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Selective Detection of the Reduced Form of Glutathione (GSH) over the Oxidized (GSSG) Form Using a Combination of Glutathione Reductase and a Tb(III)-Cyclen Maleimide Based Lanthanide Luminescent 'Switch On' Assay

Brian K. McMahon and Thorfinnur Gunnlaugsson*

School of Chemistry, Center for Synthesis and Chemical Biology, Trinity College Dublin, Dublin 2, Ireland

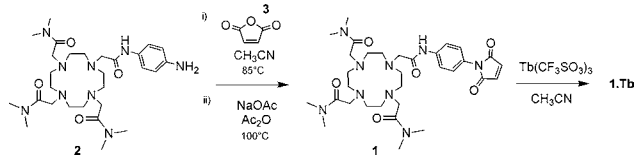
Supporting Information

ABSTRACT: The synthesis of a novel Tb(III) luminescent probe for the detection of thiols is presented. The probe **1.Tb**, possessing a maleimide moiety, as its sulfhydryl acceptor, was poorly emitting in aqueous pH 7 solution in the absence of a thiol. However, upon addition of thiols such as glutathione (GSH), large enhancements were observed, particularly within the physiological pH range. In contrast no enhancements were observed in the presence of the oxidized form of glutathione (GSSG), except in the presence of the enzyme *glutathione reductase* and NADPH which enabled **1.Tb** to be used to observe the enzymatic reduction of GSSG to GSH in real time.

Sulfhydryl-containing amino acids and peptides play a pivotal role in many biological processes such as in reversible redox reactions and in important cellular functions like detoxification and metabolism.[1,2] Low cysteine (Cys) levels have been linked to many health issues such as hematopoiesis reduction, retarded growth, hair depigmentation, liver damage, skin lesion development, and cancer.[3] In contrast, homocysteine (HCys) is a common risk factor for disorders such as Alzheimer's disease, osteoporosis, thrombosis, and cardiovascular disease.[4,5] Hence, there currently exists a significant interest within the field of supramolecular chemistry to develop selective sensors and probes for the detection of such species. The development of fluorescent probes for such detection has recently been highlighted by Yoon et al.[1a] However, while being highly sensitive, these sensors/probes usually consist of short wavelength emitting and short lifetime-based fluorophores, which is a real drawback for use in competitive biological media. The development and use of specific analyte targeting lanthanide based luminescent sensors/probes has recently emerged for various biological applications.[6,7] The lanthanides possess many desirable photophysical properties, such as long wavelength emissions (within the visible and the near-infrared (NIR) regions), sharp line-like emission bands and long-lived excited states (μs−ms time frame), allowing them to be easily distinguished from shorter-lived (ns-based) autofluorescence from biological material. This greatly improves the signal-to-noise ratio of such time delayed sensors/probes, making them desirable alternatives to fluorescence based systems.

Glutathione (γ-L-glutamyl-L-cysteineglycine, or *GSH*) is the most predominant tripeptide thiol found within the human cellular system. Of all the thiol containing components of blood, the GSH content makes up 90% of its entire composition with previous literature stating that, due to this extremely high concentration of GSH, all other potential thiol molecules can be disregarded.[8] Existing essentially in its reduced form, GSH can, however, be rapidly oxidized to its dimeric GSSG form in response to oxidative stress within cells. Therefore changes in the intracellular GSH concentration or in the GSH/GSSG ratio have become a key indicator in monitoring the cells' overall health and their ability to resist oxidative damage.[9] Various analytical methods have been developed to date for GSH detection, including the use of HPLC,[10] UV−vis/calorimetric assays[11] and capillary electrophoresis.[12] In the past, we have developed many examples of lanthanide luminescent sensors, probes, and imaging agents for biological applications.[13,14] However, to the best of our knowledge, the development of such systems for the detection of thiol specific, or biothiols, and in particular GSH has not been achieved to date. Consequently, we set out to develop such a probe, which could display both high sensitivity and selectivity for thiols and, in particular, enable the selective detection of GSH over GSSG. Herein, we present **1.Tb**, an

Scheme 1. Synthesis of 1 (Free Ligand) and the Corresponding Tb(III) Complex 1.Tb



octadentate macrocyclic Tb(III) cyclen conjugate possessing a maleimide functionality, which, upon 1,4-Michael addition of a thiol group to the alkene bond, displays large changes in the Tb(III) centered emission. Moreover, we demonstrate the high selectivity of **1.Tb** for GSH and that, in combination with the enzyme glutathione reductase, the formation of GSH from GSSG can be monitored in real-time with high sensitivity.

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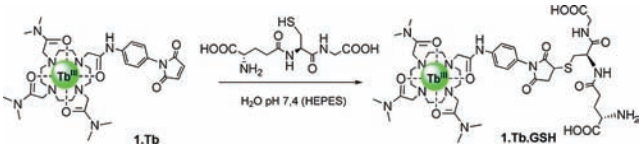
The synthesis of **1.Tb** (Scheme 1) was achieved in three steps from **2** (previously developed in our laboratory[15]), which involved reaction with maleic anhydride, **3**, in refluxing CH_3CN solution for 12 h. The resulting crude acid amide derivative was treated with NaOAc in acetic anhydride at 100 °C for 2 h, and the desired product **1**, isolated in 27% yield after aqueous–organic workup (see Supporting Information (SI)). The corresponding Tb(III) complex, **1.Tb**, was formed in 93% yield by refluxing **1** with 1 equiv of $Tb(CF_3SO_3)_3$ in freshly distilled CH_3CN for 15 h (see ^{1}H SI Figure S1a-S1c)

The design of **1.Tb** allows for sensitized excitation of the Tb(III) $^5D_4$ excited state *via* the covalently attached phenyl antenna, which upon relaxation to the ^7F_J (J = 6, 5, 4, 3) ground states gives rise to the characteristic line-like Tb(III) emission bands occurring at long wavelengths. Indeed, upon excitation of **1.Tb** at 256 nm (λ_{max} of phenyl antenna) in an aqueous buffered pH 7.4 (20 mM HEPES, 135 mM KCl) solution, metal centered emission was observed with bands appearing at 490, 545, 586, and 622 nm (Figure S2). The excited state decay was measured in both H_2O and D_2O (λ_{ex} = 256 nm), and best fit to a monoexponential decay profile, from which the excited state lifetimes τ_{H2O} = 1.31 ms and τ_{D2O} = 1.99 ms, were determined. From these lifetimes, the hydration state for **1.Tb** (the q value)[16] was determined as 1 (Figure S3).

The principal reaction mechanism behind the luminescence response of **1.Tb** to GSH is shown in Scheme 2. As stated

Scheme 2. Reaction of 1.Tb with GSH Yielding the Adduct 1.Tb.GSH



above this reaction involves a 1,4-Michael addition of the thiol functional group to the electron deficient alkene of the maleimide moiety. We envisaged that this reaction and the concomitant formation of the succinimide derivative would effect the sensitization process of the Tb(III) excited state, thus directly modulating the photophysical properties of the Tb(III) ion. We consequently carried out several screening studies with **1.Tb**, using various biologically relevant thiols. We initially used GSH using the aforementioned experimental conditions. This showed that changes in the absorption spectrum of **1.Tb** (see SI, Figure S4) upon addition of the thiol were minor and too small to analyze accurately. Similarly the changes in the fluorescence emission spectra of **1.Tb** were minor. However, and as anticipated, significant changes were seen in the metal centered emission, where the Tb(III) emission was greatly enhanced as a function of added GSH concentration. The changes in the Tb(III) emission are shown in Figure 1, where the Tb(III) emission was enhanced, or '*switched on*' by *ca.* 500% upon addition of GSH, signifying the formation of **1.Tb.GSH**. More importantly, the emission did not enhance after the addition of 1 equiv of GSH as demonstrated as an inset in Figure 1, where the intensity changes measured for the 550 nm transition are plotted as a function of added equivalents of GSH, signifying that the reaction was stoichiometric.

While this reaction has been widely used within the literature, where it takes place, under physiological conditions, in real time and with high yields, it was necessary to verify that

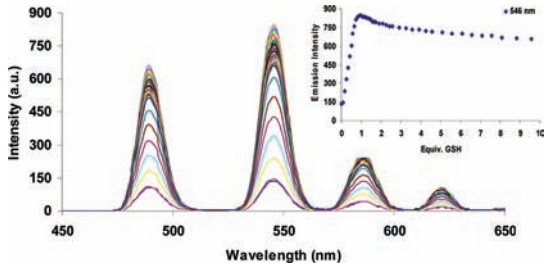


Figure 1. Changes in the Tb(III) luminescence of **1.Tb** (10 μM) upon excitation of the phenyl antenna (λ_{max} = 256 nm) as a function of GSH in H_2O (20 mM HEPES, 135 mM KCl, pH = 7.4). Inset: Plot of intensity at 545 nm with equivalents of GSH added.

the observed emission changes in Figure 1 were indeed due to the formation of the reduced form of **1.Tb** (assigned here as **1.Tb.GSH**) in solution, as it could be possible that GSH was in fact interacting with **1.Tb** in a noncovalent manner. To investigate this, a small scale reaction, where **1.Tb** was reacted with 1 equiv of GSH in H_2O, at rt for 2 h, was carried out. The reaction mixture was then analyzed using ESMS without carrying out any workup on the crude reaction mixture. The results indeed confirmed that **1.Tb** was converted successfully under these experimental conditions to the desired **1.Tb.GSH** product, where the ESMS (+ mode) showed the presence of a peak at m/z = 1119.38, corresponding to a species with the chemical formula corresponding to the [**1.Tb.GSH**-3CF_3SO_3-2H]$^+$ complex, and an isotopic distribution pattern that matched the calculated species (see SI, Figures S5 and S6).

To further analyze the changes in the Tb(III) emission spectra in the presence of 1 equiv of GSH, the Tb(III) excited state decay was determined and found to be best fit to a monoexponential profile. From these lifetimes, the q value was recalculated and shown to be unchanged (q = 0.9) (see SI, Table S1), confirming that the observed increase in Tb(III) emission of **1.Tb** was not due to displacement of the bound H_2O molecule by GSH.

We next evaluated the photophysical behavior of **1.Tb** (10 μM solutions) and its ability to react with GSH over a range of pH values. As shown in Figure 2, the Tb(III) emission of **1.Tb**

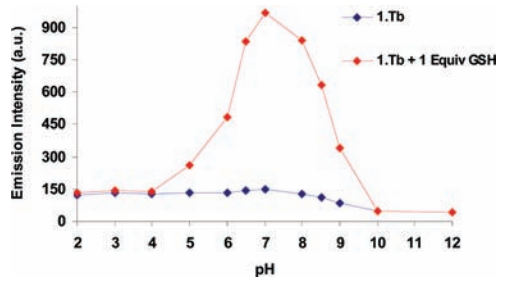


Figure 2. Effect of **1.Tb** (10 μM) and its response to GSH as a function of pH (individual 10 μM solutions of **1.Tb** at each pH value in 20 mM HEPES were used, followed by addition of 10 μM GSH).

was found to be relatively independent of pH, with only small changes being observed within the pH range of 7–9 (in blue). However, when the Tb-emission from **1.Tb** was monitored in the presence of 1 equiv of GSH it was shown to be highly pH dependent (in red, Figure 2). The effect of pH on the Tb(III) emission in the presence of GSH can be best described as displaying a bell-shaped '*Off–On–Off*' emission behavior[17] for the 550 nm transition (similar changes were seen for all the $^5D_4$

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→ ^7F_J deactivations), which mimics that often seen for biological processes such as enzymatic reactions. As shown in Figure 2, the Tb(III) emission was not affected by the presence of GSH in either acidic (<pH 4) or basic (>pH 10) media. However, within these two pH envelopes, the Tb(III) emission was highly sensitive to the presence of GSH. With a maximum Tb(III) emission enhancement being observed at pH ~7, it was demonstrated that this system could be employed as a probe for the detection of GSH within the physiological pH range.

Having demonstrated that the Tb(III) emission was highly sensitive to the presence of GSH within the physiological pH range, we next investigated the response of **1.Tb** to several other thiol based biological molecules as well as amino acids, as the latter could also potentially interact with the maleimide unit in **1.Tb** using the same 1,4-Michael addition. As expected, the Tb(III) emission of **1.Tb** (10 μM) was found to be modulated in the presence of the thiol based amino acids cysteine (Cys) and homocysteine (HCys) (see SI, Figures S7–S8). However, no significant changes were observed in the Tb(III) emission using other naturally occurring amino acids (40 μM) (Figure 3). Further investigations also showed very little variation in the

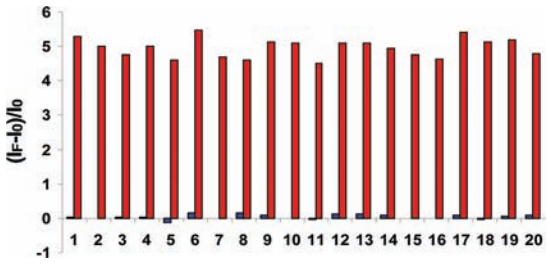

Figure 3. Tb(III) luminescence response of **1.Tb** (10 μM) in the presence of various physiologically important amino acids (40 μM) in H_2O (20 mM HEPES, 135 mM KCl, pH 7.4). Red bars represent the final emission intensity (I_F) subtracting the initial intensity (I_0) over I_0 at λ_{em} = 545 nm. Blue bars represent the addition of the amino acids: (1) Ala, (2) Asp, (3) His, (4) Arg, (5) Phe, (6) Ser, (7) Val, (8) IIe, (9) GSSG, (10) GSH, (11) Pro, (12) Thr, (13) Tyr, (14) Gly, (15) Leu, (16) Lys, (17) Met, (18) Glu, (19) Sar, (20) Asn. Red bars represent the subsequent addition of GSH (40 μM) to these respective solutions.

1.Tb response to GSH in the presence of any of these amino acids (see SI Figure S9a –S9b).[18] However, the most significant result emerging from this screening process was the ability of **1.Tb** to selectively respond to GSH over the oxidized GSSG form, which lacks the nucleophilic thiol moiety. As shown in Figure 3, in the presence of a large excess of GSSG (40 equiv), the Tb(III) emission was within experimental error, unchanged, demonstrating that **1.Tb** could be used for monitoring GSH in the presence of GSSG.

The above results also suggested that **1.Tb** could potentially be employed to observe the conversion of GSSG to its reduced GSH form in real time in competitive media; an event which is highly desirable as being able to monitor such enzymatic reactions in real time[19] is of great current interest. The shift in the concentration of the reduced form to the oxidized glutathione form is often used as an indicator of cellular well-being *in vivo*, as it can give a good indication of overall health and any oxidative damage occurring.[9] To demonstrate the ability of **1.Tb** to monitor the reduction of GSSG in solution, a series of experiments were carried out, by using the enzyme *glutathione reductase* and the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH). This enzyme catalyzes the reduction of GSSG to GSH under physiological pH conditions, and it was anticipated that **1.Tb** could be used successfully to monitor the kinetics of this redox process. This could be achieved by following the enhancement in the delayed Tb(III) emission intensity upon GSH formation, as it would be subsequently trapped by reacting with the maleimide unit as shown above (Scheme 2). Indeed, this was found to be the case where we observed the changes in the Tb(III) emission as a function of GSH formation in the presence of the enzyme and NADPH. Furthermore, optimization studies on this reaction (see SI Figure S10) showed that **1.Tb** could monitor this conversion of GSSG to GSH at low NADPH concentrations, with the optimum luminescence response being observed at 3.3 μM of NADPH. We next studied the kinetic effect on the reduction of GSSG as a function of enzyme concentration, the results of which are shown in Figure 4. These results

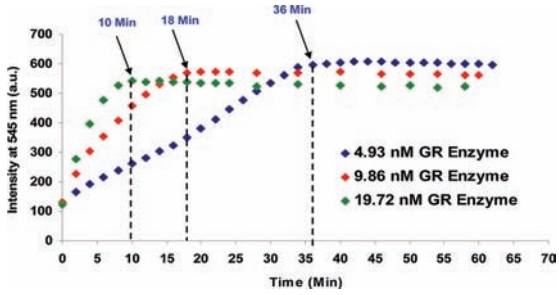

Figure 4. Luminescence response of **1.Tb** (10 μM) as a result of the enzymatic conversion of GSSG to its reduced GSH form using NADPH (3.3 μM) and various concentrations of the enzyme glutathione reductase. All enzymatic titrations were carried out in aqueous pH 7.4 buffered solutions (0.1 M TRIS, 0.135 M KCl) at 37 °C.

demonstrate that when the concentration of glutathione reductase was doubled, the time associated with the redox process was halved. Therefore, using an enzyme concentration as low as 19.72 nM ensured that **1.Tb** maximized its response over a short period of 10 min, Figure 4. By monitoring the changes in the UV–vis absorption spectra for these titrations only minimal changes were observed for the π–π^* absorbance band of **1.Tb**. However, a decrease in the absorption of the NADPH band at 340 nm confirmed that the redox process was occurring and that the modulation of the Tb(III) emission was indeed a direct result of the subsequent formation of GSH and the trapping by **1.Tb** (Figures S11a–S11c, S12), which concomitantly switched the Tb(III) emission on.

With the view of evaluating the competitiveness of this reaction, the enzymatic reduction of GSSG, in the presence of **1.Tb**, was also carried out in the presence of 40 equiv of each of the amino acids tested above (excluding any thiol based amino acids) at pH 7.4. The results (see SI Figure S13a–S13d) showed on all occasions an intensity enhancement of *ca.* 400% in the Tb(III) emission, clearly demonstrating the ability of **1.Tb** to monitor the above process, even in competitive media. Therefore, we decided to lower the concentration of the enzyme in the presence of **1.Tb** (10 μM) with the view of further maximizing the potential of using this unique combination of an enzyme and probe to observe a biological process in real time. Various titrations indeed demonstrated the feasibility of an enzyme concentration of 4.93 nM. The reaction was observed to be completed within 12 min compared to the

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36 min observed previously, demonstrating the success of **1.Tb** in monitoring the enzymatic conversion in real time.

In summary, we have designed and developed the first example of a lanthanide based GSH sensor, through functionalization of the cyclen framework with a pendant arm incorporating the maleimide moiety. We have demonstrated the ability of **1.Tb** to be used in monitoring thiol specific and in particular the GSSG to GSH redox process using glutathione reductase and the reducing agent NADPH. We are currently developing similar systems using NIR emitting probes which will allow for the sensitization of the lanthanide excited state within the visible region.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of **1** and **1.Tb**; Figures S1−S14. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

gunnlaut@tcd.ie

Notes

The authors declare no competing financial interest.

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(18) It is worth pointing out that, due to spectral overlap of the $\pi-\pi^*$ absorption bands of **1.Tb** and the amino acid tryptophan, an initial decrease in the Tb(III) emission intensity of *ca.* 76% was observed (see SI Figure S9a−S9b).

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