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Rational design of biotin–disulfide–coumarin conjugates: a cancer targeted thiol probe and bioimaging†

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Biotin–disulfide–coumarin conjugates are designed and synthesized as novel fluorescent sensors for cancer targeted intracellular thiol imaging in living organisms. *In vitro* experiments disclose that probe 6 is preferentially taken up by biotin receptor-positive A549 tumor cells through receptor mediated endocytosis.

Cellular thiols including glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) are essential biomolecules which play pivotal roles in many biochemical and physiological processes, such as cell proliferation, antioxidant defence, and cell signaling.[1] Among these metabolites GSH (in its free, reduced form) is the most plentiful, which plays an important role in maintaining the oxidation–reduction (redox) state of the cell homeostasis and is the vital source of reducing equivalents for enzymes such as superoxide, dismutase, catalase, and glutathione peroxidase.[2] In cells, the deviations from optimum ratios of GSH/GSSG are closely related to various diseases including Alzheimer's disease, cardiovascular disease, Parkinson's disease, liver damage and cancer.[3] Moreover, the efficacy of chemotherapy also depends on GSH levels in cancer cells.[4] Thus, the quantitative detection of these intracellular thiols has drawn a great deal of attention for early identification of diseases, assessment of disease development and therapeutic effectiveness of new prospective drugs.

Fluorescent molecules possess excellent light stability, high sensitivity, less toxicity and real-time monitoring ability, which have been shown to be essential for the quick and precise detection of intracellular organic biomolecules.[5] To date, a small number of rationally designed fluorescent probes have been developed for the detection of biologically free or intracellular thiols.[6] However, the maximum number of probes have exhibited poor water solubility, high background signal, narrow pH span, and slow response, and lack cell specificity with limited biological applications. In addition, some fluorophores often show aggregation-caused quenching (ACQ) properties, which diminishes their brightness and sensitivity in cellular imaging.[7] Considering the significant role of thiols in cellular functions, it is highly desirable to develop simple, non-invasive, and specific probes with high signal-to-noise ratios, and good water solubility for targeted imaging of intracellular thiols. Therefore, in view of the demand, we wish to design, synthesise and perform the spectroscopic characterization of intracellular thiol sensing systems with precision as well as fluorescence-On, for *in vivo* and *vitro* use.

One of the major challenges in modern research is to develop target specific probes which can be used to detect particular cellular components that are associated with disease states *in vitro*. Generally these probes could be used to rapidly test the therapeutic effects. In this context, the use of target specific ligands and conjugation strategies that allow for the selective targeting of a probe have emerged as attractive approaches. Biotin, also known as vitamin B7 and vitamin H, or coenzyme R is a water soluble B-complex vitamin that plays an important role in helping the body metabolize proteins and process glucose. Biotin is very much well known as a cancer targeting unit and also a growth promoter at the cellular level because in the cancer cells biotin receptors such as avidin, streptavidin are much more than that of normal cells.[8] Moreover, biotin receptors are overexpressed more than the folate or vitamin B-12 receptors in many cancer cells, such as leukemia (L1210FR), ovarian (Ov2008, ID8), colon (Colo-26), lung (M109), and breast (4T1, MMT06056) cancer cell lines respectively.[8,14] Therefore the biotin-appended coumarin unit can go to cancer cell specifically than that of normal cells through the strong interaction between biotin receptor avidin and biotin. The coumarin unit has the ability of the two-photon photophysical property, which provides the advantages of deeper tissue penetration and less photodamage.[9]

One photon fluorescent spectroscopy is not recommended for use in living systems because the shorter wavelength light is dispersed and absorbed by native cellular components. In addition, from a synthetic point of view, the coumarin moiety is also easily accessible and has structural tractability that allows for various modifications.

Therefore, the biotin-appended coumarin unit (**6**) is expected to monitor intracellular thiols level in cancer cells through fluorescence

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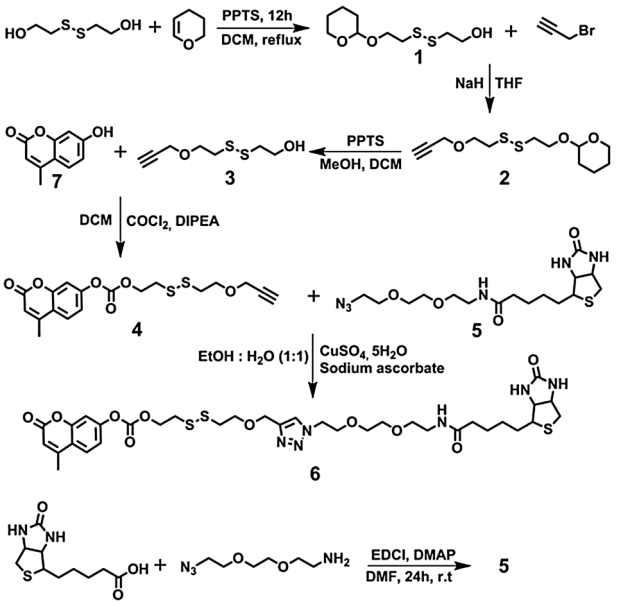


Scheme 1 Synthetic routes to **6**.

imaging, which can be used in practical applications. Here, we report a cancer targeting fluorescent probe **6** which contains a biotin-appended coumarin unit that allows for the real-time fluorescence imaging of reduced thiols such as GSH, Cys, and Hcy respectively in cells. Conjugates **6** are combined with a cancer targeting unit as biotin, a disulfide linker cleavable by the intracellular thiols, a coumarin moiety which gives rise to enhance the fluorescence intensity after disulfide bond cleavage.

Our aim to develop an intracellular thiol-specific probe relies on the use of a specific receptor-mediated endocytosis approach. Biotin-appended coumarin conjugates (**6**) were synthesized according to the following synthetic pathway shown in Scheme 1.

Compounds **1** to **3** were synthesized by reported methods.[10] Compound **3** was reacted with phosgene and diisopropylethylamine (DIPEA) followed by reaction with coumarin (**7**) to provide **4**. The biotin-azide derivative (**5**) was prepared from the previously published procedures.[11] Finally, our desired compound **6** was obtained from compounds **4** and **5** by the click reaction in good yield. The chemical structures of all compounds (**1–6**) were confirmed by ^{1}H NMR, ^{13}C NMR, and ESI-MS data.

Before proceeding to cell imaging experiments, we make clear whether biologically relevant thiols were able to induce cleavage of the disulfide bond in probe **6**. These experiments were performed by reacting **6** with glutathione (GSH) under physiological conditions and monitoring the changes in the optical features using UV-Vis and fluorescence spectroscopy. As shown in Fig. 1a, probe **6** revealed a strong absorption band at λ_{max} = 325 nm. This absorption peak intensity increases by ~1.4-fold with some red shift upon the addition of (5.0 mM) GSH. The solution of **6** displayed very weak fluorescence centred at 450 nm on excitation at 325 nm. The fluorescence emission signal of **6** (5.0 μM) at λ_{max} = 450 nm in aqueous PBS buffer was enhanced by ~5-fold following exposure to GSH (5.0 mM) as shown in Fig. 1b.

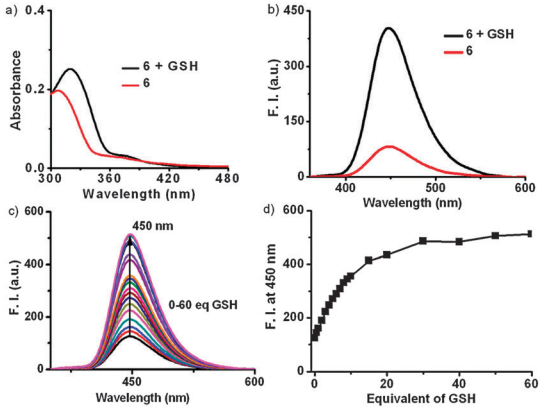


Fig. 1 (a) Absorption spectra of **6** (25.0 μM, 5% DMSO, and 95% PBS buffer) recorded in the presence and absence of GSH (5.0 mM). (b) Fluorescence spectra of **6** (5.0 μM) recorded in the presence and absence of GSH (5.0 mM). (c) Fluorescence changes of **6** (5.0 μM) seen upon treatment with increasing concentration of GSH (0–60 equiv.). (d) Changes in fluorescence intensity at 450 nm as a function of GSH concentration.

The absorption and emission nature of the solution **6** after GSH treatment was indistinguishable with that of reference **7** (Fig. S1, ESI†). We also speculated that on gradual addition of GSH (0–50 equiv.) to the solution of probe **6** (5.0 μM) in aqueous PBS buffer, the solution's fluorescence intensity at λ_{max} = 450 nm reached saturation (Fig. 1c and d). These results firmly demonstrated that the disulfide bond of **6** can be immediately cleavable by GSH, the most essential thiols among other reduced thiols in cancer cells.

Whether the projected GSH-mediated cleavage reaction of probe **6** is affected by the other biologically relevant analytes in cells, the response of **6** with various thiols, thiol-free amino acids, monovalent, and bivalent metal ions were investigated under the same reaction conditions. Similar spectroscopic changes of probe **6** were observed upon the addition of cysteine (Cys) or homocysteine (Hcy) to those encountered by GSH (Fig. S22, ESI†). In addition, no substantial spectroscopic changes were noticed upon disclosure to thiol-free amino acids such as Asp, Arg, Ala, Val, Tyr, Gly, *etc.* respectively and biologically relevant metal cations, including Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} respectively (Fig. 2). Only thiol containing amino acids specifically react with disulfide bonds because the nucleophilicity character of thiol containing amino acids along with redox potential

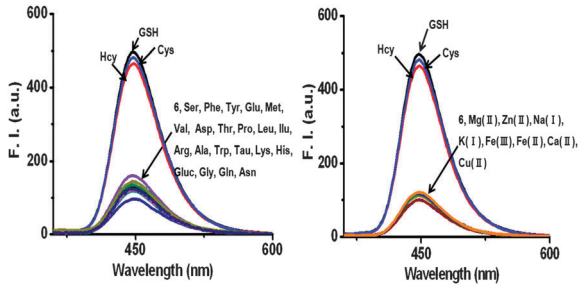


Fig. 2 Fluorescence spectra of **6** (5.0 μM) recorded in the absence and presence of GSH, Cys, Hcy (5.0 mM, respectively) and various metal cations (1.0 mM: monovalent metal ions; 0.1 mM: divalent metal ions, respectively) with excitation effected at 325 nm. All spectra were recorded in 5% DMSO & 95% PBS buffer (pH 7.4) at 37 °C.

play a vital role in the physiological environment.[6] In the cancer cells generally reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), super oxide radicals (O_2^-), and nitric oxide (NO) are present as interfering particles. But this ROS species does not break the disulfide linkage. These outcomes of the above experiment indicate that the disulfide bond in **6** will undergo specific thiol-mediated cleavage without substantial interference from other chemical analytes which might be ubiquitous in a biological system.

Furthermore, we have also inspected the pH-dependent changes in fluorescence of **6** (5.0 μM), with and without addition of 5.0 mM GSH. As shown in Fig. S2 (ESI†), in the absence of GSH, **6** is stable over a pH range from 4 to 8. However, in the presence of GSH, a substantial enhancement of fluorescence intensity was observed over a wide pH range from 5 to 8. Therefore, these results lead us to suggest that the biotin-coumarin conjugates **6** can be used to sense the existence of cellular thiols without interference from pH effects in biological environment.

To rationalize the fluorescence enhancement of **6** after the cleavage of vulnerable S–S bond, we performed time dependent fluorescence spectra with a different GSH concentration (Fig. S3, ESI†). In the presence of 5.0 mmol GSH, the enhancement of fluorescence intensity of **6** reached a saturation point within 15 min. It was observed that the rate of reaction is faster when we used 5.0 mmol GSH rather than that of 3.0 mmol, 1.0 mmol GSH. This observation suggested that free coumarin units could be generated within a short span of time once it entered into the cells. In contrast, the fluorescence intensity of **6** was not enhanced in the absence of GSH.

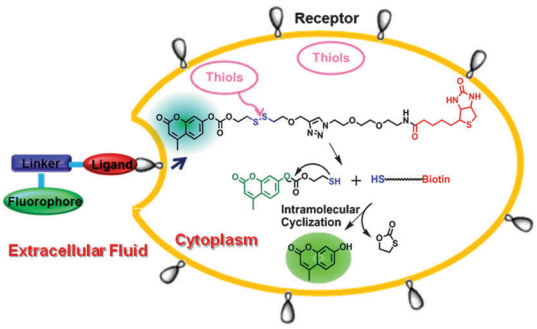
We performed another set of investigations, to justify whether the fluorescence enhancement of **6** as shown in Fig. 1, is an indication of free coumarin unit formation. Probe **6** was treated with 5.0 mmol GSH at 37 °C for 2 h, then aliquot was subjected to mass analysis. From the mass spectra, the main peak of the biotin moiety ([M + Na] = 539.10) (Fig. S21, ESI†) was observed. Together, these results lead us to suggest that the disulfide bond present in **6** was cleaved upon the treatment of GSH and thiol related enzymes. This cleavage occurred through an intramolecular nucleophilic substitution at the neighbouring carbonate bond to form a 5-membered thio lactone ring. This, in turn, will lead to the release of free coumarin units as well as fluorescent intensity increases which are described in Scheme 2. This type of self-emulative disulfide linker can be readily used in a variety of cancer-specific drug conjugates suitable for further applications in biological systems.

For the promising bio-application toward thiol-mediated cleavage of the vulnerable S–S bond and concomitant fluorescence-On *in vitro* test, the conjugate **6** was applied to living cells. Initially, we used one photon fluorescence spectroscopy to take images in living cells using probe **6**. Due to shorter wavelength light scattered and autocaptivated by native cellular components the cellular imaging was not good. Thus, we implemented two photon fluorescence spectroscopy, which has the following advantages such as minimized light scattering, reduced photodamage to living biological samples and fluorophores, and improved spatial resolution, and sensitivity as well as the ability to image thicker specimens.[9]

In order to apply probe **6** to the living systems, firstly we investigated whether the biotin moiety can lead the probe especially to biotin receptor-positive tumor cells. Probe **6** was treated with biotin receptor-positive A549 and biotin receptor-negative WI-38 cells. The A549 cells with high levels of the biotin receptor, exhibit strong fluorescence intensity within 20 min of incubation with **6** (5.0 μM), whereas very little or no fluorescence signal was observed in the biotin receptor-negative WI-38 cancer cells, under similar experimental conditions, as shown in Fig. 3. We performed another cell experiments where **6** and compound **4** without biotin were treated in the two cell lines (A549 and WI38), Fig. S4 (ESI†). In these two experiments we found that noticeable difference in the fluorescence intensity inside the cells only in the presence of the biotin group. Therefore, we concluded that the selective uptake of **6** by A549 cells can be accredited by the interaction of its biotin moiety with a biotin receptor on the tumor cells, possibly through receptor-mediated endocytosis.

To provide further confirmation for thiol-induced disulfide bond cleavage as well as concomitant fluorescence-On, probe **6** was treated with NEM (*N*-ethylmaleimide). NEM is well known to react with thiol groups.[12] Therefore, in the presence of this thiol inhibitor, the fluorescence intensity of cells treated with **6** was expected to be relatively decreased. The results are shown in Fig. 4. It is obvious that the fluorescence intensity of **6** in A549 cells decreases as the concentration of NEM is increased (0–0.5 mM). This phenomenon provided another confirmation that the fluorescence emission intensity of **6** depends on the intracellular thiol levels.

To prove our design expectation as well as receptor mediated endocytosis process, we carried out the cell viability experiments of compound **6** with biotin positive A549 cells and biotin negative WI 38 cells (Fig. S5, ESI†). We observed that at low to high concentration **6** showed less cell viability in the A549 cells



Scheme 2 Reaction mechanism of **6** with thiols under physiological conditions.

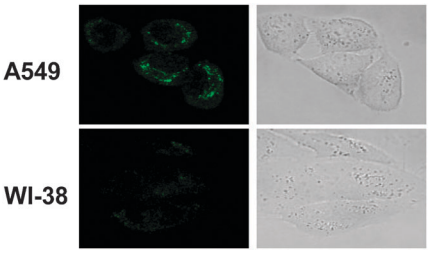


Fig. 3 The left side panels show the confocal microscopy images of A549 and WI-38 cells treated with 5.0 μM of **6** in PBS buffer, total incubation period of 20 min. The right side panels show contrast images. Cell images were obtained using two photon excitation wavelengths at 740 nm and emission wavelengths of 400–500 nm.

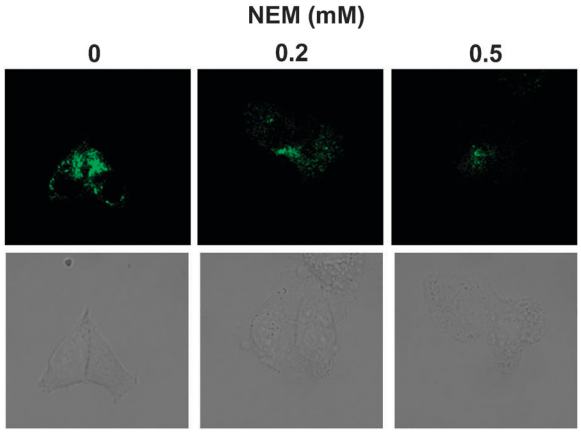


Fig. 4 Confocal laser fluorescence microscopic images of A549 cells treated with 5.0 μM of **6** in PBS buffer. The cells were pre-incubated with media containing *N*-ethylmaleimide (NEM) of various concentrations (0, 0.2, and 0.5 mM) for 30 min at 37 °C. Cell images were obtained using two photon excitation wavelengths of 740 nm, and emission wavelengths of 400–550 nm, green signal, respectively.

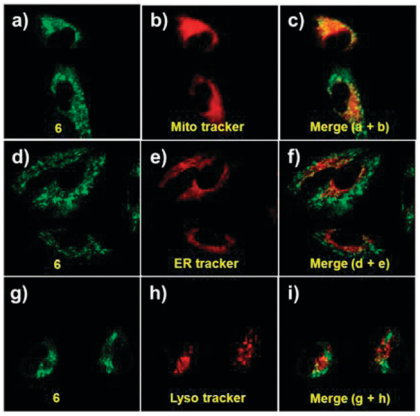


Fig. 5 Confocal laser fluorescence microscopic images of A549 cells incubated with 5.0 μM of **6** containing (b) Mito tracker deep red (0.5 μM) excited at 633 nm, collected at 650–750 nm; (e) ER-tracker red (0.5 μM) excited at 514 nm, collected at 570–600 nm; (h) Lyso tracker red DND-99 (1.0 μM) excited at 514 nm, collected at 570–650 nm. (a, d, g) are the fluorescence images of **6** (5 μM) for 20 min at 37 °C, excited at 740 nm, collected at 400–500 nm. (c, f, i) overlay of the merged images, respectively.

rather than WI38 cells. This is because **6** can easily enter into the high levels of the biotin receptor positive A549 cells through the strong interaction between avidin and biotin. Together these results provide support that cellular uptake of probe **6** into A549 cells takes place through receptor-mediated endocytosis and gives rise to fluorescence enhancement as the result of thiol-induced disulfide bond cleavage. Therefore, it is clearly confirmed again that the biotin moiety in **6** obviously plays as a targeting unit to tumor cell in this fluorescence probe.

To recognize the intracellular location of coumarin moiety released from **6**, after get into the cells, colocalization experiments were performed using selective fluorescent trackers like as mitochondria (Mito-), lysosome (Lyso-) and endoplasmic reticulum (ER). The results from these studies are shown in Fig. 5. The fluorescence image of probe **6** was mainly overlapped with Mito-, ER markers. On the other hand, the fluorescence describable to **6** does not exactly co-localize with Lyso Tracker. As the ER membrane is closely associated with the inner nuclear membrane (INM), it may be assumed that molecules intended for the INM diffuse through the ER membrane.[13] We, therefore, conclude that thiol mediated disulfide cleavage of **6** occurs in the mitochondria as well as ER; this scission process assists in the release of the free coumarin moiety and concomitant fluorescence-On.

In conclusion, we have designed and synthesized novel coumarin–disulfide–biotin conjugates (**6**) for the precise detection of intercellular thiols including GSH, Cys and Hcy and its cell viability as well as biological applications *in vivo*. Upon addition of thiols, the fluorescence intensity of **6** increases by about 5-fold. Moreover, probe **6** shows good water solubility, is inactive towards other biologically relevant analytes such as various thiols, thiol-free amino acids, monovalent, and bivalent metal ions, is easy-to-visualize, and is reactive over a wide pH range. From confocal microscopic experimental studies, we revealed that our system goes to precisely biotin receptor-positive A549 cells rather than WI 38 cells (biotin negative) through receptor mediated endocytosis. Furthermore, the fluorescence image of probe **6** was mainly overlapped with Mito-, ER markers. Therefore, our probe, described above, could afford a powerful new approach for the specific tumor targeting thiol sensor and bio imaging. In addition this system may have a role in opening new possibilities for the screening of new potential drug agents, diagnosis and bioimaging applications *in vitro* and *in vivo*.

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