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A Selective Luminescent Probe for the Direct Time-Gated Detection of Adenosine Triphosphate

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Supporting Information

ABSTRACT: A molecular probe for the luminescent detection of adenosine nucleotides is presented. The probe, Tb-DOTAm-Phen, readily distinguishes among the three adenosine nucleotides in buffered aqueous conditions at neutral pH, a requirement for the direct monitoring of enzymatic reactions converting adenosine triphosphate (ATP) to adenosine diphosphate or adenosine monophosphate. The probe is most efficient under millimolar concentrations of ATP which are relevant to intracellular conditions. Moreover, the long luminescence lifetime of the probe readily enables time-gating experiments.

Kinases currently represent one of the two largest classes of targets for drug discovery.[1,2] In the field of oncology, the FDA's approval of the first kinase inhibitor, Herceptin, in 1998 started an extensive search for cancer drugs that target cellular signaling pathways.[3] The search for new kinase inhibitors, critical tools in cancer therapy, often begins with the high-throughput screening (HTS) of libraries followed by the evaluation of the potency of potential leads and their selectivity for a desired kinase. These studies require the widespread availability of efficient and affordable screens for kinase activity. A recent survey highlighted the most important features coveted by researchers in such a platform.[4] It must concurrently (1) be affordable, (2) be non-radioactive, (3) be label-free, (4) be antibody-free, (5) be generic (able to screen all classes of kinases), and (6) enable the study of large protein substrates. In addition, (7) time-resolved or time-gated luminescence detection is highly preferred, as are (8) assays that measure the accumulation of adenosine diphosphate (ADP) as opposed to the phosphorylation of a peptide or the displacement of a biomarker. None of the current commercial assays fulfill all eight of these requirements.[5−13] Moreover, none can be performed at higher concentrations of adenosine triphosphate (ATP), closer to the intracellular concentrations of 1−10 mM needed to study inhibitors of low-affinity kinases.[14]

In terms of design, in order to fulfill each of the above parameters, the probe must consist of a small molecule which is luminescent and non-radioactive. Lanthanide complexes such as terbium or europium are preferred as their long luminescence lifetimes, typically in the ms range, enable facile time-gated experiments. Time-gating is a favored technique in drug discovery and chemical biology because the delay between the excitation pulse and the emission measurement enables the complete removal of background fluorescence, including those arising from fluorescent drug candidates.

The generic aspect and the need to screen large protein substrates can both be met by designing a probe that binds reversibly and with differential affinity to ATP, ADP, and adenosine monophosphate (AMP). If the probe is designed in such a way that binding of the nucleotide affects its luminescence, then the different binding affinities can advantageously be used to monitor conversion from one nucleotide to another. A turn-on response for ATP to ADP conversion can be achieved if both nucleotides quench the luminescence of the probe and if the probe has higher affinity for ATP than ADP. Lastly, for the probe to be efficient at the high millimolar concentration of ATP typically found intracellularly, the probe must have weak affinity for the nucleotides in the millimolar range.

Herein, we present a small terbium molecular probe that, by making use of this design strategy, can detect with different binding affinity ATP, ADP, and AMP in buffered aqueous media. It fulfills all nine of the user-defined requirements. More specifically, our probe takes advantage of three parameters: (1) the necessity, for practical application, to sensitize terbium luminescence with a nearby antenna whose triplet state is slightly higher in energy than the ^{5}D state of the lanthanide;[15,16] (2) the ability of adenosine to quench the luminescence of certain chromophores;[15,17−24] and (3) differential weak electrostatic interactions between a positively charged metal complex and negatively charged nucleotides.

Our probe, Tb-DOTAm-Phen (**1**), consists of a macrocyclic polyaminocarboxylate terbium complex conjugated to a phenanthridine antenna which is designed as follows (Figure 1): The macrocyclic DOTA-type chelate gives the complex its high thermodynamic and kinetic stability, thereby minimizing transmetalation with enzymes or peptides.[25] The three remaining ligand arms not conjugated to the phenanthridine antenna are converted to amides, thereby giving the complex an overall +3 charge. In the "off" state, that is in the absence of any nucleotide, excitation of the phenanthridine antenna at 346 nm, followed by intersystem crossing from its singlet state to its triplet state and subsequent energy transfer to the terbium ^{5}D state yields characteristic terbium phosphorescence with four sharp bands, the most intense centered at 545 nm (Figure 2).

In the presence of ATP, stacking of the purine nucleobase on the antenna favors photoelectron transfer (PET) from the adenosine to the phenanthridine, thereby preventing energy

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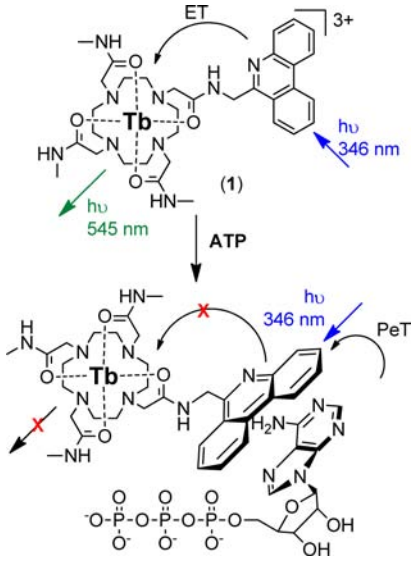


Figure 1. Chemical structure and mode of action of the ATP sensor Tb-DOTAm-Phen (**1**). In the absence of the nucleotide, excitation of the phenanthridine followed by energy transfer to the lanthanide enables bright luminescence centered at 545 nm. Stacking of the adenosine on the phenanthridine favors PET quenching of the antenna and, consequentially, also that of the Tb luminescence.

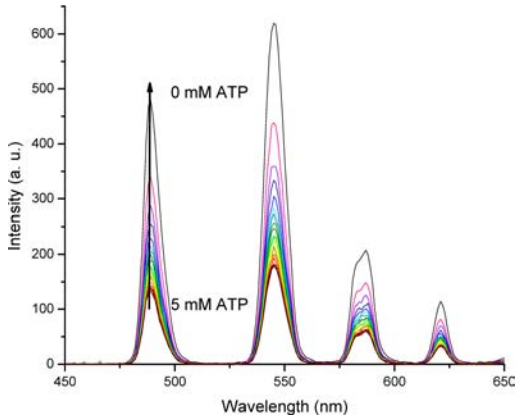
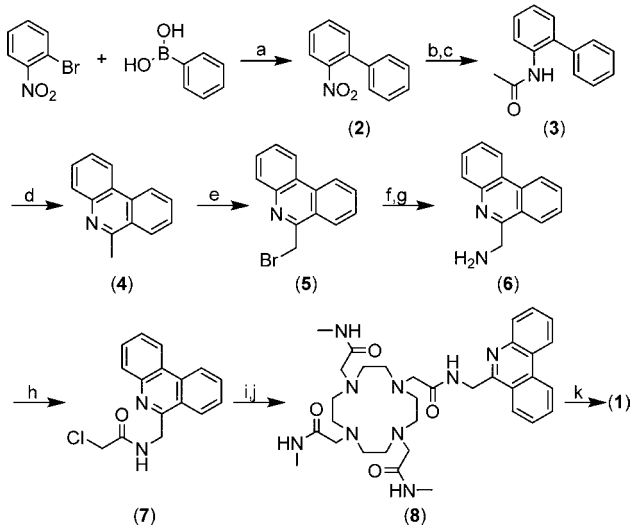


Figure 2. Time-delayed luminescence spectra of Tb-DOTAm-Phen·ATP. Experimental conditions: excitation at 346 nm, time-delay = 0.1 ms, [Tb-DOTAm-Phen] = 10 μM, water, [Tris] = 10 mM, pH 7.0, T = 20 °C.

transfer from the antenna to the lanthanide and consequently quenching terbium luminescence. All three adenosine nucleotides were expected to quench the probe's luminescence. Differentiation among ATP, ADP, and AMP is achieved via weak electrostatic interactions between the +3 charged terbium complex and the negatively charged nucleotides. The higher the number of phosphates, the more negative the charge of the nucleotide, and the higher its affinity for the positively charged probe. Although these interactions are weak in water, we postulated that they would be sufficient to distinguish the three nucleotides at their millimolar intracellular concentrations.[17−20,26]

The molecular probe Tb-DOTAm-Phen (**1**) was synthesized according to Scheme 1. Suzuki coupling of 2-bromonitrobenzene with phenylboronic acid yielded the nitrobiphenyl (**2**) which was subsequently reduced to an amine and acetylated to the acetamide (**3**). The amide was subsequently cyclized to 6-

Scheme 1. Synthesis of Tb-PhenDOTAm (1)[a]



[a]Experimental conditions: (a) $(PPh_3)_4Pd$, Na_2CO_3, H_2O, dimethoxyethane, reflux, 3 h; (b) Pd/C, H_2, CH_3OH, 12 h, rt; (c) $CH_3C(O)Br$, LiOH, CH_2Cl_2, 0 °C→rt, 2 h; (d) H_3PO_4, 150 °C, 2.5 h; (e) *N*-bromosuccinimide, $h\nu$, rt, 45 min; (f) NaN_3, acetone, rt, 1 h; (g) Pd/C, H_2, CH_3OH, 3.5 h, rt; (h) $ClCH_2C(O)Cl$, CH_2Cl_2, NEt_3, rt, 2 h; (i) cyclen, Cs_2CO_3, CH_3CN, DMA, 90 °C, 4 h; (j) 2-chloro-*N*-methylacetamide, Cs_2CO_3, CH_3CN, 80 °C, 34 h; (k) $TbCl_3$, LiOH, H_2O, 70 °C, 17 h.

methylphenanthridine (**4**) in the presence of polyphosphoric acid. Bromination under standard radical conditions, followed by reaction with sodium azide and reduction yielded phenanthridin-6-ylmethanamine (**6**) which was then conjugated to the macrocycle following standard conditions in two steps. First, reaction with chloroacetyl chloride yielded the chloride (**7**) which was then conjugated to the cyclen backbone under basic anhydrous conditions. Addition of the remaining three acetamide arms yielded the hygroscopic ligand DOTAm-Phen (**8**). The lanthanide complex, Tb-DOTAm-Phen (**1**), was formed quantitatively by refluxing the ligand and 1 equiv of $TbCl_3$ in water at neutral pH.

A typical time-gated titration of the probe with ATP is shown in Figure 2. The probe works most efficiently with ATP concentrations between 1 and 10 mM, concentrations which are within the intracellular range and which are therefore more relevant to drug screening. Moreover, the long luminescence lifetime of the terbium complex readily enables time-delayed experiments, whereby the background luminescence of the sample (enzyme, peptide and/or fluorescent drugs) can be readily gated out, thereby yielding more accurate measurements, not influenced by the autofluorescence of the system.

The Stern−Volmer plot of the time-gated luminescence of the terbium probe in the presence of the three adenosine nucleotides is shown in Figure 3. Each nucleotide quenches both terbium phosphorescence (Figure 2) and the antenna's luminescence (Supporting Information, Figure S2) efficiently. The fact that the antenna's luminescence is also quenched strongly suggests that terbium phosphorescence is quenched via a mechanism that involves PET from the purine to the phenanthridine (Figure 1). Importantly, the three nucleotides, ATP, ADP, and AMP, can be readily distinguished and present noticeably different Stern−Volmer constants, K_{SV} (Table 1). At the intracellularly relevant concentration of 1 mM, ATP

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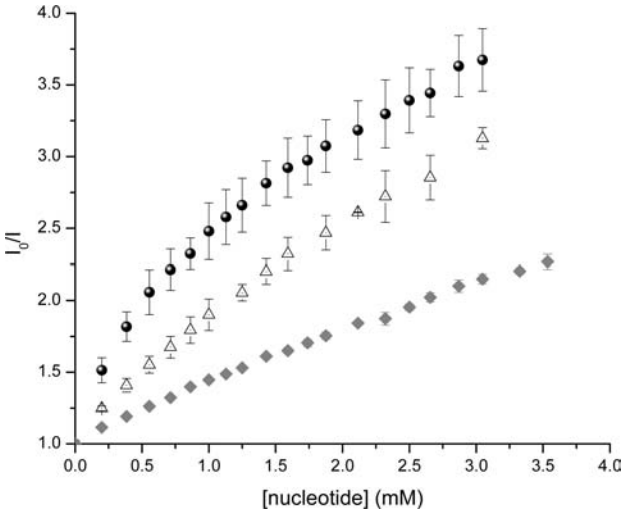

Figure 3. Stern–Volmer plot of the time-delayed luminescence of Tb-DOTAm-Phen (**1**) by the adenosine nucleotides ATP (filled circles), ADP (open triangles), and AMP (gray diamonds). Experimental conditions: excitation at 346 nm, emission integrated from 450 to 650 nm, time-delay = 0.1 ms, [Tb-DOTAm-Phen] = 10 μM, water, [Tris] = 10 mM, pH 7.0, T = 20 °C, error bars represent SD, n = 3.

Table 1. Stern–Volmer Constants of Tb-DOTAm-Phen (1) with Adenosine Nucleotides[a]

adenosine nucleotide	K_{SV}
ATP	1.28 ± 0.05
ADP	0.85 ± 0.02
AMP	0.397 ± 0.005

[a]Experimental conditions: excitation at 346 nm, emission integrated from 450 to 650 nm, time-delay = 0.1 ms, [Tb-DOTAm-Phen] = 10 μM, water, [Tris] = 10 mM, pH 7.0, T = 20 °C, error represents SD, n = 3.

quenches 59.7% of Tb-DOTAm-Phen's time-gated luminescence. At that same concentration, ADP quenches 47.4% of the probe's phosphorescence, whereas AMP quenches only 31.0%.

The Stern–Volmer relationship observed does not curve upward as the concentration of nucleotide increases. This indicates that quenching is either purely dynamic or purely static in nature, but not both.[27] Since quenching involves the adenosine moiety (phosphates and sugars do not quench terbium luminescence), a purely dynamic (collisional) mechanism would have resulted in identical Stern–Volmer constants for each nucleotide. The significantly different Stern–Volmer constants of the linear plots, however, strongly suggests formation of 1:1 complexes between the probe and the nucleotides, likely involving stacking of the purine base on the aromatic antenna in a manner similar to that observed by Lehn.[17−20] Differentiation between the three nucleotides is likely the result of electrostatic interactions between the positively charged terbium probe and the negatively charged nucleotides: the greater the negative charge of the nucleotide, the higher its Stern–Volmer constant. A similar terbium probe which is instead charged neutral is also efficiently quenched by adenosine nucleotides, but it does not differentiate ATP from ADP and AMP. Electrostatic interactions, albeit weak, have already been used in molecular recognition in water.[17−20,26]

In conclusion, Tb-DOTAm-Phen (**1**) is an efficient molecular probe for the time-gated molecular detection of adenosine nucleotides. Its ability to readily distinguish ATP from ADP and AMP under physiologically relevant conditions renders it an attractive candidate for direct monitoring of enzymatic reactions involving ATP hydrolysis such as those of kinases and ATPases. We are currently investigating its application in high-throughput screening and kinetic studies of inhibition of kinases.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, characterization of Tb-DOTAm-Phen (**1**), and fluorescence spectra of Tb-DOTAm-Phen·ATP titration. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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