorescence quenching only occurred slightly after one month of storage at room temperature (Fig. 1F).

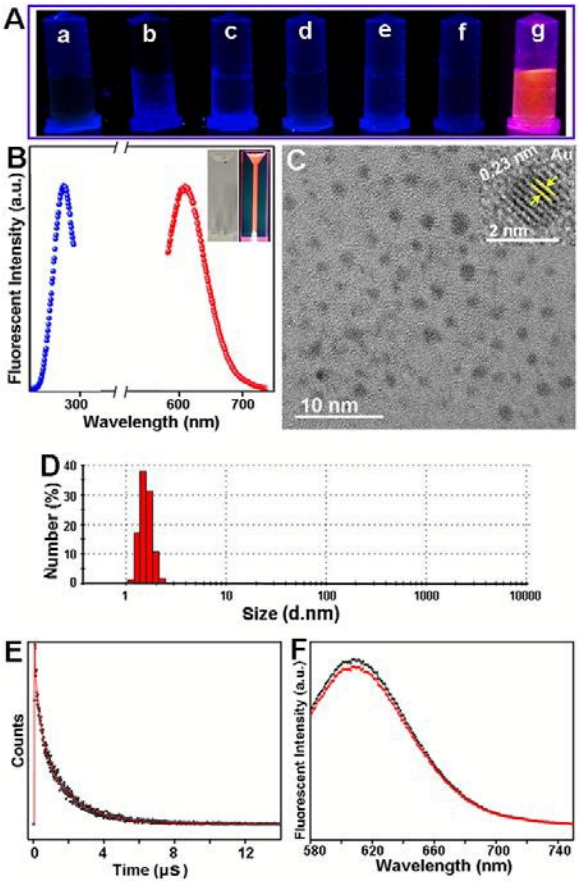


Fig. 1 (A) Photographs of (a) $HAuCl_4$, (b) MUA, (c) Met and the mixture of (d) $HAuCl_4$ + Met, (e) MUA+ Met, (f) $HAuCl_4$ + MUA and (g) $HAuCl_4$ + MUA + Met under UV light, the mixing time is 2 h. (B) Excitation spectra (blue line) and emission (red line) spectra of the Au NCs, inset shows photographs of the Au NCs under sunlight (left) and 365 nm UV light illumination (right). (C) Typical TEM image of the Au NCs, inset displays the HRTEM image of the Au NCs. (D) DLS histogram of the Au NCs. (E) Fluorescence lifetime of the Au NCs. (F) Emission spectrum of the Au NCs freshly prepared (black line) and after one months storage (red line) at room temperature.

In order to achieve more information about the as-prepared Au NCs, IR spectra was performed. As shown in Fig. 2A, the IR spectroscopy analysis indicated that two chief functional groups (–COOH and $–NH_2$) were on the surface of the Au NCs, with the fact: asymmetric and symmetric stretching vibration peaks of COO^- at 1600 and1400cm^{-1} and the $–NH_2$ and –NH wagging band around 702 cm^{-1}. [33,34] This demonstrated that both Met and MUA collaboratively on the surface of the Au NCs. For in-depth investigation of the as-prepared Au NCs, XPS was performed and the result was shown in Fig. 2B. The binding energies of Au 4f, located at 88.38 eV and 84.68 eV, respectively, definitely demonstrates that both Au(0) and Au(I) exist in the Au NCs.[35] The Au(I) surrounded on the surface of gold core is reported to be beneficial for the stabilization of thiol-stabilized Au NCs,[36] and it is proved that the as-prepared dual ligand co-functionalized fluorescent Au NCs really have high stability under various pH values, different concentrations of NaCl and various temperatures (Fig. S2). In addition, the S $2p_{3/2}$ peak located at 162.8 eV (Fig. 2C) corresponding to a gold thiolate confirms the covalent interaction of AuNCs with the sulfhydryl group.[37] Moreover, C(1s), N(1s), O(1s),

and S(2p) core-level photoemission spectra all appeared on the XPS spectrum (Fig. 2D), further reflecting that both MUA and Met collaboratively stabilized the as-prepared Au NCs. Besides, a systematic study was carried out to explore the optimal conditions to synthsize the as-prepared Au NCs (Fig. S3).

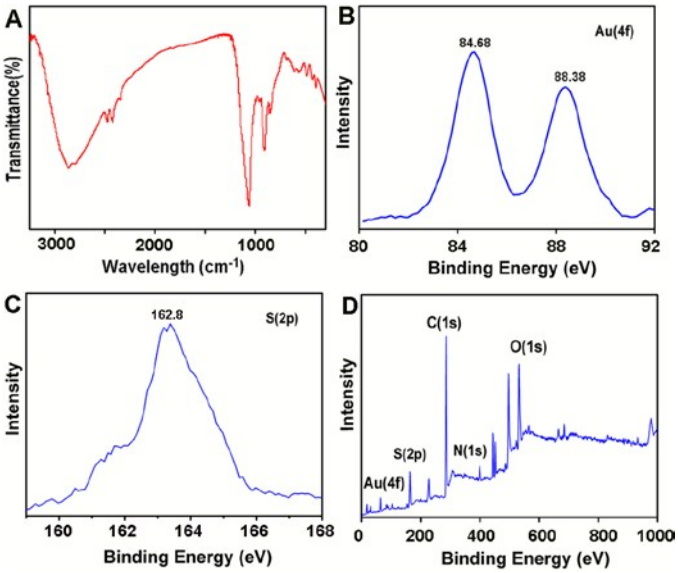
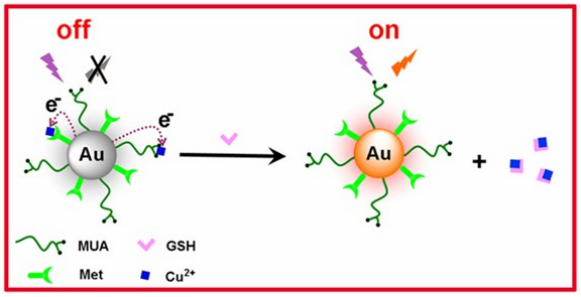


Fig. 2 (A) Infrared spectroscopy of the as-prepared Au NCs. (B) Au 4f electron region of the XPS of the as-prepared Au NCs. S(2p) XPS spectrum (C) and the whole XPS spectrum (D) of the as-prepared Au NCs, C(1s), N(1s), O(1s), S(2p) and Au (4f) core-level photoemission spectra all appeared on the XPS spectrum.

3.2 Principle of the fluorescent "turn on" assay

It is reported that Cu^{2+} is inclined to quench the fluorescence of the thiol protected Au NCs owing to its high coordination ability to the special carboxyl or thiol groups on their surface, and this coordination establishes effective charge transfer from Au NCs to Cu^{2+} and blocks the Au-S charge transfer in the interface of the Au NCs. [38] After the addition of a analyte which has a the higher binding affinity with Cu^{2+}, the Au-S charge transfer in the interface of the Au NCs reverted, and thus the quenched fluorescence could be recovered (Scheme 1). According to this, we engineered a highly sensitive assay of GSH by the competitive binding of Cu^{2+} between the as-prepared Au NCs and GSH, accompanied by a fluorescence "off-on" process. As shown in Fig. 3A, the Au NCs (1/20) exhibited intense orange-red fluorescence which could be immediately quenched in the presence of Cu^{2+} (10 μM), because of the coordination of Cu^{2+} with the special carboxyl group on the surface of Au NCs. [39] Thus, effective charge transfer from Au NCs to Cu^{2+} was established and the Au-S charge transfer in the interface of the Au NCs might be blocked. [40] However, the fluorescence could be partially recovered within seconds by addition of GSH (20 μM), because GSH would preferentially bind to Cu^{2+} with higher affinity, and effectively recapture the Cu^{2+} from the surface of the Au NCs. Therefore, the quantitative detection of GSH could be realized based on the fluorescence signal recovery.



Scheme 1 Schematic representation of the"turn on"assay for GSH.

3.3 Fluorescent "turn on" for GSH detection

The influence of Cu^{2+} concentration on the assay system was firstly investigated. As shown in Fig. 3B, the titration of Cu^{2+} into the AuNCs solution (1/20) results in a gradual quenching of the AuNCs fluorescence with the increasing concentration of Cu^{2+} (0-35 μM). It was demonstrated that Cu^{2+} with a final concentration of 18.75 μM was able to induce the fluorescence quenching approximately 85% of the initial fluorescence intensity of the AuNCs solution (1/20), and this Cu^{2+} concentration would be adopted in the following detection, taking the low probe background signals and wide linear range into consideration. After adding GSH to the AuNCs solution (1/20) containing Cu^{2+} (18.75 μM), the fluorescence signal was found to be gradually recovered with increasing the concentration of GSH within seconds (Fig. 3C). An excellent linear correlation (R^2=0.9927) exists based on the recovering effects (I/I_0) on the concentration of GSH over the range from 0.03 to 22.5 μM (Fig.3D), with a detection limit of 9.7 nM based on a signal-to-noise ratio of three. The sensitivity of the proposed assay is comparable to, or even better than, those of previously reported approaches for GSH detection. [14, 41,42]

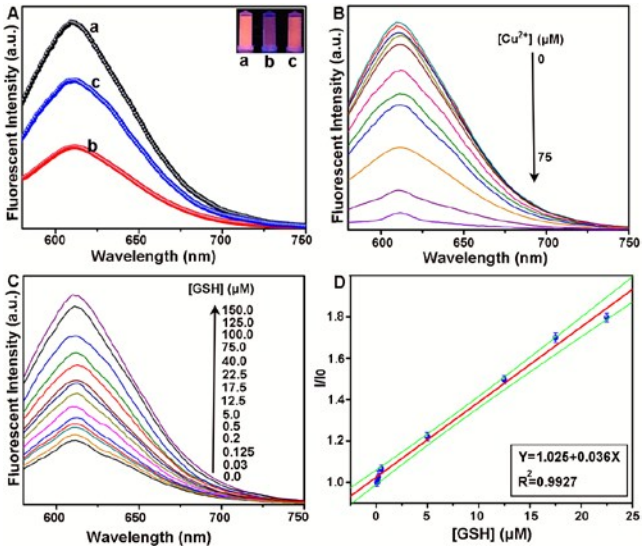


Fig. 3 (A) Emission spectra and photographs (inset) under UV light of (a) the as-prepared AuNCs, (b)AuNCs + Cu^{2+} and (c) AuNCs + Cu^{2+} + GSH. (B) Emission spectra of the as-prepared AuNCs in the presence of different concentrations of Cu^{2+}. (C) Emission spectra of the as-prepared AuNCs-Cu^{2+} complexes with addition of different GSH concentrations. (D) Linear relationship between the I/I_0 and the concentration of GSH, where I_0 and I are the corresponding fluorescence intensities in the absence and presence of GSH, respectively

Besides a good sensitivity, a highly special response to the analyte over potentially competing species is a necessity for real applications. Therefore, considering the complexity of the bio-matrix, the selectivity of the current assay to GSH over other electrolytes and biological species including some common proteins, amino acids, glucose, uric acids, dopamine and metal ions was investigated. As shown in Fig. 4, it was found that the present assay exhibited high fluorescence recovery selectivity towards GSH, other common proteins, amino acids, glucose, uric acids, dopamine and metal ions had minor or negligible recovery effects.These results demonstrated that the present assay approach owned high sensitivity and excellent selectivity towards GSH.

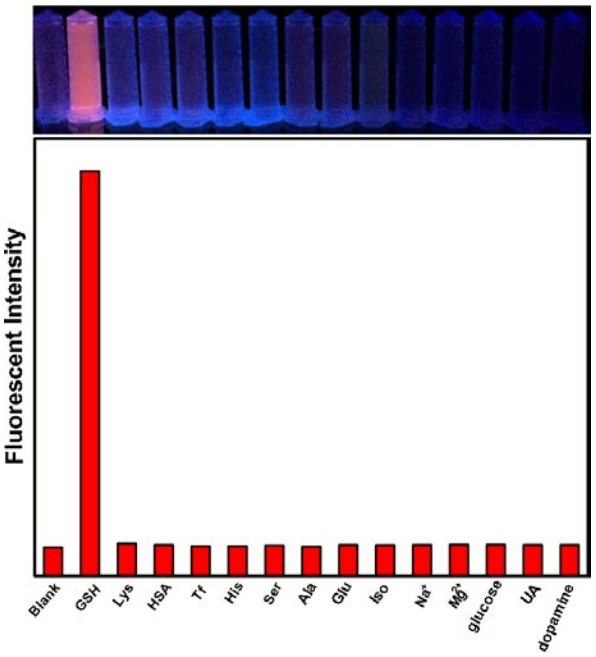


Fig. 4 Fluorescence response of the AuNCs solution (1/20) containing Cu^{2+} (18.75 μM) toward GSH (20 μM) and different electrolytes and biomolecules (0.5 mg/mL for Lys, HSA and Tf, 40 μM for His, Ser, Ala, Glu, Iso, 0.1 mM for Na^{+} and Mg^{2+} and 5 mM for Glucose, UA and dopamine.

3.4 Monitoring the changes of the intracellular GSH levels

Sensing the intracellular GSH level is extremely attractive because of its important functions in biological systems. Consequently, we examined the ability of the present assay approach for monitoring changes of the intracellular GSH level in Hep G2 cells. The Hep G2 cells were first incubated with AuNCs solution (1/20) containing Cu^{2+} (18.75 μM) for 10 min and orange-red fluorescence emission could be readily observed (Fig. 5B), whereas almost no fluorescence was observed for control Hep G2 cells without the incubation of AuNCs solution (1/20) containing Cu^{2+} (Fig. 5A). This result demonstrates that the present assay approach can be rapidly sensing the high expressed level of GSH in Hep G2 cells. In order to investigate whether the present assay approach can monitor the changes of the intracellular GSH level, we pretreated the Hep G2 cells with N-methylmaleimide (a thiol-reactive reagent for decreasing the GSH level) for 10 min, an obvious decrease of the fluorescence intensity can be seen in Fig. 4C after adding AuNCs solution (1/20) containing Cu^{2+}. It was concluded that the present assay approach could not only for the "turn on" imaging of the intracellular GSH but also to monitor the changes of the intracellular GSH in tumor cells.

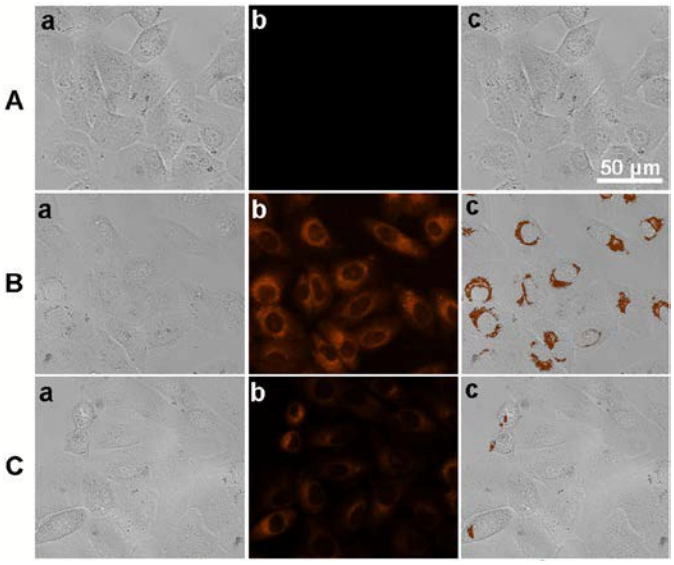


Fig. 5 (A) Bright field (a) and fluorescence microscopic images (b) of Hep G2 cells before incubation with AuNCs solution (1/20) containing Cu^{2+}. (B) Bright field (a) and fluorescence microscopic images (b) of Hep G2 cells after incubation with AuNCs solution (1/20) containing Cu^{2+}. (C) Bright field (a) and fluorescence microscopic images (b) of Hep G2 cells pretreated with N-methylmaleimide after incubation with AuNCs solution (1/20) containing Cu^{2+}. (c) in (A), (B) and (C) are the overlay of fluorescence and bright field images, respectively.

4. Conclusions

In summary, a facile and one-step method to accelerate fabricate dual ligand co-functionalized fluorescent Au NCs at room temperature was firstly demonstrated. Inspired by the competitive coordination of Cu^{2+} between the as-prepared Au NCs and GSH, we have established a simple "turn on" fluorescent approach for monitoring the changes of the intracellular GSH level in Hep G2 cells. We expect that the present work may not only offer a general route for the synthesis of other dual ligand co-functionalized fluorescent Au NCs, but also provide a new approach to study the biological roles of glutathione in pathophysiological situations.

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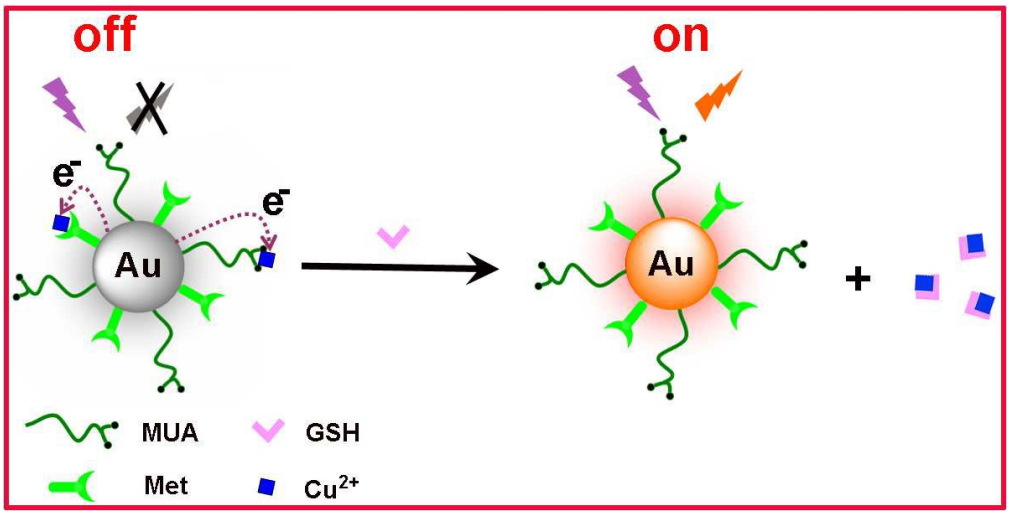
Graphical Abstract



A “turn on” approach was established for GSH sensing in tumor cells based on dual ligand co-functionalized fluorescent Au NCs.