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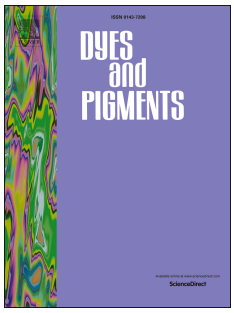

A “turn-on” near-infrared fluorescent probe with high sensitivity for detecting reduced glutathione based on red shift *in vitro and in vivo*

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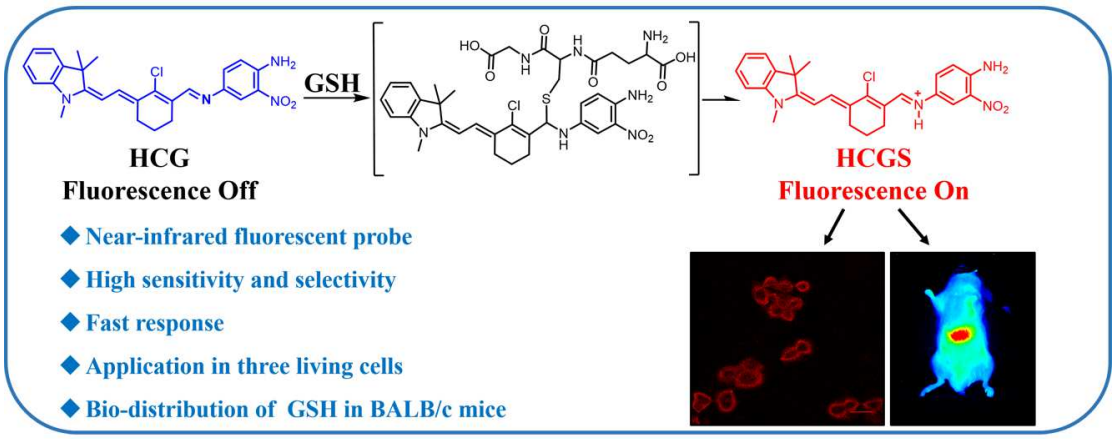

In this manuscript, a “turn-on” near-infrared fluorescent probe was developed and applied to monitor glutathione (GSH) with excellent sensitivity and selectivity *in vitro*. The novel probe HCG was successfully applied to detect endogenous and exogenous GSH in three living cells and the bio-distribution of GSH in BALB/c mice.

A "turn-on" near-infrared fluorescent probe with high sensitivity for detecting reduced glutathione based on red shift *in vitro and in vivo.*

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Abstract

Fluorescence imaging has become a powerful tool for detecting reduced glutathione (GSH) to comprehend the physiological and pathological roles of GSH and the potential clinical diagnosis of GSH-related diseases, such as AIDS, liver damage, cancer, and leucocyte loss. High sensitivity and high selectivity remain challenges for near-infrared fluorescent probes to monitor GSH. Herein, a turn-on near-infrared fluorescent probe (HCG) was designed and developed in a convenient synthetic procedure, which had high sensitivity and selectivity to detect GSH based on

the red shift of Schiff base. HCG could discriminate against amino acid that resemble GSH to monitor GSH *in vitro*. The fluorescence emission intensity linearly increased with an increasing concentration of GSH in the range of 0-16 μM, with a limit detection of 252 nM. HCG exhibited a diminutive detection limit (0.5 μM in actual experiments), a fast response time (30 s) and low cytotoxicity. The detection mechanism was confirmed by HPLC and HRMS spectra. Furthermore, HCG exhibited an excellent capacity for fluorescence imaging and has been successfully applied to detect endogenous GSH in three living cells. Finally, the bio-distribution of GSH in BALB/c mice was studied by using HCG. The results suggest that HCG has potential utility in biological science research.

Keywords: *Near-infrared fluorescent probe; Reduced glutathione detection; High sensitivity and selectivity; Schiff base's red shift; Fluorescence imaging; Facile synthetic procedures.*

1. Introduction

Intracellular biothiols, including reduced glutathione (GSH), cysteine (Cys), and homocysteione (Hcy), act as significant roles in many biological processes [1-3]. It is deserved to be mentioned that GSH is widely distributed in living organisms and can serve as a biomarker in the biological system among intracellular bio-thiols [4]. As reported in literatures [5-8], GSH can contribute to an improvement in immunity and anti-aging among physiological processes and GSH is a great scavenger for reactive oxygen to maintain the appropriate physiological redox state . However, aberrant GSH levels have been associated with a variety of diseases, such as AIDS, liver damage,

cancer, and leucocyte loss [9-11]. Based on these disease features, traditional analytical method has been developed for detecting GSH by high-performance liquid chromatography and mass spectrometry [5]. However, it is difficult to prepare biological samples and monitor GSH in real time. Thus, many new strategies have been gradually used to monitor GSH in biological models [12-15]. Among these strategies, fluorescence imaging has been paid great attention for the detection of GSH in biological systems [16-18]. Different kinds of fluorescent probes have been developed for detecting GSH on the basis of different reaction mechanisms, such as Michael addition [19, 20], nucleophilic substitution [21], cyclization with an aldehyde [22], Se-N cleavage by thiols [23], the elimination of disulfide [24] and others [25-27]. In the reported fluorescent probes for the detection of GSH, there are strong interference from Cys and Hcy, due to their similar molecular structures and nucleophilicity. So, it is still a challenge to develop a highly specific fluorescent probe for detecting GSH. [28-31].

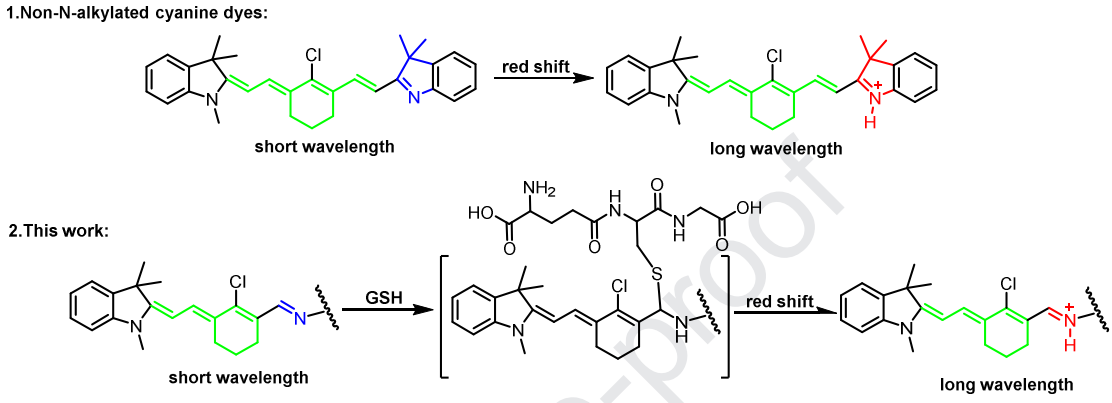
In recent years, near-infrared fluorescent probes have increasingly been applied to detect GSH, due to the reduced biological damage and deeper tissue penetration of the light in this wavelength region [21, 28, 31-34]. Near-infrared fluorescent probes contain low photon absorption and can provide high-resolution fluorescence intensity to produce effective fluorescent signals *in vitro and in vivo* without fluorescent interference. Compared with non-near-infrared fluorescent probes, near-infrared fluorescent probes have greater sensitivity and more extensive applications [35]. Taking advantage of these near-infrared fluorescent probes, several turn-on and

ratiometric near-infrared fluorescent probes have been developed for detecting GSH [32-34]. The detection moieties of these near-infrared fluorescent probes still utilize the nucleophilicity of GSH to lead to low selectivity for discriminating against amino acids that resemble GSH. Moreover, these probes exhibited slow response time and their synthetic routes were complicated. Based on the shortcomings of the existing fluorescent probes, it is still of great importance to develop a more sensitive and selective near-infrared probe to detect intracellular GSH through a convenient synthetic procedure.

As reported in the literature [36-41], non-N-alkylated cyanine dyes include a protonatable amino group with an indole group. When the indole nitrogen atom deprotonates, it absorbs in the short wavelength region. The dye will have a strong red fluorescence when the indole nitrogen atom has a proton. This change occurs over a very short time, which means that a more sensitive probe with an indole nitrogen atom moiety may be designed to detect GSH, and suggests an excellent spectroscopic window to monitor the turning off or on of a visual optical signal (Scheme 1). This strategy offers a new way to design near-infrared fluorescent probes. Encouraged by this mechanism, we hope to develop a highly sensitive and selective near-infrared fluorescent probe with a similar structure to detect GSH.

Herein, making use of this shift strategy, we report the turn-on hemi-cyanine fluorescent probe HCG with excellent sensitivity and selectivity based on a Schiff base redshift reaction for the detection of GSH *in vitro*. Moreover, HCG was successfully applied to three living cells and the BALB/c mice to detect GSH by

fluorescence imaging. The fluorescent images showed that using HCG to detect GSH is helpful to understand the possible biogenesis approaches of endogenous GSH *in vivo*. The results indicated that HCG was an effective tool for monitoring GSH in bioscience research.



Scheme 1. Schiff base redshift

2. Experimental

2.1 Reagents and apparatus

All commercial chemicals were of analytical reagent grade and used without further purification. All reactions were monitored by TLC and the TLC plate was detected by ultraviolet light (254 nm or 365 nm). Silica gel column chromatography was used to purify the compound. A reduced glutathione (GSH) assay kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China). ^{1}H-NMR and ^{13}C-NMR spectra were acquired on a Bruker NMR 400MHz spectrometer. The high resolution mass spectra of all compounds were recorded by FT-MS (Bruker Daltonics SolariX 7.0T). The UV spectra were obtained with UV/VIS (Jena , Specord 210) spectrophotometer in 1 cm quartz cells. Fluorescence excitation and emission spectra

were measured on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescent images of cells were obtained with a confocal laser scanning microscope (Nikon A1). Fluorescent images of BALB/c were recorded by a small-animal *in vivo* imaging system (IVIS).

2.2 Synthesis of HCG

The synthesis procedures of the other compounds are shown in Scheme S1. Cy7 (300 mg, 1.037 mmol) and 2-nitrobenzene-1,4-diamine (190 mg, 1.244 mmol) were dissolved in 5 mL of anhydrous DMSO. Then, N,N-diisopropylethylamine (100 μL) was added to the reaction mixture. The reaction was kept at 60 ^{o}C for 24 h under nitrogen atmosphere. Next, the solvent was removed under reduced pressure to obtain the crude product, which was purified by column chromatography on silica gel with a gradient of ethyl acetate ; petroleum ether from 10:1 to 3:1 (v/v), and the black solid HCG was finally obtained (153.3 mg, 32%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.02 (d, J = 2.4 Hz, 1H), 7.65 (d, J = 12.7 Hz, 1H), 7.41 (dd, J = 8.8, 2.4 Hz, 1H), 7.26 – 7.18 (m, 2H), 6.95 – 6.83 (m, 2H), 6.70 (d, J = 7.8 Hz, 1H), 6.13 (s, 2H), 5.47 (d, J = 12.7 Hz, 1H), 3.22 (s, 3H), 2.77 (t, J = 6.1 Hz, 2H), 2.67 – 2.60 (m, 2H), 1.91 – 1.83 (m, 2H), 1.69 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 160.8 (s), 158.5 (s), 144.8 (s), 142.8 (s), 142.8 (s), 141.9 (s), 139.1 (s), 132.2 (s), 130.8 (s), 128.9 (s), 127.8 (s), 124.5 (s), 121.7 (s), 120.3 (s), 119.3 (s), 117.1 (s), 106.3 (s), 92.9 (s), 46.1 (s), 29.3 (s), 28.3 (s), 26.7 (s), 26.6 (s), 21.4 (s). HRMS (ESI^+): m/z found $[M+H]^+$ 463.1889. molecular formula $C_{26}H_{28}ClN_4O_2^+$, requires $[M+H]^+$ 463.1895.

2.3 Cell culture and fluorescent imaging

MCF-7, HT-29 and HepG2 cells were incubated in DMEM supplemented with 10 % (v/v) FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C under a humidified atmosphere containing 5 % CO_2. For fluorescence imaging, cells were pretreated with varying concentrations of GSH or other analytes in 12-well plates (1× 10^6 cells/well) at 37□ for the appropriate time. Then, the cells were washed with HEPES (10 mM, pH=7.4) and incubated with a mixture of fresh DMEM and an administered the probe HCG (10 μM) at 37 ºC for 15 min. Fluorescence images were acquired with a confocal laser scanning microscope. The fluorescence detection setting were kept constant throughout all imaging experiments.

2.4 General flow cytometry methods

In general, for flow cytometry, the cells were incubated with different concentrations of GSH, and GSH scavengers in 12-well plates (1× 10^6 cells/well) at 37□ for a certain amount of time. Then, the cells were washed with HEPES (10 mM, pH=7.4) and incubated with a mixture of fresh DMEM without fetal bovine serum and administered HCG (10 μM) at 37º C for 15 min. Subsequently, the cells with HEPES were analyzed by flow cytometry (BD, Accuri™ C6) equipped with 640 nm excitation laser light line source.

2.5 Fluorescence imaging in BALB/c mice

BALB/c mice were divided into two groups. The control group mice were pre-injected with saline for 5 min and GSH group mice were pre-injected with concentration of GSH (1 mM) for 5 min. Next, all mice were injected with HCG (10 μM, 100 μL in 1:99 DMSO/saline, v/v). The probe was incubated in BALB/c mice for

30 min and the fluorescent images of the BALB/c mice were obtained by a small-animal *in vivo* imaging system (IVIS).

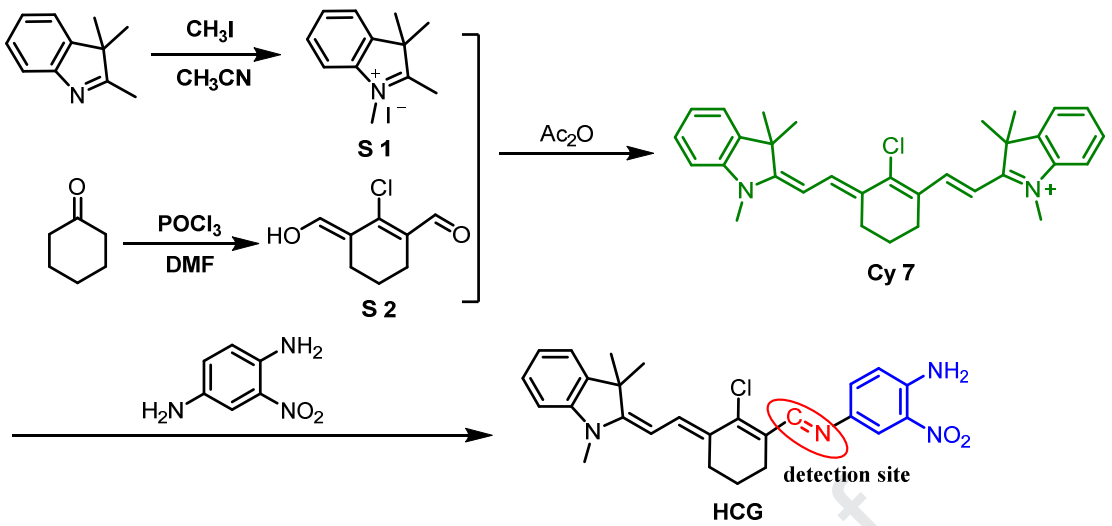
2.6 High-performance liquid chromatography (HPLC) analysis

The HPLC technique was employed in the reaction mechanism experiment. HPLC was used to monitor the process by which the probe HCG (10 μM) reacted with 500 μM GSH for 15 min. The retention time of each compound was confirmed so that a better understanding of the reaction mechanism could be obtained.

3. Results and discussion

3.1 Design and synthesis of probe HCG

Because protonatable amino groups can undergo a red shift, we hoped to design a similar structure. The Schiff base also contains a protonatable nitrogen atom and can produce a red emission optical signal. Meanwhile, the Schiff base moiety can be synthesized conveniently. Thus, for the purpose of constructing a turn on fluorescent probe, 1,2,3,3-tetramethyl-3H-indole and (E)-2-chloro-cyclohex-1-ene-1-carbaldehyde were synthesized by known conditions initially. Next, compound **S1** reacted with compound **S2** under aldol condensation conditions to give Cyanine 7. The probe HCG was obtained in one step by the combination compound Cy7 and 2-nitrobenzene-1,4-diamine [42, 43]. HCG contains a Schiff base structure, which is the core of the probe and can serve as the recognition group for GSH. Moreover, the withdrawing group of probe HCG is beneficial to produce a large red shift emission in the fluorescence spectrum signal. Compared with traditional synthetic approaches, this procedure was more convenient. (Scheme 2).



Scheme 2. The synthetic procedure of probe HCG

3.2 Spectroscopic properties and sensitivity of the probe HCG

Our studies began by examining the UV/Vis absorption and fluorescence spectra of HCG and 10 μM HCG reacted with 500 μM GSH. The results indicated that the maximum absorption wavelength of HCG was located at 450 nm. Interestingly, after reacting with GSH, its maximum absorption wavelength was observed in the 653 nm region (Fig 1a). In the fluorescence spectra, there is a weak fluorescence emission from HCG at 720 nm. After 500 μM GSH was added followed by incubation for 1 min, the fluorescence intensity increased 8-fold at 720 nm (Fig 1b). This result indicated that HCG is a turn on near-infrared fluorescent probe and has a short detection time. The fluorescent quantum yield of HCG reacted with GSH was 17% relative to Oxazine 1 (Φ_{oxz}=0.14 in EtOH) [44]. To test the sensitivity of HCG, the fluorescence spectra of HCG (10 μM) reacted with varying concentrations of GSH (0-1000 μM) were recorded by fluorescence spectrophotometry. The results showed that the fluorescence intensity increased and reached a gradual plateau with increasing concentration of GSH (Fig 1c and S1c). Moreover, it was found that the fluorescence

emission intensity linearly increased with the concentration of GSH in the range of 0-16 μM and the detection limit (DL) of HCG was calculated to be 252 nM (signal-to-noise ratio (S/N) = 3). In the real detection experiment, the DL of HCG was 0.5 μM (Fig 1d). There is a reduced glutathione (GSH) assay kit from Jiancheng Bioengineering Institute (Nanjing, China), whose DL is 451 nM and the real detection limit is 1.5 μM (Fig S1). Compared with this commercial kit, HCG has higher sensitivity.

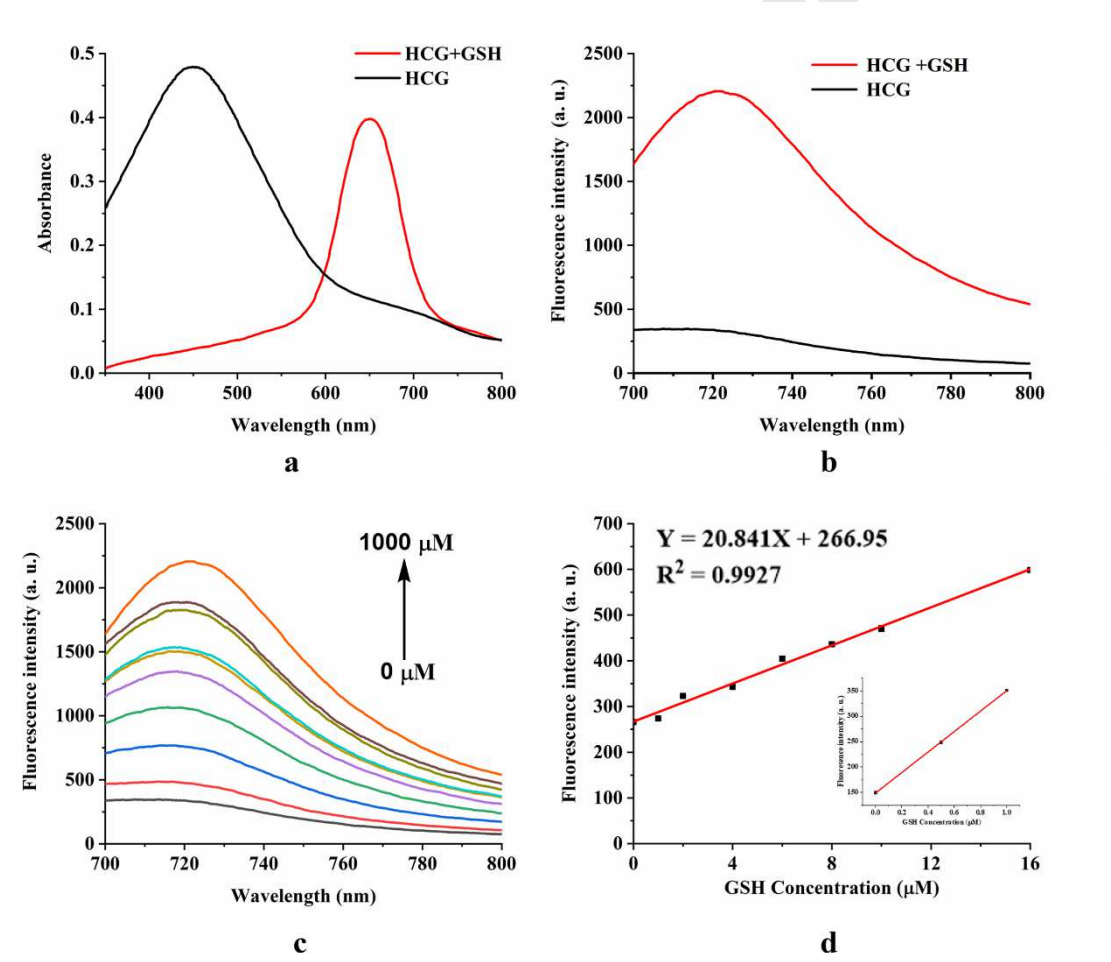


Fig 1. The UV spectra and fluorescence emission spectra of HCG in response to GSH. a) Black: Absorbance spectrum of HCG (10 μM) in DMSO/HEPES buffer (10 mM, 1:10, v/v, pH 7.4) at 25 °C, Red: Absorbance spectrum of 10 μM HCG reacted with 500 μM GSH in DMSO/HEPES buffer (10 mM, 1:10, v/v, pH 7.4) at 25 °C for 15 min; b) Fluorescence emission spectra of HCG,

and 10 μM HCG with 500 μM GSH in DMSO/HEPES buffer (10 mM, 1:10, v/v, pH 7.4) at 37 °C for 1 min; c) Fluorescence emission spectra of HCG (10 μM) with various concentrations of GSH (0, 10, 20, 40, 60, 80, 100, 200, 500, 1000 μM) at 37□ for 15 min; d) Linear correlation of fluorescence emission intensity towards GSH concentration and the real experimental detection limit. (λ_{ex} = 653 nm).

3.3 Selectivity of the probe HCG

To examine the selectivity of HCG, different amino acids and other species were introduced to HCG, such as Cys, GSH, Ala, Pro, Lys, Thr, His, Val, Tyr, Ser, Phe, Arg, Trp, Met, Leu, Asp, Gly, Ile, Gln, Hcy and others. As shown in Fig 2a, only GSH can be monitored by HCG among all the amino acids tested. It is worth mentioning that the fluorescence intensity of the reaction of HCG with Cys, Hcy and GSH showed a large difference, indicating that there is good anti-interference and high selectivity for HCG to react with GSH compared with similar amino acids. In addition, the time-dependence was measured to explore the time required for HCG to respond to GSH. Excitingly, when GSH was added into the HCG buffer solution, the fluorescence intensity increased immediately and achieved a balance within 30 s (Fig 2b), suggesting that HCG has a great response to GSH and exhibits excellent sensitivity. A possible interpretation is that the Schiff base redshift emission leads to a rapid response to GSH.

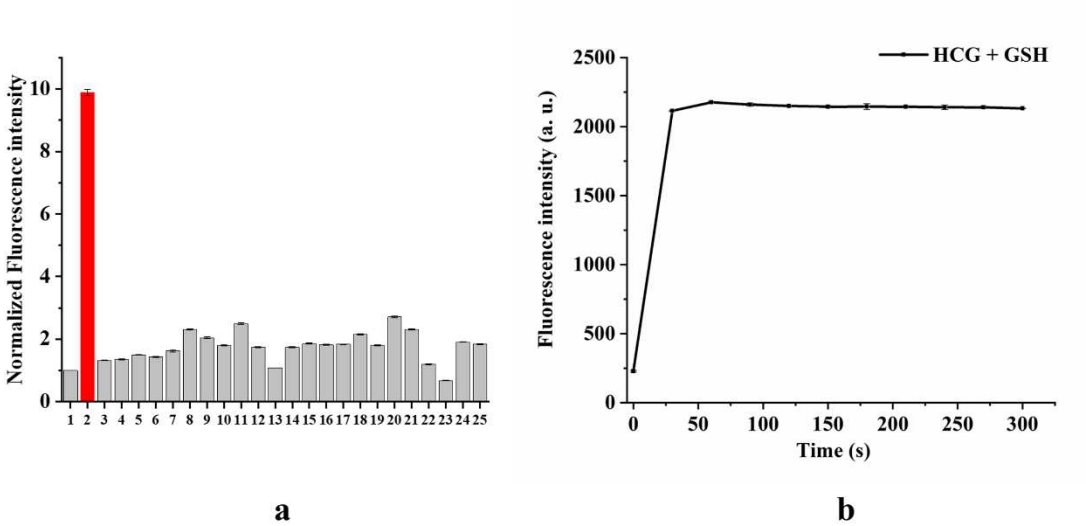


Fig 2. The selectivity and kinetics of the HCG response to GSH. a) Fluorescence responses of 10 μM HCG to 500 μM of biologically relevant species in DMSO/HEPES buffer (10 mM, 1:10, v/v, pH 7.4) at 37 °C for 15 min: (1) probe, (2) GSH, (3) Cys, (4) Ala, (5) Pro, (6) Lys, (7) Thr, (8) His, (9) Val, (10) Tyr, (11) Ser, (12) Phe, (13) Arg, (14) Cystine, (15) Trp, (16) Met, (17) Leu, (18) Asp, (19) Gly, (20) Ile, (21) Gln, (22) HSO_3^-, (23) SO_3^{2-}, (24) H_2O_2, (25) Hcy; b) Kinetics of 500 μM GSH detected by 10 μM HCG at 37°C in HEPES (pH 7.4) (λ_{ex} = 653 nm).

3.4 Flow cytometry analysis of GSH and confocal microscopy images in living cells using HCG

Based on the good fluorescent properties of HCG in buffer, HCG was applied to detect GSH in living cells. The cytotoxicity of HCG was established firstly, using MTT assays with MCF-7, HT-29 and HepG2 cells. The cell viabilities exceeded 92% when incubated with 10 μM HCG. In the range of 0-100 μM HCG, the cell viabilities were still over 82%, demonstrating the low cytotoxicity of the probe HCG in living cells (Fig S2). Furthermore, the fluorescence intensity of HCG was weak and stable in the range of pH 5-10. When added GSH into the solution of HCG, the fluorescence intensity of HCG response to GSH had a significant improvement in the range of pH

5-10 (Fig S3). As shown in Fig S2 and S3, the results above demonstrated that HCG was stable and suitable for cell imaging under biological conditions. On the basis of these results, flow cytometry was used to analyze of the fluorescence intensity of HCG in response to GSH in MCF-7, HT-29 and HepG2 cells. HT-29 cells were pretreated with different concentrations of GSH (0, 200, 500, 1000 μM) for 30 min, followed by, incubation with 10 μM HCG for 15 min and analysis by flow cytometry. As shown in Fig 3a, the fluorescence intensity of the HCG response to GSH improved with increasing GSH concentration. N-ethylmaleimide (NEM, 2 mM), a well-known scavenger of biothiols [45, 46], was pretreated with HT-29 cells for 30 min, and then subsequently, incubated with 10 μM HCG for 15 min and analyzed by flow cytometry. The flow cytometry data showed that the fluorescence intensity of HT-29 cells incubated with NEM decreased to half that of the control group (Fig 3b). The flow cytometry results in MCF-7 cells and HepG2 cells were analogous to those in HT-29 cells (Fig S4). Encouraged by the results above, confocal microscopy imaging was applied to HT-29, MCF-7 and HepG2 cells. HT-29 cells were incubated with different concentrations of GSH (0, 200, 500, 1000 μM) for 30 min. Next, the cells were treated with 10 μM HCG for additional 15 min, and fluorescent images were acquired by confocal microscopy. As shown in Fig 3c, the fluorescence intensity of the HCG response to GSH gradually increased in a GSH concentration dependent manner. When the control group only was incubated with 10 μM HCG, it was obvious that a red fluorescence was observed in HT-29 cells. This result indicated that HCG could detect endogenous GSH in living cells. When 2mM NEM was introduced in HT-29

cells prior to, and all other procedures were consistent with the above mentioned conditions, only weak red fluorescence was observed the confocal microscopy image of HT-29 cells. These results were consistent with the flow cytometry results. Similar results were achieved with MCF-7 cells and HepG2 cells (Fig S5). Therefore, according to these results, it has been suggested that the probe HCG can be an effective tool for detecting GSH in living cells.

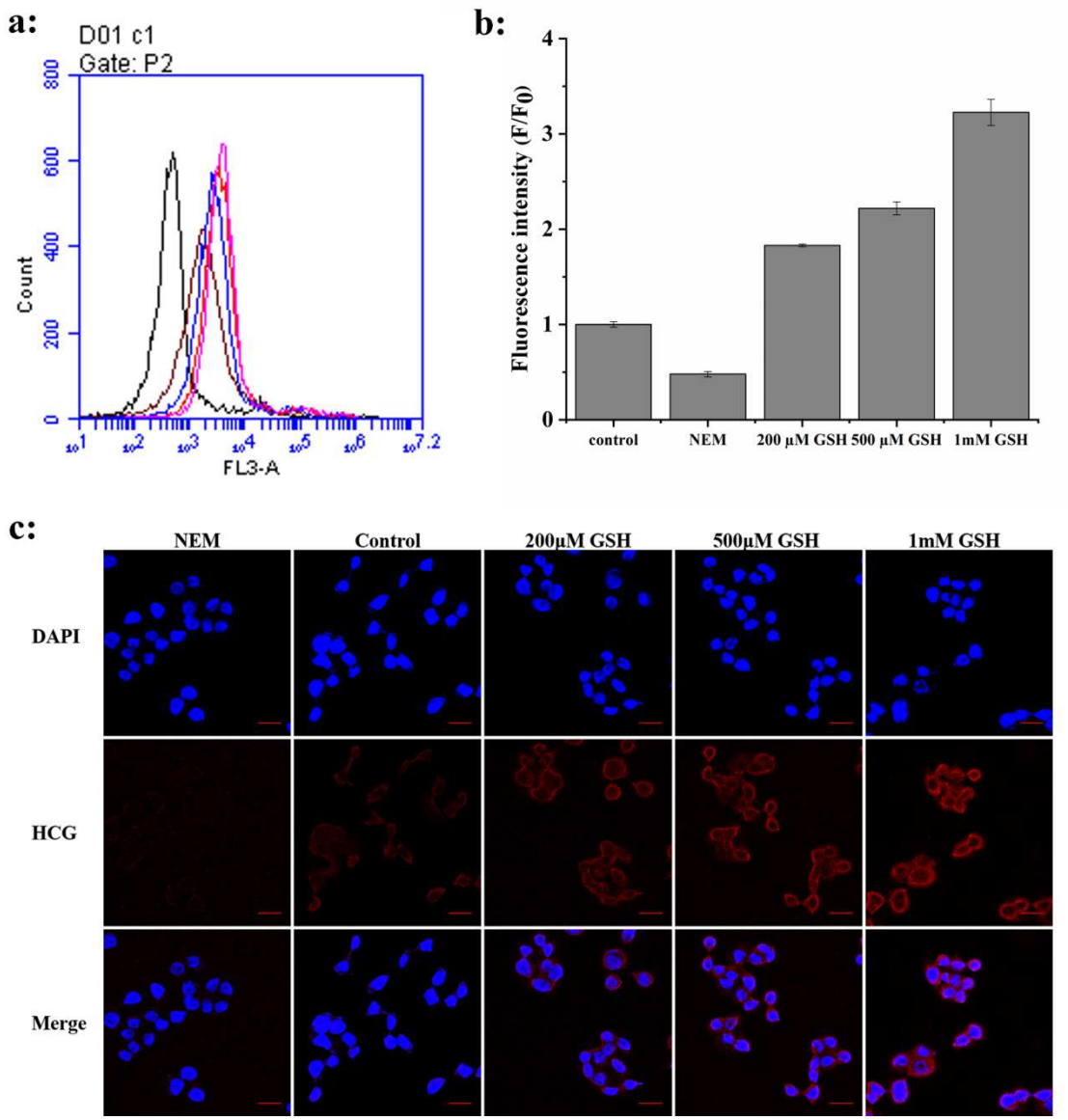


Fig 3. Flow cytometry analysis of GSH and confocal microscopy images of GSH in HT-29 cells using HCG. a) Cells were pretreated with GSH (0, 0.2, 0.5, 1.0 mM) at 37□ for 30 min and then stained with 10 μM HCG for 15 min before flow cytometry analysis; b) Normalized

fluorescence intensity of flow cytometry data; c) Cells were pretreated with varying concentrations of GSH or NEM at 37□ for 30 min, and then treated with 10 μM HCG for 15 min before obtaining the confocal microscopy images. Scale bar: 20 μm. Representative images from replicate experiments (n = 5) are shown.

3.5 The plausible mechanism

To gain insights into the mechanism of the HCG fluorescent response to GSH, HCG (10 μM) was reacted with GSH (500 μM) in 5 mL of methanol. The reaction mixture was measured by high-performance liquid chromatography (HPLC). The retention time of the HCG, GSH and the reaction product were recorded. As it is shown in Fig 4, the retention time of the HCG was 8.0 min (Fig 4a), and the retention time of GSH was 2.5 min (Fig 4b). However, the reaction product has a strong signal at 6.0 min (Fig 4c). Additionally, the reaction mixture was surveyed with a high-resolution liquid chromatograph mass spectrometer (HR-LC-MS). In positive ion mode of the HRMS analysis, the thiol-imine Michael adduct was found to have a m/z of 770.27025 (Fig S6) and HCGS was found to have a m/z of 463.19006 in the mass spectra. However, in negative ion mode of the HRMS analysis, the HCGS wasn't found in mass spectra and the probe HCG could be found to have a m/z of 461.17547 in the mass spectra. This indicated that HCG reacted with GSH to form a thiol-imine adduct and then the thiol-imine adduct decomposed into a protonatable Schiff base, which couldn't be measured in negative ion mode on the mass spectrometer (Fig S6 and Fig 5). The plausible mechanism was speculated that firstly, the non-red fluorescent HCG reacted with GSH to gain a thiol-imine adduct [47]. Because the thiol-imine Michael addition were unstable, the thiol-imine adduct was easily

decomposed into a protonatable Schiff base of HCG. The protonatable Schiff base of HCG was generated in the reaction mixture and led to a red shift in the optical signal (Scheme 3).

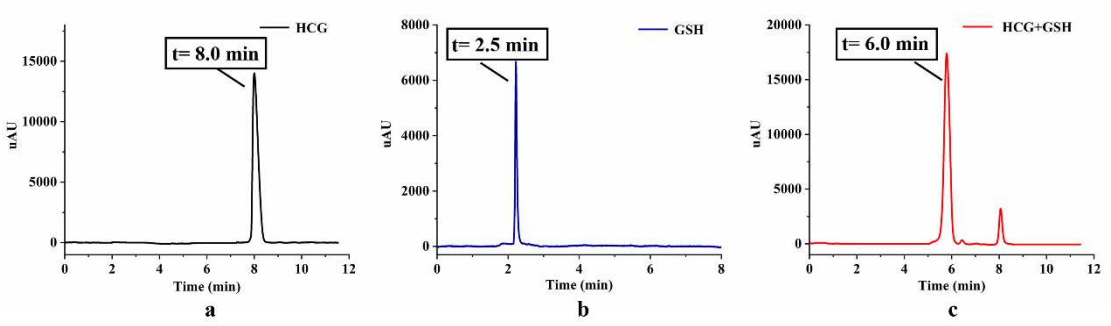


Fig 4. The reaction between 500 μM GSH and 10 μM HCG was tracked by HPLC. a) the retention time of HCG; b) the retention time of the GSH; c) the retention time of the reaction product.

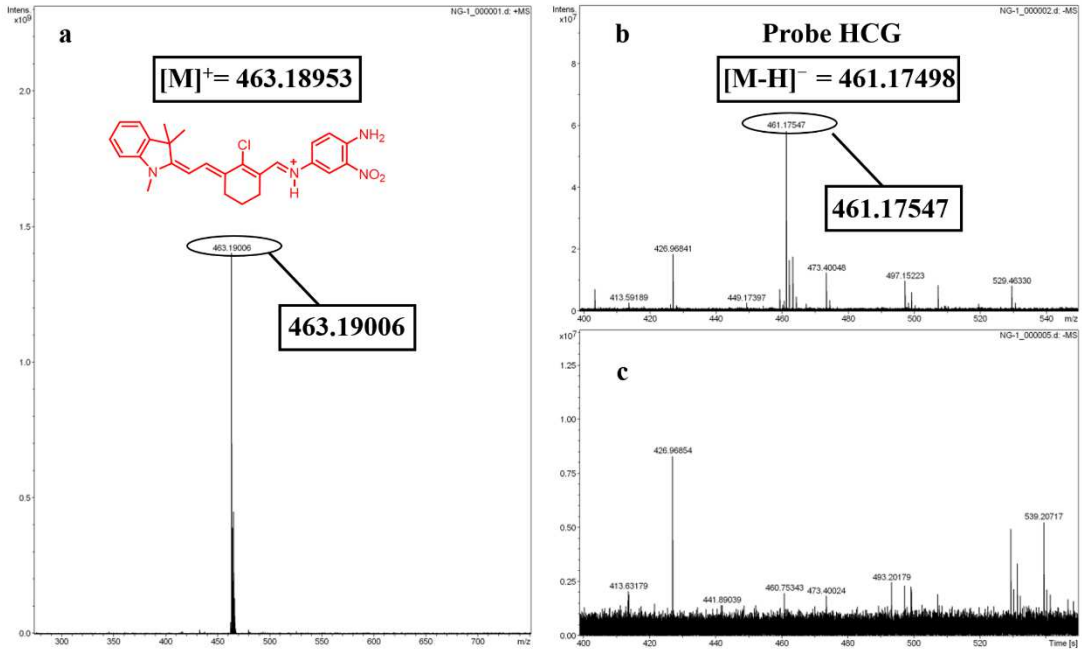
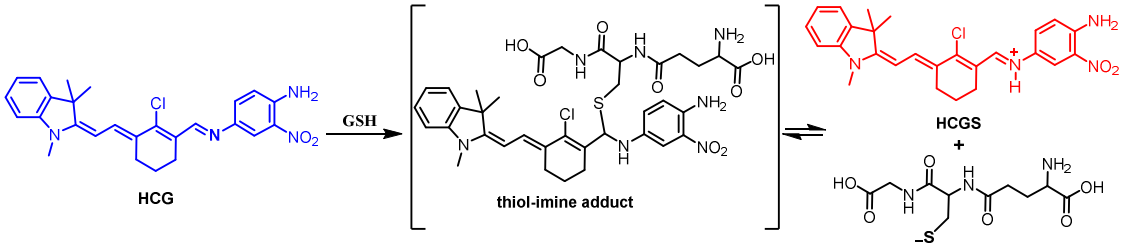


Fig 5. High resolution mass spectrum of 10 μM HCG reacted with 500 μM GSH. a) the positive ion mode of reaction product; b) the negative ion mode of probe HCG; c) the negative ion mode of the reaction product.



Scheme 3. Proposed detection mechanism of probe HCG for GSH.

3.6 The application of HCG *in vivo*

To investigate the application of HCG to respond to GSH *in vivo*, the BALB/c mice were pretreated with GSH (1 mM) and saline for 5 min, and then injected with HCG (10 μM, 100 μL in 1:99 DMSO/saline, v/v). After 30 min, fluorescence images were obtained by a small-animal *in vivo* imaging system (IVIS). There was a fluorescence signal in the control group. GSH was introduced into the mice ahead of time, and the fluorescence signal was greatly improved in the liver of the mice (Fig 6), indicating that the probe HCG can detect endogenous GSH by the blood circulation in mice. Because GSH is gathered and metabolized first in the liver of the mice by the blood circulation [48-50], the probe HCG enters the liver and detects GSH to lead to a fluorescence signal *in vivo*. In GSH group mice, the liver had a more obvious fluorescence signal than the other organs, because the liver contains abundant GSH. In this regard, HCG is an effective probe to detect changes in the concentrations of GSH *in vivo* and can provide a visual signal from IVIS.

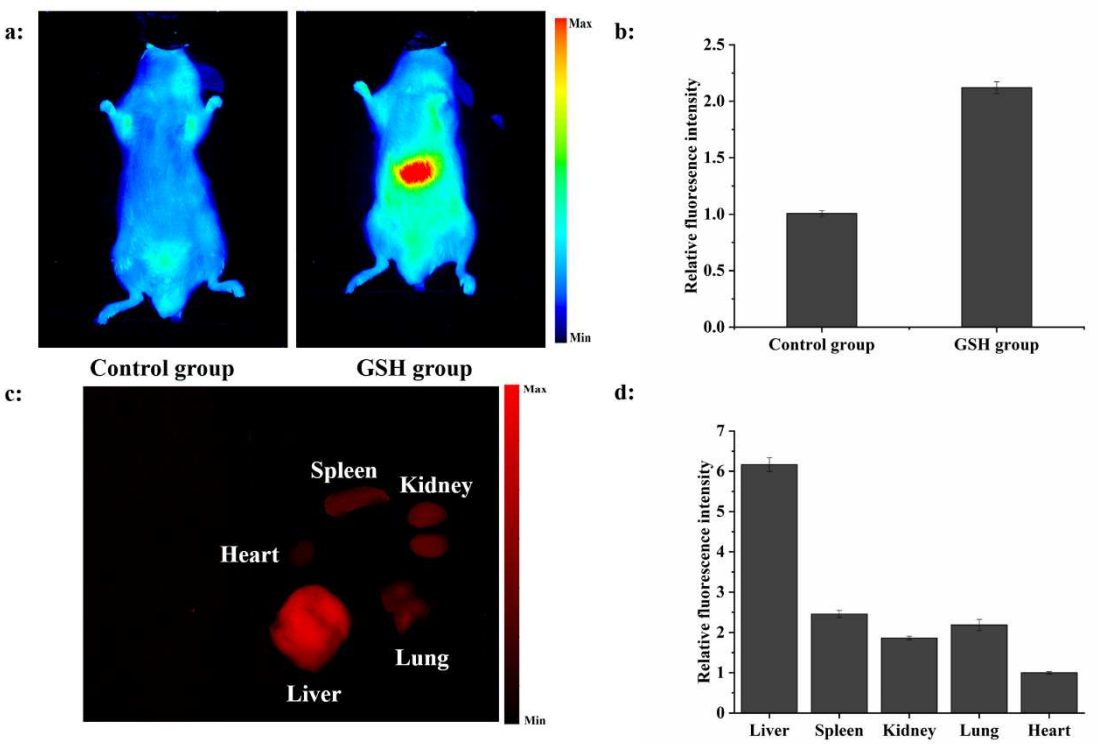


Fig 6. Fluorescence imaging of BALB/c mice. a) HCG was applied to a control group and a GSH group with IVIS; b) Relative fluorescence intensity of the control group and GSH group; c) *Ex vivo* fluorescence imaging: the separated organs from the sacrificed GSH group mice; d) Relative fluorescence intensity of the separated organs from the GSH group.

4. Conclusions

In summary, the near-infrared fluorescent probe HCG was designed and developed successfully based on the Schiff base red shift for the detection of GSH. HCG shows high selectivity to discriminate between GSH and similar amino acids, shows a fast response time (30 s) and has a low actual detection limit of 0.5 μM *in vitro*. Due to the low cytotoxicity and great near-infrared properties, HCG was successfully applied to the fluorescence imaging of endogenous GSH in HT-29, MCF-7 and HepG2 cells. Last, we studied the bio-distribution of GSH in BALB/c mice through fluorescence imaging of HCG. With facile preparations, this strategy

can provide an effective tool to detect GSH levels. The results suggest that HCG has potential utility in biological science research.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://xxxxxxxxxxxxx.

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Highlights

- **A turn-on near-infrared fluorescent probe was developed for monitoring reduced glutathione (GSH) by facile synthetic procedures**
- **The probe can detect GSH based on the red shift of Schiff base, which had high sensitivity, high selectivity, low detection limit and fast response time *in vitro*.**
- **The probe served as bioimging tool for detecting endogenous and exogenous GSH in three living cells.**
- **The bio-distribution of GSH in the BALB/c mice was studied through fluorescent imaging of HCG**