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An ultra-sensitive fluorescent "Turn On" biosensor for glutathione and its application in living cells

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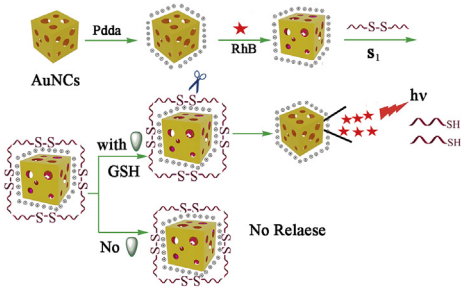
HIGHLIGHTS

- An effective controlled-release biosensor based on Au nanocages was developed for ultra-sensitive detection of GSH.
- The system can successfully achieve the controlled release of the loaded molecules in response to intracellular target molecules without any external stimuli.
- A detection limit of as low as 4.8×10^{-13} M with an excellent selectivity toward GSH could be achieved.
- The fabricated system was successfully employed for fluorescence microscopy imaging of GSH in living cells.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, an effective controlled-release biosensor based on Au nanocages (AuNCs) capped with disulfide-containing DNA molecular gates was developed for ultra-sensitive and highly selective detection of glutathione (GSH). Oligonucleotides containing the S-S bonds were assembled on the surface of the AuNCs by means of electrostatic interactions in order to inhibit the release of fluorescent molecules such as Rhodamine B (RhB) loaded by AuNCs. In the presence of GSH, due to the specific cleavage of S-S bonds in disulfide-containing single-stranded DNAs (ssDNAs) as well as their subsequent departure from the surface of AuNCs, the pores could be opened, and then the dye molecules would be released from AuNCs. The concentration of GSH ranged from 1.0×10^{-12} to 6.0×10^{-10} M could be detected. The developed amplification strategy based on the controlled-release of fluorescent molecules reached an extraordinary sensitivity of GSH. A detection limit of as low as 4.8×10^{-13} M with an excellent selectivity toward GSH could be achieved. The results of fluorescence microscopy imaging of GSH in living cells indicate that the fabricated system is an efficient controlled-release biosensor in response to intracellular target molecules and predict its potential use for *in situ* molecular imaging in living systems.

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1. Introduction

Gold nanostructures of different sizes, shapes and composition provide a multifaceted, functional platform can be widely used in bio-medical and imaging field [1]. Compared with other potential nanomaterials used in sensing protocols, Au nanocages (AuNCs) are getting more and more attention due to their significantly physical and unique chemical properties, excellent bio-compatibility and bio-inert nature in bio-medical applications [2,3]. Thanks to their hollow interiors, porous walls, and highly tunable localized surface plasmon resonance (LSPR) into the near infrared (NIR) region ranging from 700 to 900 nm, AuNCs as an attractive platform for optical imaging applications can be widely used in the field of cancer diagnostics [4] and treatment [5]. In addition to the above properties, AuNCs still have many superiorities, such as a compact size, extraordinarily large scattering and absorption cross-sections (almost five orders of magnitude greater than those of conventional organic dyes), low cytotoxicity, excellent bio-compatibility, as well as a robust and straightforward procedure for surface modification using the well-established thiolate-Au chemistry [6–8]. For *in vivo* applications, these significant features of AuNCs can make them as a noticeable material for biomedical applications including imaging [9], diagnosis [10] and treatment [11]. In order to meet both early diagnostic and therapeutic requirements of cancer, it is necessary to develop a composite system that combines all the features of AuNCs.

Recently, AuNCs have been received intensive research as a novel class of controlled-release delivery vehicles by taking advantage of their hollow interiors, porous walls, as well as significant bio-compatibility such as non toxicity and non immunogenicity [12]. It can be functionalized with bio-chemical or supramolecular ensembles than acted as “molecular gates” for the development of stimuli-responsive systems able to release guest molecules due to the presence of certain and well defined stimuli. For example, phase-change material was used as the medium to help load the drug, which can also serve as a “gate-keeper” to control the release of drug in response to temperature up [13,14]. Different external stimuli such as pH or temperature can be used to trigger and regulate the release of filler. In addition, it is possible to vary the power or the duration of exposure to high-intensity focused ultrasound (HIFU) to control the release [15].

Relative to the controlled-release systems triggered by external stimuli, those not requiring any external stimuli can provide more benefits and advantages, including simplicity and practicality, especially *in vivo* biomedical applications. In recent years, our group has proposed a number of controlled-release systems based on AuNCs [16,17], which not requiring any external stimuli and showing promising performance in the field of controlled-release systems. In our reported systems, the controlled release of the guest molecules from the gated AuNCs could be achieved successfully by the challenge with the target biological molecules rather than external stimuli such as pH, temperature, NIR laser irradiation or high-intensity focused ultrasound, and so on. Despite these advances, it is still a challenge to develop more simple and efficient controlled-release AuNCs systems. In present study, we focus on the design and assembly of molecular gates by selecting glutathione (GSH) as a target molecule.

GSH and its oxidized glutathione disulfide (GSSG) as the molecules containing sulfur are known to be closely related with various physiological activities. In particular, GSH as the most abundant cellar thiol is critical for combating oxidative stress and maintaining redox homeostasis, this is because GSH exists in redox equilibrium between sulfhydryl (reduced form GSH) and disulfide (oxidized form GSSG) forms [18]. Recent studies have also shown that the changes in physiological concentrations of GSH are related to a variety of diseases. For example, Alzheimer's disease [19], Parkinson's disease [20–22], arthritis [23], diabetes mellitus [24,25], atherosclerosis [26], epilepsy [27], aging [28] as well as numerous types of cancers. In view of the close relationship between the abnormal concentration of GSH and these diseases development in body fluids, many methods for quantitative detection of GSH have been greatly developed in recent years [29,30], such as electrochemical methods [31], colorimetric methods [32,33], quantum dots-based fluorescence methods [34], and mass spectroscopy [35]. Since GSH can be oxidized, the detection of GSH based on redox reaction has been proposed. In most cases, the processing method requires sophisticated instruments, complicated operation, but with low detection accuracy. To solve these problems, a novel controlled-release biosensor based on AuNCs was designed for accurate and convenient detection of GSH by coupling the controlled-release of cargo molecules from gated AuNCs and the cleavage of S-S bonds in disulfide-containing DNAs by GSH.

Herein, a functional nucleic acid, that is a designed disulfide-bond-modified single-stranded DNA (ssDNA) recognition probe specific for GSH was used as a pore blocker to make AuNC a controllable release system. Based on the design, a novel fluorescent biosensor for GSH was constructed by filling the hollow interiors of AuNCs with fluorescent molecules such as Rhodamine-B (RhB). To the best of our knowledge, a controlled-release biosensor based on signal amplification by the RhB-loaded AuNCs caped with GSH-specific recognition probes and its application in living cells have not yet been reported. Owing to the distinct advantages such as simplicity in molecular gate design and biosensor fabrication, cost-effective and high sensitivity, it thus holds a huge potential for the development of ultra-sensitive bio-sensing platform both *in vitro* and *in vivo* biomedical applications and has a promising future in the fields of cancer theranostics including imaging, diagnosis, drug delivery, therapy and so on.

2. Experimental

2.1. Materials

Sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$), polyvinylpyrrolidone (PVP, average Mr ≈ 55,000), silver nitrate ($AgNO_3$), ethylene glycol, sodium chloride (NaCl), poly diallyldimethylammonium chloride (Pdda), N-ethylmaleimide (NEM), hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich (Shanghai, China) and used without further purification. The oligonucleotides used for the detection of GSH were synthesized and purified by Sangon Biotech Co. Ltd. (Shanghai, China), their sequences are as follows: 5′-ACG AGT CA S-S TTT TGT TTG TTC CCC CCT TCT TTC TTA-3' (S1, recognized by GSH), the random DNA, 5′-ACA AGC GCC AAC CCA GAG ACC GAG GAG G-3' (S2, not recognized by GSH). Rhodamine B (RhB) was obtained from Shanghai Aladdin Chemistry Corp (China). Glutathione (GSH), sodium hydrosulfide (NaSH), mercaptohexanol (MCH), thioglycolic acid (TA), cysteine (CYS) and dithiothreitol (DTT) were obtained from Sigma-Aldrich. Silver nanocubes were prepared according to literature procedures [36]. Hela cells were obtained from Chinese Academy of Medical Sciences and Ocean University of China. The 0.01 M PBS buffer (pH 7.4) was prepared by standard methods. Deionized and doubly distilled water was used throughout the experiments.

2.2. Apparatus

Magnetic nanoparticles (MNPs) modified with sulfhydryl groups (3.0–4.0 μm, 10 mg mL^{-1}) (SH-MNPs) and a magnetic rack were obtained from BaseLine Chrom Tech Research Centre (Tianjin,

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China). UV-visible spectra were recorded with a Cary 50 UV-vis-NIR spectrophotometer (Varian, Agilent). Transmission electron microscopy (TEM) image was taken with a JEM-2000EX/ASID2 instrument (Hitachi, Japan). Fluorescence spectra were recorded using a F-4600 fluorescence spectrophotometer (Hitachi, Japan) with a scan rate at 1200 nm/min. The excitation wavelength was set at 535 nm. The slits for excitation and emission were set at 10 nm/10 nm.

2.3. Synthesis of AuNCs

AuNCs were synthesized by the galvanic replacement reaction between Ag nanocubes and chloroauric acid ($HAuCl_4$) with some modifications [36]. Briefly, 200 μL of silver nanocubes was dispersed in 10 mL PVP (1 mg mL^{-1}) in a 50 mL round-bottomed flask that was equipped with a condenser under magnetic stirring. The dispersion was then refluxed for 10–15 min before 6.0 mL of 0.2 mM $HAuCl_4$ aqueous solution was added. As the $HAuCl_4$ solution was added to the flask, a series of color changes would be observed and can be used to estimate the position of the LSPR peak of the AuNCs. The mixture was refluxed for another 10 min until its LSPR peak had reached about 780 nm. After temperature dropped to room temperature, the sample was centrifuged and washed with saturated NaCl solution. The obtained AuNCs were dispersed in deionized water and stored in dark for further use.

2.4. Fabrication of the controlled-release biosensor

20 μL of MNPs (10 mg mL^{-1}), washed extensively with PBS buffer (pH 7.4), was added into 400 μL of the as-prepared AuNCs suspension, and the mixture was agitated overnight at room temperature. Magnetic separate and wash the obtained MNPs-combined AuNCs with PBS. Then, 200 μL of 11.7 mg mL^{-1} Pdda solution was added and incubated for 12 h at 37 °C.

After being washed three times with PBS buffer through magnetic separation, the obtained positively surface-charged AuNCs were soaked in 100 μL of PBS solution containing RhB (1.0×10^{-5} M). The mixture was incubated for 12 h at 37 °C.

In order to cap the holes of the RhB-loaded AuNCs, 10 μL of disulfide-containing recognition probes S1 (1.0×10^{-5} M) for GSH, used as pore blockers, were added into the above solution. The mixture was incubated for 12 h at 37 °C. The obtained controlled-release biosensor was completed by washing away the residual RhB and the unbound probes.

2.5. Detection of GSH

A GSH solution was introduced into the proposed sensing system and the mixture was subjected to incubation for 2 h at 37 °C to ensure the full cleavage reaction between the recognition probe S_1 and GSH. The supernatant was collected by magnetic separation for fluorescence detection with an excitation wavelength at 535 nm and an emission wavelength at 573 nm.

2.6. Detection of intracellular non-protein thiols in Hela cells

Hela cells (a human cervical carcinoma cell line) were applied to evaluate the capability of the biosensor system presented here in biological samples. A 1.0 mL suspension of Hela cells (approximately 1.0×10^6 cells mL^{-1}) was diluted to a final volume of 3.0 mL using ice-cold PBS (pH 7.4, 10 mM) and centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the cells were washed twice more with the same cleaning conditions. The treated cells were suspended in 3.0 mL ice cold PBS-EDTA (pH 7.4, 10 mM) buffer solution, after homogenization, 3% perchloric acid was added to precipitate the proteins in the cells. The whole operation is carried out on ice. Subsequently, centrifugation was performed at 1,4000 rpm for 5 min at 4 °C, the supernatant was collected for detection of non-protein thiols.

2.7. Fluorescence microscopy imaging of Hela cells

The supernatant of 1.0 mL suspension of Hela Cells was removed by centrifugation at 3000 rpm for 5 min. The fluorescence microscopy imaging was performed by incubating the treated Hela cells with the RhB-loaded AuNCs caped with GSH-specific recognition probes S1 in PBS for 15 min at 37 °C. The RhB-loaded AuNCs capped with random ssDNAs S2 were used as a control under the same conditions. A laser confocal microscope (Leica TCS SP5 II) was employed for fluorescence microscopy imaging.

3. Results and discussion

3.1. Characterization of the synthesized AuNCs

The synthesized AuNCs had a near-infrared LSPR peak at 780.0 nm (Fig. S1), indicating that the hollow structure of AuNCs. It was verified by transmission electron microscopy (TEM). As shown in Fig. 1, AuNCs with a clear hollow interior and thin wall were observed. The morphology and particle size of the synthesized AuNCs were consistent with the literature reports [36].

3.2. Design of strategy

In this paper, a simple amplification strategy based on the controlled-release AuNCs was developed for the achievement of ultra-sensitive detection of GSH, which was schematically illustrated in Fig. 2. A specific recognition probe S1 for GSH, which was a disulfide-containing ssDNA, was cleverly designed and employed as a molecular gate to block the pores of AuNCs, making which a controllable release system. RhB fluorescent molecules used as guest molecules were filled into the hollow interiors of AuNCs and could be released in a controlled manner in response to GSH target molecule. The formation of molecular gates can be achieved by assembling the electronegative probes S1 onto the surface of AuNCs through electrostatic interaction, which was pre-modified using an ordinary and water-soluble cationic polyelectrolyte, poly (diallyl dimethylammonium) chloride (Pdda) to give the positively surface-charged AuNCs. The release of RhB from the gated AuNCs could be triggered by the challenge with GSH target molecule. The proposed biosensor was characterized by TEM (Fig. S2) and UV–visible spectroscopy (Fig. S3).

In the presence of GSH molecules, contributing to the efficient cleavage of S-S bonds in disulfide-containing probes S1 as well as their subsequent departure from the surface of AuNCs, the pores could be opened, and then the loaded RhB molecules would be bio-responsive released from AuNCs. The departure of probe S1 from the surface of AuNCs was confirmed by the Zeta potential measurements (Table S1).

In order to improve the separation process, the magnetic nanoparticles (MNPs) modified with sulfhydryl groups (SH-MNPs) were used to combine with the as-prepared AuNCs. The feasibility of the proposed sensor for GSH detection was firstly verified. Fig. 3 showed the fluorescent signals of dye molecules RhB released from the hollow interiors of AuNCs caped with 1.0×10^{-6} M disulfide-containing S1. In the absence of GSH (curve b), the probes immobilized on the surface of AuNCs *via* the electrostatic interaction could block the pores and prevent the release of RhB from AuNCs. As indicated by curve a, a significantly enhanced fluorescent signal was indeed observed in the presence of 6.0×10^{-10} M GSH due to

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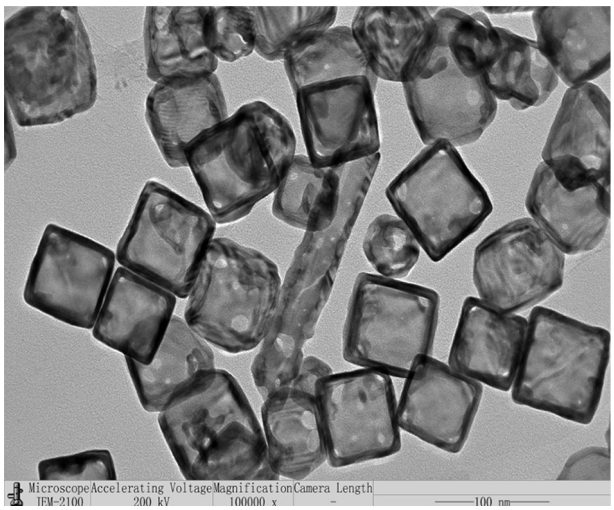

Fig. 1. Transmission electron microscopy (TEM) image of the synthesized AuNCs.

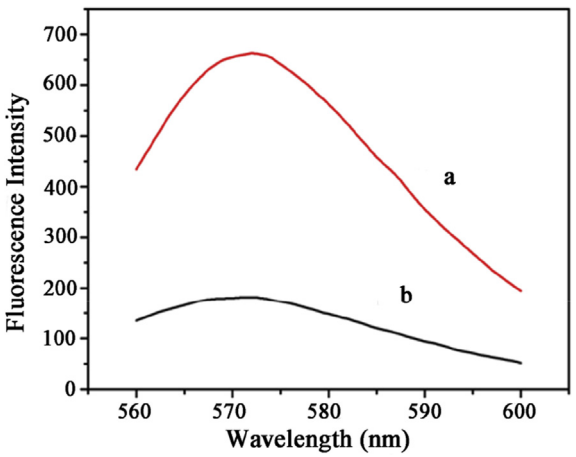

Fig. 3. Fluorescence intensity of the sensing system in the absence (b) and in the presence of 6.0×10^{-10} M of GSH (a).

the cleavage of disulfide-containing probes into two SH-DNA fragments and the resulting removal of the probes from the surface of AuNCs.

The results indicated that the fluorescence intensity of released RhB was sensitive to GSH target molecules. Since AuNCs were loaded with a large number of RhB fluorescent molecules, as long as a molecular gate is opened, there will be a lot of fluorescent molecules are released. So, based on the amplification by the controllable-release AuNCs, the ultra-sensitive detection of GSH can be achieved.

3.3. Optimization of reaction conditions

In order to obtain the optimal sensing performance, the experimental conditions including the incubation temperature, the concentration of the disulfide-containing probes S1, and the reaction time were optimized. It could be found that the fluorescence enhancement of the sensing system in the presence of 6.0×10^{-10} M GSH (Fig. S4) at 37 °C was higher than that at 25 °C evidently. Thus, 37 °C was chosen as the optimal incubation temperature.

The concentration of the disulfide-containing probes S1 was also investigated (Fig. S5). It could be seen that the maximum fluorescence response of the sensing system in the presence of 6.0×10^{-10} M GSH was obtained at the concentration of 1.0×10^{-6} M S1. Thus, the optimized S1 concentration was chosen as 1.0×10^{-6} M.

The results of the optimization of the reaction time showed that the fluorescence response of the sensing system in the presence of 6.0×10^{-10} M GSH increased continuously with the reaction time ranging from 0 to 2 h and almost reached the plateau after about 2 h. It suggested that the release process triggered by the cleavage of S-S bonds in disulfide-containing probes S1 reached to equilibrium at about 2 h (Fig. S6). As a result, the optimal reaction time was chosen as 2 h.

3.4. GSH detection

The sensitivity of the fabricated controlled-release biosensor for quantitative analysis of GSH was investigated under the optimized experimental conditions. As shown in Fig. 4, the fluorescence intensity increased with the concentration of GSH ranged from 0 to 10.0 nM, indicating that the release of RhB was highly dependent

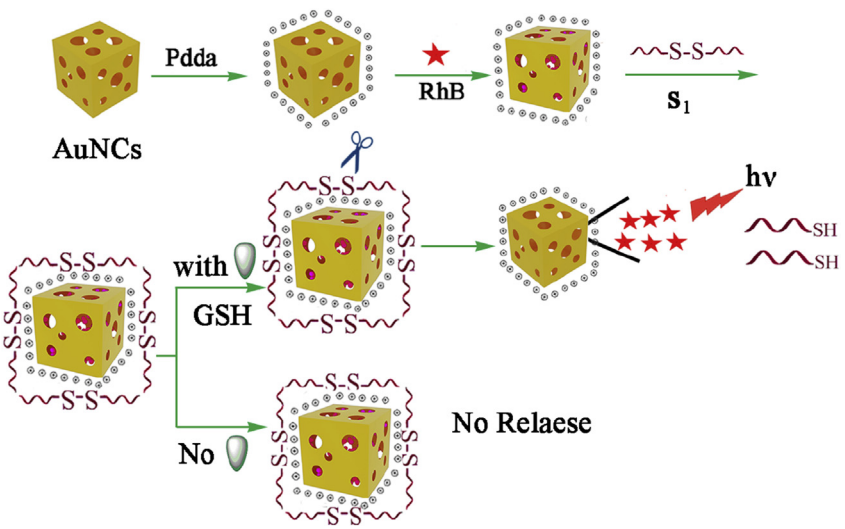

Fig. 2. Schematic illustration of the controlled-release biosensor system for ultra-sensitive detection of GSH.

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on the concentration of GSH. The results confirmed that the disulfide-containing probes S1 assembled on the surface of AuNCs through electrostatic interaction can be used as molecular gates effectively, which can be bio-responsively opened due to the cleavage of disulfide-containing probes into two SH-DNA fragments by GSH. The inset of Fig. 4 showed a good linear correlation between the fluorescence intensity and the concentration of GSH in the range from 1.0×10^{-12} to 6.0×10^{-10} M with a correlation coefficient of 0.9939. The linear regression equation could be expressed as $FL = 220.464 + 0.805C_{GSH}$ (10^{-12} M) with a detection limit of as low as 4.8×10^{-13} M, which was calculated by three times signal-to-noise ratio. The present detection limit is far superior to that of our previously reported electrochemical detection [37], which was 83 pM, compared with it, the sensitivity of the present method was enhanced about 173-fold. The results demonstrated that the proposed signal amplification strategy based on the controlled-release AuNCs was efficient for ultrasensitive detection of GSH.

3.5. Specificity of the controlled-release biosensor

Under the same experimental conditions, GSH and several non-protein thiols including sodium hydrosulfide (NaSH), mercaptohexanol (MCH), thioglycolic acid (TA), cysteine (CYS) and dithiothreitol (DTT) were utilized to investigate the specificity of the developed sensing system at the same conentration of 4.0×10^{-10} M, respectively. As shown in Fig. 5, the developed biosensor was found to show a best response to GSH than any other studied non-protein thiols.

3.6. Detection of intracellular non-protein thiols in Hela cells

To further evaluate the capability of the sensing system presented here in biological samples, the detection of intracellular non-protein thiols in the extracts of Hela cells was performed. A 10 μL supernatant was introduced into the proposed biosensor system and the mixture was subjected to incubation for 2 h at 37 °C. The experimental results demonstrated that the fluorescence signal was significantly enhanced when compared to the PBS control (Fig. S7). The detected concentration of intracellular non-protein thiols in the extracts of Hela cells is 3.0×10^{-8} M per 1.0×10^{6} cells. The results indicates that this facile and robust method is suitable for the quantification of non-protein thiols in cells.

3.7. Cell imaging

GSH is well known to be the most abundant intracellular non-protein thiol and the intracellular concentration of GSH in mammalian cells is in the millimolar range (1–10 mM) [38]. To further demonstrate the possibility of the proposed sensing system for monitoring GSH levels in living cells, Laser confocal microscopy imaging was performed on Hela cells by employing the RhB-loaded AuNCs caped with GSH-specific recognition probes S1. As shown in Fig. 6A and E, after Hela cells were incubated with the AuNCs for 15 min at 37 °C, strong red fluorescence signal from RhB in cells could be seen clearly, indicating cellular uptake of the RhB-loaded AuNCs by the living Hela cells and the bio-responsive release of

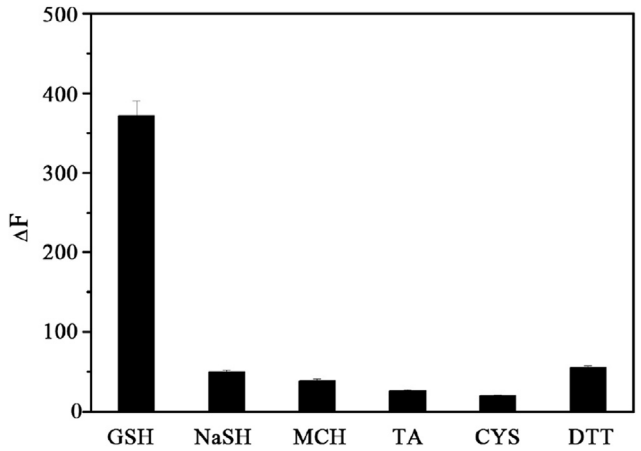

Fig. 5. Selectivity of the fabricated biosensor for GSH compared to other non-protein thiols including sodium hydrosulfide (NaSH), mercaptohexanol (MCH), thioglycolic acid (TA), cysteine (CYS) and dithiothreitol (DTT) at the same concentration of 4.0×10^{-10} M. ΔF is the fluorescence intensity after blank deduction.

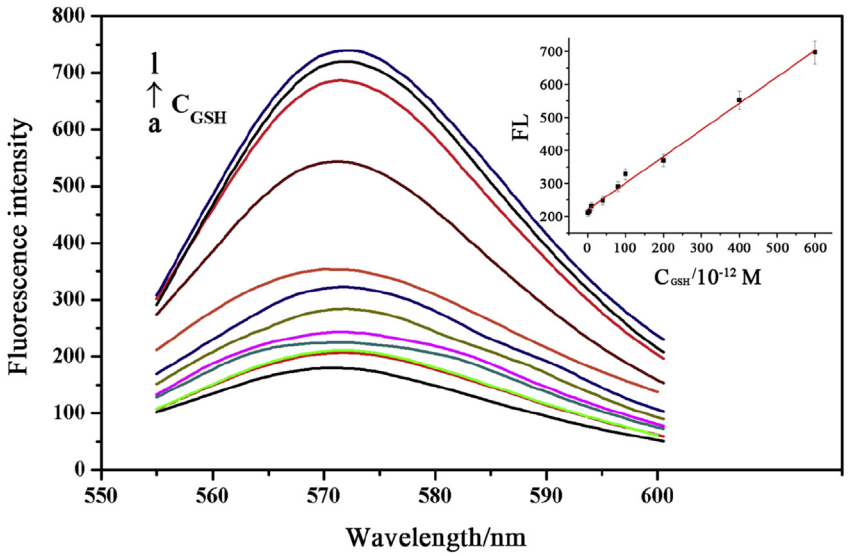

Fig. 4. Fluorescence spectra of the sensing system in the absence and presence of different concentrations of GSH (a→l: 0, 1.0×10^{-12}, 5.0×10^{-12}, 1.0×10^{-11}, 4.0×10^{-11}, 8.0×10^{-11}, 1.0×10^{-10}, 2.0×10^{-10}, 4.0×10^{-10}, 6.0×10^{-10}, 2.0×10^{-9}, 1.0×10^{-8} M). Inset: The linear relationship between fluorescence intensity and GSH concentrations from 1.0×10^{-12} to 6.0×10^{-10} M. Error bars show the standard deviations of measurements taken from three independent experiments.

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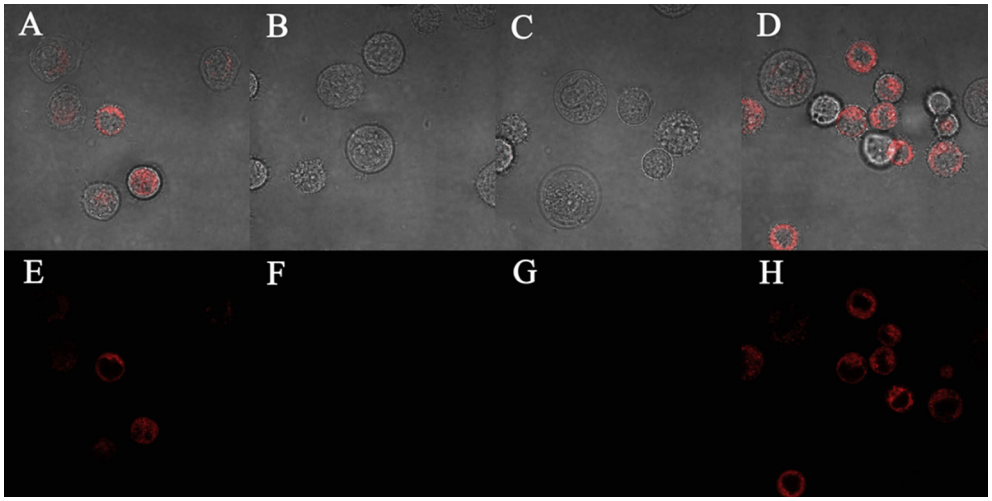

Fig. 6. Confocal fluorescence microscopy images of Hela cells: (A) cells incubated with the developed sensing system, (B) cells as a control by using the system capped with arbitrarily ssDNA S2, (C) cells pretreated with NEM (500 μM) for 20 min, followed by the developed sensing system, (D) cells pretreated with GSH (5 mM) for 20 min, followed by the developed sensing system. Top row of images show the dual channel (optical and fluorescence) microscopy images (A, B, C, D) and bottom row of images show the corresponding fluorescence microscopy images (E, F, G, H), respectively.

the loaded RhB in response to intracellular GSH. In order to prove it, a control experiment was further performed by using the RhB-loaded AuNCs blocked with arbitrarily ssDNA S2 containing no disulfide bonds under the same conditions (Fig. 6B and F). Obviously, Hela cells showed no appreciable fluorescence because of containing no disulfide bonds in random ssDNA S2, which was not recognized by intracellular GSH, so no release occurs naturally. These results indicate that the developed RhB-loaded AuNC is an efficient controlled-release transporter for guest molecules in response to intracellular target molecules and predict its potential use for *in situ* molecular imaging and targeted cancer treatment in living systems.

To validate that the control-release system is able to respond to changes of the intracellular GSH level in living cells, two control experiments were also performed. In one experiment, Hela cells were pretreated with 500 μM of N-ethylmaleimide (NEM), a known thiol trapping reagent for reducing the GSH level, and then incubated with the system for 15 min at 37 °C. As shown in Fig. 6C and G, no fluorescence was observed due to a decrease in the GSH concentration. In another experiment, Hela cells were treated with 5 mM GSH prior to incubation with the system for 15 min at 37 °C. As shown in Fig. 6D and H, Hela cells showed a clear increase in the intracellular fluorescence intensity. These results imply that the developed RhB-loaded AuNC system can be applied not only for the fluorescence enhanced imaging of the intracellular GSH but also to monitor change of the intracellular GSH in living cells.

4. Conclusions

In summary, an efficient fluorescent biosensor was developed for GSH detection using cleavage-induced controlled-release amplification strategy. It took fully advantage of the efficient and specific cleavage of S-S bonds in disulfide-containing molecular gates by GSH target molecules to achieve the controlled-release of fluorescent molecules from the RhB-loaded AuNCs, which guaranteed the ultra-sensitive detection of GSH with a detection limit of as low as 0.48 pM. It also exhibited the distinct advantages of simplicity and flexibility in molecular gate design and biosensor fabrication. Obviously, the proposed strategy should be easily extended to detect a lot of analytes including protein, nucleic acid, metal ions and a variety of biological small molecules, and thus it opens a promising avenue for the development of fluorescent biosensors by combining specific molecular recognition with controlled-release technology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.10.024.

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