

# A ratiometric and non-enzymatic luminescence assay for uric acid: differential quenching of lanthanide excited states by anti-oxidants†

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The excited states of Tb and Eu complexes of a common macrocyclic ligand are quenched preferentially by electron transfer from the urate anion, allowing the creation of a new assay to measure uric acid in biological fluids.

Uric acid is the final breakdown product of purine (*e.g.* adenine, guanine) metabolism. Purines from nucleic acid breakdown are converted to uric acid in the liver. The body uric acid level is determined by the balance between synthesis and urinary elimination. Elevated levels of uric acid (hyperuricaemia) are most commonly associated with gout, increased breakdown of cell nuclei and renal disease. Patients on chemotherapy for proliferative diseases such as lymphoma, leukaemia or myeloma often exhibit hyperuricaemia and levels must be monitored to avoid kidney damage. Treatment involves administration of allopurinol, an inhibitor of uric acid synthesis.<sup>1</sup> In gout patients, precipitation of uric acid in the joints leads to pain and inflammation and in many examples, this is directly linked to over-production of uric acid. Plasma uric acid levels in such patients are usually high (above 60 mg l<sup>-1</sup> [0.36 mmol] *cf.* 1.5 to 4.5 mmol l<sup>-1</sup> in urine); these patients are also more susceptible to the formation of kidney stones. Hypouricaemia is much less common but may result from under-production of uric acid or from a reduction in uric acid excretion, as may occur in AIDS, diabetes mellitus and various malignant diseases. Measurement of uric acid in urine and plasma is therefore an important marker in clinical diagnosis and is essential in the treatment regime for hyperuricaemia, involving either controlled use of uricosuric drugs, to enhance renal excretion or administration of allopurinol to suppress purine synthesis.<sup>2</sup>

Current *in vitro* clinical assays used to measure uric acid in urine and serum are predominantly based on the use of the uricase enzyme, which catalyses the hydrolysis of uric acid leading to formation of the more water-soluble compound, allantoin, and hydrogen peroxide.<sup>3</sup> The hydrogen peroxide is detected either directly *via* oxidation of a phenolic dye to produce a strongly coloured compound, or indirectly by the action of a peroxidase enzyme, leading to formation of an intensely coloured chromogen or a strongly emissive fluorophore. These enzymatic methods are typically subject to interference from ascorbate (requiring co-administration of ascorbate oxidase) and bilirubin. Typically, clinical kits give a linear response for the range 0.12 to 6 mmol l<sup>-1</sup> for urine. The analysis requires incubation for at least 30 minutes

and needs careful control of pH.<sup>4</sup> The enzymes must be handled carefully to avoid protein denaturation and the organic dyes used are often light and temperature sensitive, requiring storage in the dark at low temperatures.

Lanthanide ions afford considerable scope for the development of new chemical entities that can be used as analytical or imaging probes, as components of optoelectronic devices, or as key sensor materials. The advantages of f-block ions have been demonstrated irrefutably:<sup>5–7</sup> large Stokes' shifts; intense, line-like and long-lived luminescence at a range of wavelengths spanning the visible and near infrared (NIR) regions; time-gated rejection of unwanted signals arising from (short-lived) auto-fluorescence from biomolecules. Here, we report on the use of two different lanthanide(III) complexes of a common macrocyclic ligand, allowing the analysis of the concentration of low molecular weight reductants, exemplified by the assay of uric acid in diluted urine samples.

The <sup>5</sup>D<sub>4</sub> and <sup>5</sup>D<sub>0</sub> excited states of Tb and Eu lie 2.44 V and 2.06 V above the ground state and the associated free energy may be harnessed to drive various electron or energy transfer reactions. In preliminary work, it was noted that species such as ascorbate (*E*<sub>1/2</sub> = 0.30 V<sup>8</sup> (1e-process), 298 K, pH 7; p*K*<sub>a</sub> 4.2), iodide (0.51 V), urate (0.59 V, p*K*<sub>a</sub> 5.4<sup>9</sup>) and bromide (1.07 V) were able to deactivate the lanthanide excited state, leading to a reduction in both the emission lifetime and intensity.<sup>10</sup> This dynamic quenching process is characterised by a Stern–Volmer quenching constant, representing the concentration of reductant needed to reduce the lifetime or emission intensity to 50% of its original value. Values for the quenching by iodide, ascorbate and urate for a series of structurally related macrocyclic ligands, based on either the tetraazatriphenylene sensitising moiety or the azaxanthone chromophore are collated in the Table. Quenching requires collisional encounter, and the lifetime of the encounter complex is likely to be determined by steric factors and by the local electrostatic potential gradient. Examples selected here (complexes [Ln.1] to [Ln.8], selected synthesis and characterisation details of new complexes are given in the ESI†) serve to highlight the effects either of introducing negative charge on the complex (on the periphery of the complex or on the chromophore itself), or of varying the steric bulk of the complex, by adding different pendant donors of varying steric demand to three ring-nitrogens.

Some general trends are apparent following inspection of the data in the Table. First, the Tb complexes are more sensitive to quenching than the Eu analogues—as expected from the higher excited state energy of the Tb <sup>5</sup>D<sub>4</sub> state. Second, urate quenching is much more effective than anticipated on the basis of its one-electron oxidation potential. Indeed, with [Tb.1] and [Tb.2], quenching by urate is 50 times more effective than ascorbate.

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**Table 1** Stern–Volmer quenching constants ( $K_{SV}^{-1}/\text{mM}$ ) for dynamic quenching of the lanthanide excited state (pH 7.4, 0.1 M HEPES, 298 K)

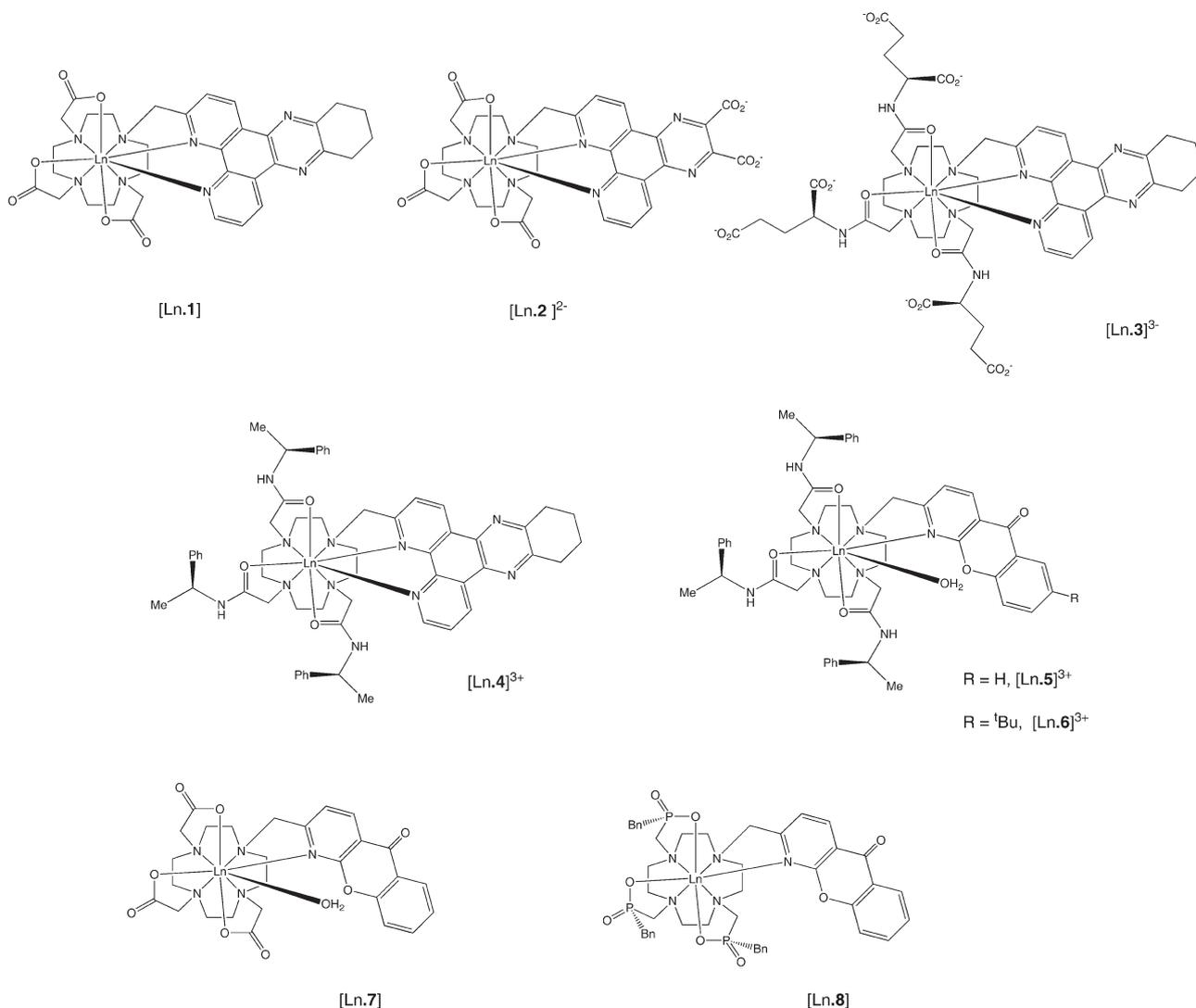
Complex	Urate	Iodide	Ascorbate
[Tb.1]	0.006	2.10	0.30
[Eu.1]	0.11	>1,000 <sup>b</sup>	2.43
[Tb.2]	0.01	6.90	0.75
[Eu.2]	0.16	>1,000 <sup>b</sup>	4.31
[Tb.3]	0.012	2.50	0.38
[Eu.3]	0.084	>1,000 <sup>b</sup>	2.55
[Tb.4]	0.25	0.90	0.18
[Eu.4]	0.93	27.0	0.39
[Tb.5]	0.04	9.20	0.37
[Eu.5]	0.60	278	1.50
[Tb.6]	0.02	38.2	0.30
[Eu.6]	0.27	250	1.52
[Tb.7]	0.012	53.5	0.57
[Eu.7]	0.28	125	8.90
[Tb.8] <sup>d</sup>	0.06	26.2	0.93
[Eu.8]	0.36	139	11.3

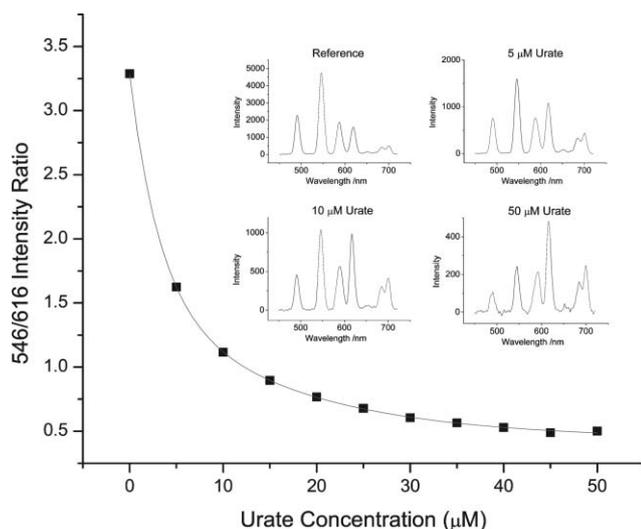
<sup>a</sup> The P-methyl analogue gave values for quenching of the terbium complex as follows: urate, 0.02; iodide, 36.6, ascorbate, 0.48 mM.

<sup>b</sup> At high quenching concentrations of iodide, quenching of the chromophore singlet excited state was evident. <sup>c</sup> For urate quenching, over the range examined, emission lifetime and total integrated emission intensity variations showed a similar dependence.

Third, for complexes with a very similar donor set but a different chromophore, (e.g. [Ln.1] vs [Ln.7]; [Ln.4]<sup>3+</sup> vs [Ln.5]<sup>3+</sup>) the more readily reduced chromophore (Me-tetraazatriphenylene **9**,  $E_{1/2} = -1.1$  V (MeCN, 0.1 M NEt<sub>4</sub>ClO<sub>4</sub>, 298 K), Me-azaxanthone, **10**,  $E_{1/2} = -1.6$  V, 298 K) is the more sensitive to quenching by iodide and ascorbate. Again, in these cases, urate quenching does not always follow the trend, suggesting a more pronounced sensitivity to factors that determine the lifetime or orientation of the encounter complex. A tentative hypothesis is that the quenching encounter complex with urate is longer-lived by virtue of  $\pi$ -complex formation between the electron-poor sensitizer and urate anion.

The quenching behaviour observed suggested an application for the direct analysis of urate concentration in aqueous samples. Using a mixture of Eu and Tb complexes with a common ligand, measurement of the ratio of intensities of a Tb emission band (e.g. at 546 nm) versus a dominant Eu emission band (e.g. at 700 nm, or 616 nm) is a direct function of the amount of urate present in solution, providing that interfering species contribute little to the overall quenching effect. Accordingly, a solution containing 5  $\mu\text{M}$  concentrations of [Tb.3]<sup>3-</sup> and [Eu.3]<sup>3-</sup> was prepared (absorbance was ca. 0.1 at 313 and 348 nm, pH 7.4 in 0.1 M HEPES) and





**Fig. 1** Variation of the Tb (546 nm) to Eu (616 nm) emission intensity ratio (pH 7.4, 0.1 M HEPES, 298 K) as a function of sodium urate concentration. The curve depicts the iterative fit to a double exponential decay (details in the ESI†) and the inset shows selected spectra recorded (Analytik Flash Scan 530 Multiwell Analyser).

diluted to a standard volume of 200  $\mu\text{L}$  with a solution of recrystallised uric acid (solution standardised by measuring absorbance at 290 nm,  $\epsilon = 1.22 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ). Aliquots were dispensed into wells of a 96-plate reader and triplicate samples were prepared over the urate concentration range 0 to 50  $\mu\text{M}$ . In parallel, samples of urine from healthy volunteers were diluted 100, 300 to 500-fold and triplicate samples prepared. For each well, 10 separate spectra were recorded (Analytik Flash Scan 530,  $\lambda_{\text{exc}}$  313 nm) and averaged, and the ratio of the 546/616 and 546/700 nm bands was determined. For the calibration solution, a plot of the ratio of emission intensities *versus* urate concentration was made and was fitted iteratively to a bi-exponential model (Fig. 1; see ESI for additional spectra†). Less than 1% variance was found in the measured intensity ratio for a given urate concentration. Such precision is an inherent feature of a ratiometric assay. Here, because the assay involves two complexes of the same ligand, other non-specific effects that may affect the observed emission intensity or lifetime for a given sample occur to the same extent for the Eu and Tb complexes. Examples of such effects include sample to sample variation of protein, light scattering due to particulates and surface adhesion. In particular, any static quenching component, operating *via* deactivation of the chromophore singlet excited state occurs to the same extent for each of the Tb and Eu complexes. It is corrected for intrinsically as the lanthanide emission intensity ratio is taken. Examples of halide anions deactivating chromophore singlet excited states are well known, and have been studied as simple halide anion sensors in lanthanide complexes.<sup>11</sup>

For the unknown samples, the urate concentration was readily computed, from the calibration curve or by applying the mathematical model. With the same samples, a commercial uricase assay kit was employed (Invitrogen, Amplex Red Uric Acid kit), that uses horseradish peroxidase. Agreement between the two methods was  $\pm 10\%$ , but it should be noted that the enzyme assay gave values that were estimated to have a precision of no better than 10% for the 3 replicates examined.

In summary, a simple, rapid non-enzymatic luminescence assay has been developed for uric acid, suitable for application in diluted urine samples and measurement in equipment commonly found in high throughput screening laboratories. The same methodology has also been applied to diluted serum samples and should be applicable to other common bio-fluids. The ratiometric luminescence method may be generically applicable for appropriately selected analytes in the determination of certain compounds, *e.g.* bioactive phenols, catechols and catecholamines, possessing a one-electron oxidation potential of less than about 0.75 V.

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