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Turning an Aptamer into a Light-Switch Probe with a Single Bioconjugation

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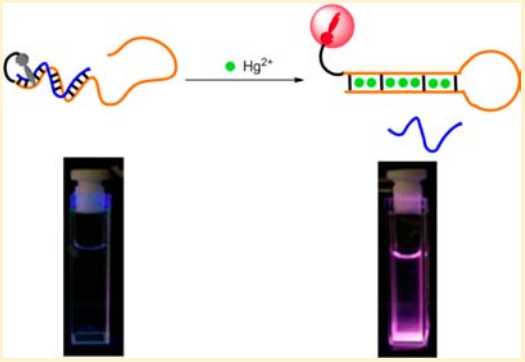
Supporting Information

ABSTRACT: We describe a method for transforming a structure-switching aptamer into a luminescent light-switch probe via a single conjugation. The methodology is demonstrated using a known aptamer for Hg^{2+} as a case study. This approach utilizes a lanthanide-based metallointercalator, Eu-DOTA-Phen, whose luminescence is quenched almost entirely and selectively by purines, but not at all by pyrimidines. This complex, therefore, does not luminesce while intercalated in dsDNA, but it is bright red when conjugated to a ssDNA that is terminated by several pyrimidines. In its design, the light-switch probe incorporates a structure-switching aptamer partially hybridized to its complementary strand. The lanthanide complex is conjugated to either strand via a stable amide bond. Binding of the analyte by the structure-switching aptamer releases the complementary strand. This release precludes intercalation of the intercalator in dsDNA, which switches on its luminescence. The resulting probe turns on 21-fold upon binding to its analyte. Moreover, the structure switching aptamer is highly selective, and the long luminescence lifetime of the probe readily enables time-gating experiments for removal of the background autofluorescence of the sample.



INTRODUCTION

Aptamers, single-stranded oligonucleotides that bind with high affinity and high selectivity to a selected target such as a protein, a small molecule, or a metal ion, are increasingly used in therapeutics, diagnosis, and detection.[1] Luminescent analogs have been prevalent for the latter two applications due to the widespread use of the technique. Indeed, significant work has already been published on the design of fluorescent aptamer-based probes, most commonly and successfully with approaches involving molecular beacons[2−5] and aptamer-based G-quadruplex systems.[6−9] Unfortunately, this technology requires multiple, often difficult, bioconjugation steps and it remains ill-adapted to multiplex detection and applications in complex aqueous or biological media. The first limitation with regard to synthesis can, in theory, be met with the use of light-switch DNA intercalators, molecules whose quantum yields vary greatly upon intercalation between base pairs. However, intercalators have so far only been used in "label-free" approaches, which consist of simply mixing the intercalators with the aptamer. In each case, most of the dyes are intercalated both in the absence and in the presence of the analyte. Consequently, the responses observed were small and also turn-off.[10−19] The other two limitations, multiplex detection and detection in a complex aqueous sample, could in theory be resolved with a lanthanide-based probe.[20] The narrow emission bands and long luminescence lifetimes of lanthanide complexes make them uniquely suited for multiplex detection and quantitative analysis in complex media via time-gated measurements. Herein we report such a light-switch lanthanide-based aptamer probe, with substantial turn-*on* and a long luminescence lifetime in the millisecond range. Its synthesis, unlike that of most aptamer-based probes,[2−5] requires only one bioconjugation. In comparison, other systems need an individual conjugation for both the fluorophore and the quencher. The application of this methodology can readily be expanded to other substrates and for multiplex detection. The key to multiplex detection is simply to ensure that each aptamer probe for each analyte has a unique reporter strand sequence and makes use of a distinct lanthanide ion such as terbium, europium, thulium, dysprosium, or samarium. Since the emission spectra of lanthanide ions mostly do not overlap, they can be readily used for multiplex detection. Multiplex detection with lanthanide ions has been extensively reported by our group and others.[21−24]

In terms of design, we postulated that the first of the aforementioned three limitations, the number of bioconjugation steps, could be resolved via a single conjugation of a light-switch DNA intercalator to a structure-switching aptamer. Structure-switching aptamers change their structure upon binding to their cognate target, most often via the release of a shorter complementary strand.[25,26] Judicious conjugation of an intercalating dye to either strand of such an aptamer/complement duplex ensures that the dye is completely intercalated only in the absence of the analyte, but is not

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intercalated in the presence of the analyte (Figure 1). If the intercalating dye itself is a light-switch, then the aptamer probe

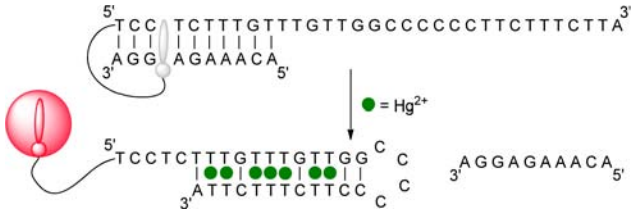

Figure 1. Proposed mode of action of aptamer light-switch, Eu-AptaSwitch (**1**). In the absence of Hg^{2+}, the Eu^{III} complex intercalates in dsDNA. Photoelectron transfer from the purines to the phenanthridine antenna quenches the luminescence of Eu^{III}. Binding of Hg^{2+} by the aptamer releases the short complementary strand, thereby preventing intercalation of the Eu^{III} complex and turning on its luminescence.

will also be one. The probe is thus devised to have a higher response than label-free intercalator-based systems,[13] while at the same time limiting bioconjugation to a single DNA strand. In this design, use of a dye that only luminesces while intercalated will result in a turn-*off* probe, while one that is quenched by stacking with base pairs will result in a turn-*on* probe. Standard intercalators, which are luminescent only when intercalated, would thus turn-*off*: in the absence of the analyte they would intercalate and be luminescent; whereas in the presence of the analyte they would not intercalate and not luminesce. Since turn-*on* probes are generally preferred for practical applications, this design excludes the use of common light-switch intercalators such as dppz-based ruthenium[27] or osmium complexes,[28] platinum complexes,[29,30] or organo-intercalators such as ethidium bromide.[31] Instead, we postulated that the desired response could be achieved with lanthanide complexes whose luminescence is nearly completely quenched upon intercalation in double-stranded DNA (dsDNA). Our complex works as a turn-*on* probe because its response to intercalation is reversed: it is not luminescent when intercalated (i.e., in the absence of the analyte) but is luminescent when not intercalated (i.e., in the presence of the analyte). A further advantage of using lanthanides is that they also solve the other two limitations mentioned above of current molecular beacons. The long luminescence lifetimes of lanthanide complexes, typically in the millisecond range for Eu^{III} and Tb^{III}, enable facile time-gating experiments whereby the background autofluorescence of the sample is removed. Lanthanides have multiple emission bands which are also narrow. These narrow emission bands with limited overlap further enable simultaneous detection of multiple analytes in the same sample. If bands which do not overlap at all are chosen, multiple probes with different lanthanides can be used simultaneously in the same medium to test several analytes.

This design thus requires a lanthanide complex not only capable of intercalating in dsDNA, but whose luminescence is substantially affected by the oligonucleotide. Our group[21,32,33] and Parker's[34−37] have previously demonstrated that the luminescence of phenanthridine-based terbium and europium complexes can be nearly completely quenched upon intercalation in dsDNA. This effect, which is likely the result of photoelectron transfer from stacked purines to the excited state of the chromophore, was the basis behind the recent design of our probe for GTP and ATP detection.[21,33] Importantly, although both purines efficiently quench the lanthanide-centered emission of this complex, neither of the two pyrimidines does. Our probe, Eu-AptaSwitch (**1**), was thus constructed by conjugating the phenanthridine-based lanthanide complex to the DNA strand of the structure-switching aptamer that is mostly composed of thymidines and cytidines (Figure 2). The shorter complementary strand is thus primarily

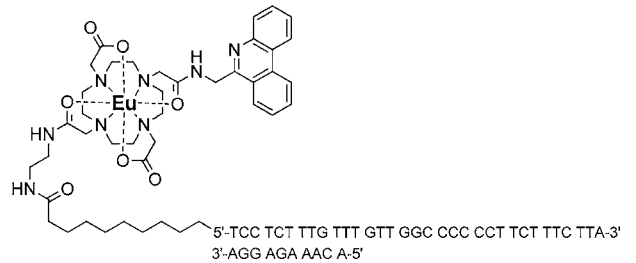

Figure 2. Chemical structure of Eu-AptaSwitch (**1**). The Eu^{III} complex is conjugated to the aptamer via a C9 alkyl chain. The two oligonucleotides are annealed prior to use.

composed of adenosines and guanosines. The probe was designed to function as follows (Figure 1). In the absence of the analyte, i.e., when the phenanthridine is intercalated in the dsDNA, the probe is in the *off* mode. Photoelectron transfer (PeT) from the stacked purine bases quenches the lanthanide luminescence. Binding of the analyte by the aptamer releases the short complementary strand. At this point, intercalation and quenching of the phenanthridine by purines are no longer possible. Therefore, the luminescence of the lanthanide complex turns *on*.

A note on the stability of the metal complex used for detection and diagnosis. The probe Eu-AptaSwitch (**1**) incorporates a macrocyclic polyaminocarboxylate ligand which renders the complex both thermodynamically stable and kinetically inert. Lanthanide complexes used for biological and medical applications must be stable and inert so as to minimize the risk of trans-metalation with Ca^{2+} and trans-ligation of the lanthanide with endogenous ligands such as phosphates.[38] Although more difficult to synthesize than their linear analogs, macrocyclic DOTA-type chelates, such as the one used in this study, are more appropriate for detection of analytes in biological or environmental aqueous systems due to their kinetic inertness. Furthermore, the overall positively charged lanthanide complexes of DOTA tetraamide (DOTAm) ligands are kinetically more inert than DOTA analogues as a result of the decreased basicity of the nitrogen atoms.[39]

As a proof of principle, we applied our concept to a structure-switching aptamer previously reported for Hg^{2+}.[40] This aptamer makes use of the ability of thymidines to coordinate Hg^{2+} in a T-Hg^{2+}-T fashion. Therefore, the lanthanide complex was conjugated to the thymidine-rich strand that binds Hg^{2+} as opposed to its shorter complementary strand. Note that in order to further increase the turn-*on* response of the probe, some of the purine bases in the mercury-binding strand reported by Lu[40] were replaced by pyrimidines (third and fifth positions, A to C and G to C, respectively). We anticipate that this design can be applied to any structure-switching aptamer, regardless of how the aptamer binds its analyte.

RESULTS AND DISCUSSION

The molecular probe Eu-AptaSwitch (**1**) was synthesized according to Scheme 1 from three advanced intermediates:

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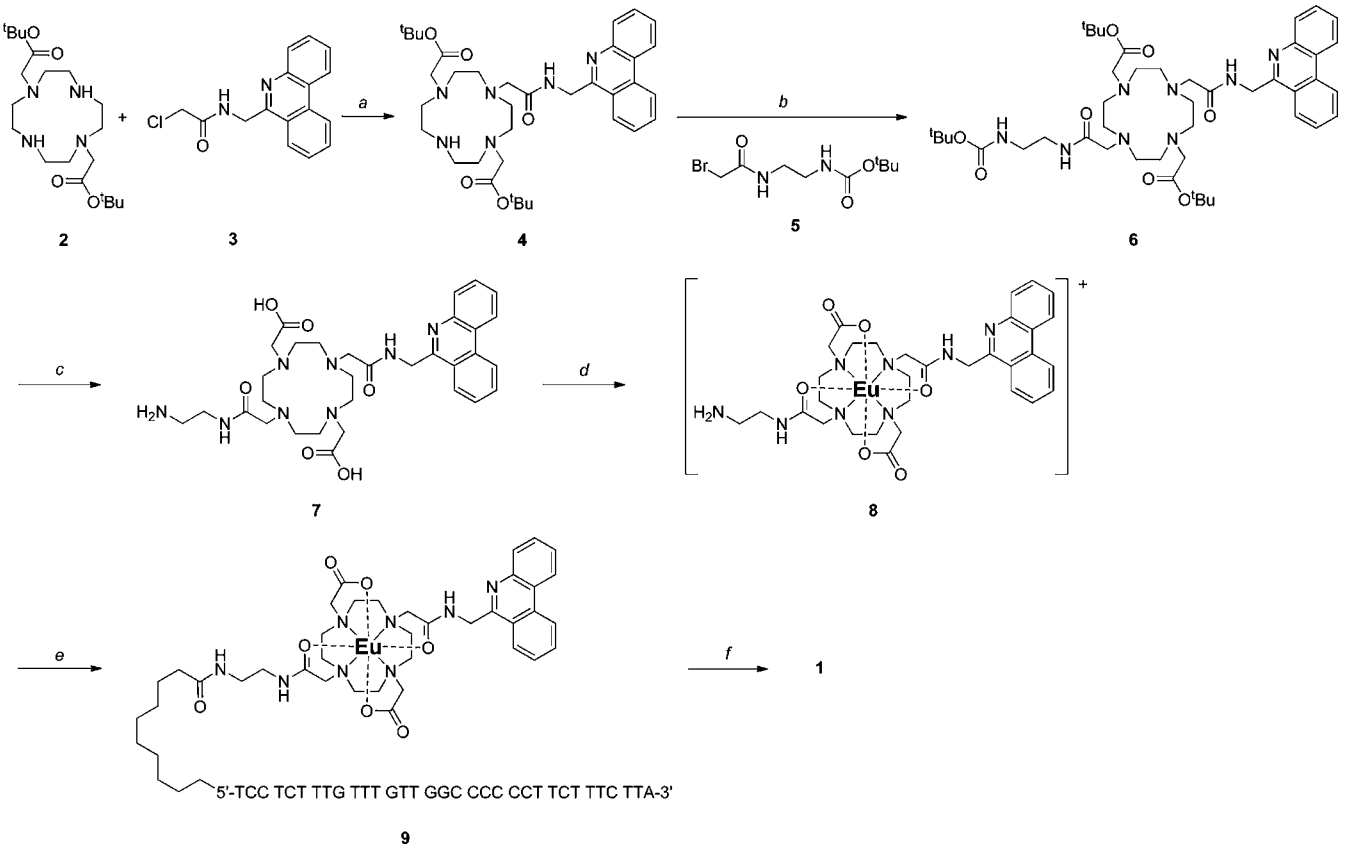
Scheme 1. Synthesis of Eu-AptaSwitch (1)[a]



[a]Experimental conditions: (a) Et_3N, $CHCl_3$, 62 °C, 18 h; (b) Cs_2CO_3, CH_3CN, 82 °C, 18 h; (c) HCl, CH_3OH, rt, 5 d; (d) $EuCl_3{\cdot}6H_2O$, LiOH, H_2O, pH 8, 70 °C, 76 h; (e) conjugation of the aptamer to the DADE linker (on solid support) in phosphate buffer, pH 8, 15 h; deprotection: 30% NH_3, 65 °C, 5 h; (f) complementary strand, PBS, 95 °C for 15 min, slow cooling.

DO2A^{t}Bu (**2**),[41] the phenanthridine acetamide arm (**3**),[33] and the bridging arm (**5**). The syntheses of the former two were previously reported in the literature. Coupling of the phenanthridine arm (**3**) to DO2A^{t}Bu (**2**) followed by that of the bridging arm (**5**). yielded the fully protected ligand (**6**). Treatment under acidic conditions deprotected the acids and the amine simultaneously, thereby yielding the free ligand which was metalated with Eu^{III} under slightly basic aqueous conditions. Note that as for any DOTA-type complex, long reaction times are needed to ensure full complexation of the lanthanide by the macrocycle.[42] Advantageously, the stability of the resulting amine-terminated complex enables subsequent conjugation to any molecule containing a carboxylate. For our intended application, the complex was reacted with the DADE-terminated aptamer oligonucleotide while on solid support. DADE (decanoic acid diester) is a commercially available preactivated carboxyl linker that enables facile conjugation to the Eu^{III}-complex all the while being long enough for the phenanthridine to intercalate in the dsDNA (Figure 2). Cleavage of the bioconjugate from its solid support, followed by deprotection of the bases and purification by reverse phase high pressure liquid chromatography (RP-HPLC), yielded the Eu^{III} ssDNA conjugate (**9**) that was annealed with the short complementary strand to yield the luminescent probe Eu-AptaSwitch (**1**). Notably, bioconjugation was only possible with a lanthanide complex containing an amine and a DNA terminated with an activated carboxylic acid. Our multiple attempts at reversing the functional groups, that is, to conjugate a Eu complex with a pendant acid or activated acid to an amine-terminated oligonucleotide, were all unsuccessful, regardless of the coupling agent used. This also highlights the difficulty of conjugating metal complexes to DNA.[2−4,43−54] The synthesis described above should be applicable to the conjugation of other types of metal complexes to DNA for therapeutic and diagnostic purposes.

The time-gated emission profile of the probe in the absence and presence of its analyte is shown in Figure 3. As expected, annealing the Eu-ssDNA (**9**) conjugate with the complementary strand quenches most of the metal-centered luminescence; the luminescence of the annealed Eu-AptaSwitch (**1**) is only 4% that of the ssDNA conjugate (**9**). This result is consistent with intercalation of the phenanthridine in the dsDNA; resulting photoelectron transfer from the purine bases to the antenna quenches the lanthanide-based emission. Importantly, the luminescence of Eu^{III} is fully recovered upon addition of Hg^{2+}. This observation is in agreement with binding of Hg^{2+} by its structure-switching aptamer concomitant with release of the complementary strand such that the phenanthridine can no longer intercalate in dsDNA. Since PeT from purine bases to the antenna is no longer possible, luminescence of Eu^{III} is recovered. This design enables not only a complete recovery of the lanthanide emission, but also a substantial 21-fold turn-*on* which is comparable to that of other light-switch probes. Support for our proposed mechanism of action comes from three observations. First, the luminescence of the unconjugated Eu complex (**8**) is identical to that of the Eu-ssDNA conjugate

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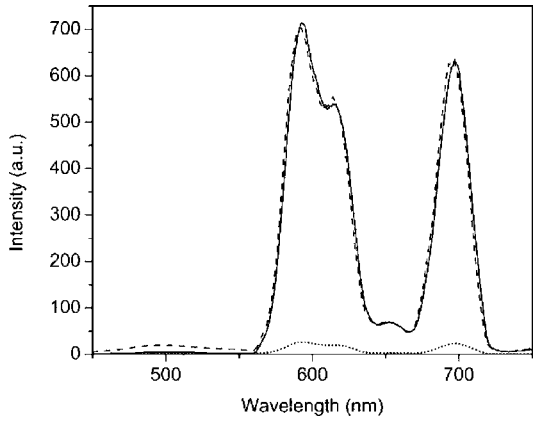

Figure 3. Time-gated emission profiles of Eu-DNA conjugate (**9**) (solid line), Eu-AptaSwitch (**1**) (dotted line), Eu-AptaSwitch·Hg^{2+}(dashed line). Experimental conditions: [Eu-DNA conjugate] = [Eu-AptaSwitch] = 20 μM, [Hg^{2+}] = 250 μM, PBS buffer, pH = 7.0, T = 20 °C, time-delay = 0.1 ms, slit widths = 20 nm, excitation at 271 nm.

(**9**); they both have the same excitation and emission profiles and the same quantum yield. Second, the luminescence of the probe after addition of Hg^{2+} is also identical to that of the Eu-ssDNA conjugate (**9**, same excitation and emission profiles, and same quantum yield). Last, heating the probe past its melting point in the absence of Hg^{2+}, which also liberates the Eu-complex by unfolding the DNA, also turns the Eu luminescence back on. The combination of these three observations strongly supports our proposed mechanism.

Notably, such a turn-*on* is readily observable with the naked eye upon illumination with a standard portable UV-lamp (Figure 4). The characteristic luminescence of the probe is only

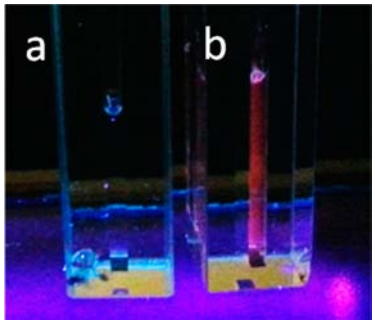

Figure 4. Luminescence of Eu-AptaSwitch (**1**) upon excitation with a portable UV lamp in the absence (a) and presence (b) of 10 equiv of Hg^{II}. Experimental conditions: PBS, pH 7.0, rt, $\lambda_{excitation}$ = 254 nm.

apparent in the presence of 10 equiv of Hg^{2+}. Although Eu emits in the red, the probe appears purple to the naked eye. Indeed, pure Eu-centered emission can only be observed with time-gating, as was performed in all of the titrations. Without time-gating and with the naked eye, one sees a combination of the Eu-centered emission (red) and the left-over emission from the phenanthridine antenna which is not transferred to the lanthanide (blue). The combination of the red Eu emission and the blue antenna emission give the overall purple color seen in the photo taken with a standard hand-held camera. This light-switch response thus bodes well for future application of this technology to the detection of other analytes without the need for a spectrophotometer. Moreover, the probe detects its analyte rapidly; the luminescence turn-*on* is observed in 1 min after adding mercury at room temperature.

Time-gated luminescence titration of Eu-AptaSwitch (**1**) in the presence of Hg^{2+} is shown in Figure 5. Turn-*on* in the

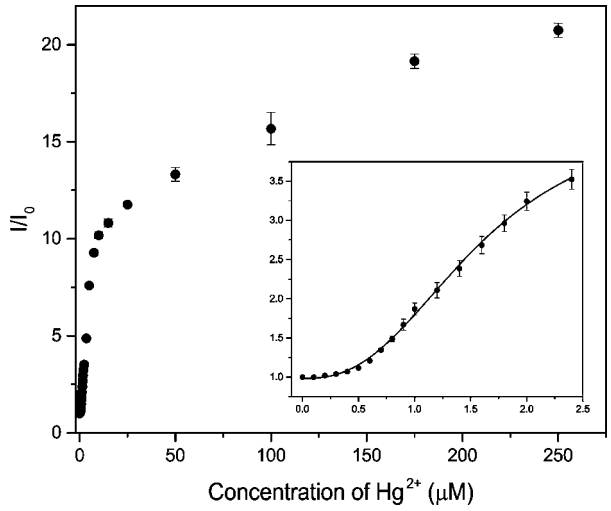

Figure 5. Time-gated luminescence of Eu-AptaSwitch (**1**) with increasing concentration of Hg^{2+}. Experimental conditions: [Eu-AptaSwitch] = 20 μM, PBS buffer, pH = 7.0, T = 20 °C, time-delay = 0.1 ms, slit widths = 20 nm, excitation at 271 nm, emission integrated from 450 to 750 nm, error bars represent SD, n = 3. Inset: data points at low concentrations of Hg^{2+}.

presence of low concentration of mercury is shown in the inset. The very long lifetime characteristic of Eu^{III}-centered luminescence, a requirement for time-gating experiments, is ideally suited for titrations in complex aqueous media. Note that, as for Lu's probe,[40] the titration curve of Eu-AptaSwitch can be fitted with a Hill coefficient of 2, suggesting a cooperative binding of Hg^{2+} to the aptamer.

Importantly, conjugating the Eu complex to the aptamer does not changes its affinity for Hg^{2+}, nor its selectivity over competing divalent metal. The selectivity of the probe toward the alkali earth and transition metals Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+}, and Cu^{2+} is shown in Figure 6. The probe does not turn on upon addition of an excess of each competing metal ions. Importantly, the presence of these metal ions also does not influence the response of the probe toward Hg^{2+}. The light-switch probe (**1**) remains highly selective, as was the original probe reported by Lu and co-workers. The fact that the metallointercalator does not influence the response of the aptamer bodes well for other applications.

A distinct advantage of lanthanides in the design of probes is their very long luminescence lifetime, in the millisecond range, that is uniquely suited for applications in complex aqueous media. With such long lifetimes, the background autofluorescence of the sample can readily be removed with time-gating experiments allowing for more accurate measurements at low turn-*on*. Indeed, as can be seen in Figure 7, the Eu-AptaSwitch probe is also efficient in Human serum. A lower turn-*on* is observed for a given concentration of Hg^{2+} in serum as compared to phosphate-buffered saline. This difference could be due to partial quenching of the lanthanide emission by serum protein and/or to binding of Hg^{2+} by serum proteins, which decreases the concentration of Hg^{2+} available to the probe.

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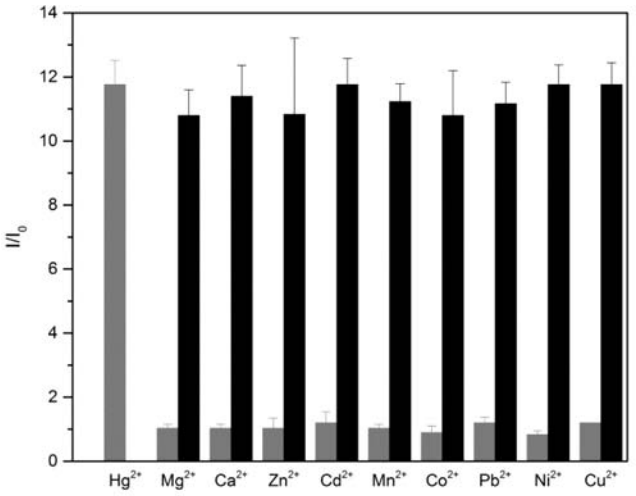

Figure 6. Selectivity of Eu-AptaSwitch (**1**). Gray bars represent the luminescence response after adding 250 μM metal ion (except Hg^{2+}, which is only 25 μM). Black bars represent the luminescence response after adding 25 μM of Hg^{2+} to the probe containing 250 μM metal ion. Experimental conditions: [Eu-AptaSwitch] = 20 μM, PBS buffer, pH = 7.0, T = 20 °C, time-delay = 0.1 ms, excitation at 271 nm, emission at 592 nm, error bars represent SD, n = 3.

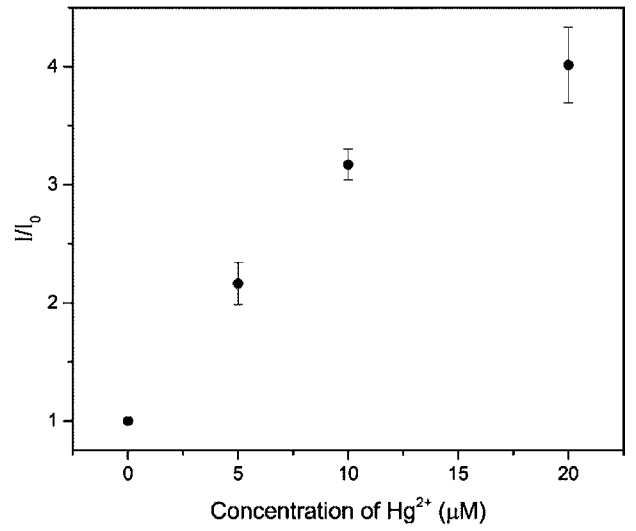

Figure 7. Time-gated luminescence of Eu-AptaSwitch (**1**) in human serum with increasing concentration of Hg^{2+}. Experimental conditions: [Eu-AptaSwitch] = 20 μM, T = 20 °C, time-delay = 0.1 ms, excitation at 271 nm, emission at 592 nm, error bars represent SD, n = 3.

CONCLUSION

In conclusion, a novel approach to the design of luminescent aptamer probes that makes use of a light-switch lanthanide intercalator is reported. Compared to beacon-type approaches, this approach only requires a single conjugation, and therefore enables a more facile synthesis. As opposed to the label-free approaches that also employ DNA intercalators, our approach yields not only a turn-*on* response but also a substantially greater response. Moreover, the structure switching aptamer ensures the selectivity of the probe, while the long luminescence lifetime and the narrow emission bands of lanthanide metals are expected to facilitate multiplex detection in a single, complex aqueous sample.

EXPERIMENTAL PROCEDURES

General Considerations. Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. DNA was purchased from Trilink Biotechnologies. Water was distilled and further purified by a Millipore cartridge system (resistivity 1.8 × 10^7 Ω). Flash chromatography was performed on Salicycle Silica Gel (230–400 mesh) or Brockmann activated aluminum oxide (neutral, 60 mesh). ^{1}H NMR spectra were recorded on a Varian 300 or Varian 500 at 300 or 500 MHz respectively, and ^{13}C NMR spectra on a Varian 300 at 75 MHz at the LeClaire-Dow Instrumentation Facility at the Department of Chemistry at the University of Minnesota, Twin-Cities. The residual solvent peak was used as an internal reference. Data for ^{1}H NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet), integration, coupling constant (Hz). Data for ^{13}C NMR are reported in terms of chemical shift (δ, ppm). Mass spectra (LR = low resolution; HR = high resolution; ESI = electrospray ionization) were recorded on a Bruker BioTOF II at the Waters Center of Innovation for Mass Spectrometry at the Department of Chemistry at the University of Minnesota, Twin-Cities. Liquid Chromatography–Mass Spectrometry, HPLC-ESI-MS were performed on an Agilent 1100 capillary HPLC-ion trap mass spectrometer (Agilent Technologies) at the Mass Spectrometry facility at the Masonic Cancer Center at the University of Minnesota. A Zorbax 300SB-C8 column (150 mm × 0.3 mm, 3.5 μm, Agilent Technologies) was eluted at a flow rate of 9 μL/min with a gradient of 15 mM ammonium acetate and 5–16% acetonitrile in over 14 min and then up to 90% over 2 min. The column was maintained at 40 °C. The mass spectrometer was operated in the negative mode for the analyses of oligonucleotide conjugates. UV–vis data was obtained on a Cary Bio 100 UV–vis spectrophotometer. Fluorescence data was acquired on a Varian Cary Eclipse Spectrophotometer using a quartz cell with a path length of 10 mm, excitation slit width of 20 nm, and emission slit width of 20 nm at 20 °C.

Di-*tert*-butyl 2,2′-(4-(2-oxo-2-((phenanthridin-6-ylmethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (4). Di-*tert*-butyl 2,2′-(1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (**2**) and 2-chloro-*N*-(phenanthridine-6-ylmethyl)acetamide (**3**) were synthesized according to previously published procedures.[21,41] A solution of 2-chloro-*N*-(phenanthridine-6-ylmethyl)acetamide (**3**) (101 mg, 0.355 mmol) in $CHCl_3$ (10 mL) was added to a solution of di-*tert*-butyl 2,2′-(1,4,7,10-tetraazacyclododecane-1,7-diyl)-diacetate (**2**) (285 mg, 0.711 mmol) and triethylamine (59 μL, 0.48 mmol) in $CHCl_3$ (10 mL) under N_2 (*g*). The reaction mixture was heated to reflux under N_2 (*g*) for 20 h then allowed to cool to room temperature. The product was purified by chromatography over alumina eluting with a gradient of CH_2Cl_2 to 10% CH_3OH/90% CH_2Cl_2. The product was obtained as a yellow oil (200 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.65 (d, J = 8.5 Hz,1H), 8.57 (d, J = 8.0 Hz, 1H), 8.36 (d, J = 7.5 Hz, 1H), 8.16 (m, 1H), 7.88 (t, J = 7.5 Hz, 1H), 7.73 (m, 3H), 5.14 (s, 2H), 2.91 (m, 22H), 1.30 (s, 18H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.8, 170.5, 143.4, 133.0, 131.2, 129.6, 129.0, 128.1, 127.3, 126.5, 125.6, 124.5, 124.3, 122.7, 122.4, 81.5, 58.0, 54.6, 52.5, 49.8, 47.9, 43.1, 28.1; HRMS (ESI) calc for $[C_{36}H_{52}DN_6O_5]^+$ ($[M+D]^+$): m/z 649.4056, found: 649.4070.

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***tert*-Butyl (2-(2-bromoacetamido)ethyl)carbamate (5).** A solution of K_2CO_3 (202 mg, 1.46 mmol) in water (20 mL) and a solution of bromoacetyl bromide (132 μL, 1.51 mmol) in CH_2Cl_2 (20 mL) were simultaneously added over 30 min to a solution of *tert*-butyl (2-aminoethyl)carbamate (200 μL, 1.26 mmol) in CH_2Cl_2 (50 mL) cooled to 0 °C. The reaction mixture was subsequently allowed to warm to room temperature and stirred for an additional 1.5 h. The reaction mixture was rinsed with water (2 × 50 mL) and saturated NaCl (*aq*) (1 × 50 mL), dried over magnesium sulfate, and filtered. The solvents were removed under reduced pressure to yield the product **5** as a white solid (315 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.21 (s, 2H), 3.61 (t, J = 6.6 Hz, 2H), 3.06 (m, 2H), 1.39 (s, 1H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 166.5, 156.5, 79.7, 53.2, 39.5, 41.2, 28.0; HRMS (ESI) calc for $C_9H_{18}NaN_2O_3Br$ ($[M + Na]^+$): m/z 303.0315, found: 303.0377.

Di-*tert*-butyl 2,2′-(4-(2-((2-((*tert*-butoxycarbonyl)-amino)ethyl)amino)-2-oxoethyl)-10-(2-oxo-2-((phenanthridin-6-ylmethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (6). Di-*tert*-butyl 2,2′-(4-(2-oxo-2-((phenanthridin-6-ylmethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (**4**, 50 mg, 0.077 mmol) and Cs_2CO_3 (50 mg, 0.15 mmol) were added to a solution of *tert*-butyl (2-(2-bromoacetamido)ethyl)carbamate (**5**, 43 mg, 0.15 mmol) in acetonitrile (25 mL). The reaction mixture was heated for 18 h at 82 °C. The reaction mixture was then allowed to cool to room temperature and the solvents were removed under reduced pressure yielding the protected ligand **6** as a yellow oil which was used immediately in the next step. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.79 (d, J = 8.1 Hz, 1H), 8.71 (d, J = 7.8 Hz, 1H), 8.30 (d, J = 8.1 Hz, 1H), 8.09 (d, J = 7.8 Hz, 1H), 7.93 (t, J = 8.1 Hz, 1H), 7.75 (m, 3H), 4.34 (s, 2H), 3.25 (m, 4H), 2.71 (m, 24H), 1.43 (s, 18H), 1.36 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.2, 175.5, 174.5, 174.1, 172.0, 144.3, 134.5, 132.5, 130.4, 130.1, 129.1, 128.6, 126.9, 125.7, 125.6, 124.0, 123.6, 118.5, 82.1, 80.1, 80.0, 64.5, 62.6, 59.8, 59.4, 57.7, 57.2, 55.9, 55.4, 53.7, 44.6, 41.8, 41.1, 40.1, 28.9, 28.7, 28.5, 28.4, 28.3; HRMS (ESI) calc for $[C_{45}H_{68}NaN_8O_8]^+$ ($[M + Na]^+$): m/z 871.5052, found: 871.5034.

2,2′-(4-(2-((2-Aminoethyl)amino)-2-oxoethyl)-10-(2-oxo-2-((phenanthridin-6-ylmethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetic acid (7). Di-*tert*-butyl 2,2′-(4-(2-((2-((*tert*-butoxycarbonyl)amino)-ethyl)amino)-2-oxoethyl)-10-(2-oxo-2-((phenanthridin-6-ylmethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetate (**6**) (65 mg, 0.077 mmol) was dissolved in CH_3OH (5 mL) and concentrated HCl (*aq*) (1 mL) was added. The reaction was stirred for 5 days at room temperature after which the volatiles were removed under reduced pressure. The resulting oil was washed with cold CH_3OH (8 × 5 mL). Yield (42 mg, 79% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (d, 2H), 8.17 (s, 1H), 7.81 (m, 5H), 4.42 (s, 2H), 2.40−3.80 (m, 28H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.2, 174.7, 158.3, 156.6, 141.6, 131.6, 130.8, 130.3, 129.2, 128.0, 127.4, 125.0, 124.4, 124.0, 123.7, 123.2,122.4, 63.5, 60.9, 56.7, 56.2, 52.3, 48.4, 45.5, 42.7, 39.5, 39.2, 38.0, 37.7, 36.6, 36.4; HRMS (ESI) calc for $[C_{32}H_{41}Na_2LiN_8O_6]^+$ ($[M+2Na+Li]^+$): m/z 687.4049, found: 687.4069. See Supporting Information Figure S1 for ^{1}H NMR spectra of the free ligand **7**.

Eu-DO2A-Phen-Amine (8). 2,2′-(4-(2-((2-Aminoethyl)-amino)-2-oxoethyl)-10-(2-oxo-2-((phenanthridin-6-ylmethyl)-amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)diacetic acid (**7**) (42 mg, 66 μmol) was dissolved in milliQ water (5 mL) and magnetically stirred. $EuCl_3{\cdot}6H_2O$ (25 mg, 66 μmol) was added to the reaction mixture and the pH was adjusted to 8 with LiOH. The reaction mixture was stirred for 76 h at 70 °C, filtered, and the solvents were removed under reduced pressure. See Supporting Information Figure S2 for the ^{1}H NMR spectra and Supporting Information Figure S3 for the HPLC trace of the complex. HRMS (ESI) calc for $[C_{33}H_{46}EuN_8O_6]^+$ ($[M + H]^+$): m/z 787.2798, found: 787.2731.

Eu-DNA Conjugate (9). Eu-DO2A-Phen-Amine (**8**, 2 mg, 5 μmol) was dissolved in phosphate buffer saline (80 μL) at pH 8. After stirring for 5 min, the amine was added to the protected aptamer (50 nmol) on beads in DMF (20 μL). The reaction mixture was mixed slowly for 15 h. The supernatant was decanted and the beads were rinsed with mQ water (2 × 1 mL), CH_3OH (2 × 1 mL), and CH_3CN (2 × 1 mL). The beads were dried under reduced pressure for 15 min and 30% NH_3 (*aq*) (750 μL) was added. The mixture was then heated at 65 °C for 5 h. The mixture was decanted and the beads were rinsed with water. The combined supernatants were concentrated under reduced pressure. The DNA conjugate was purified by high performance liquid chromatography (HPLC) using a Zorbax 300SB-C8 9.4 × 250 mm, 5 μm Agilent Technologies) column. Solvents were eluted at a flow rate of 2.5 mL/min with a gradient of 12−14% acetonitrile in 15 mM ammonium acetate over 35 min and 90% over 2 min. Conjugates were desalted by passing through NAP-5 columns and the eluent was concentrated. Capillary HPLC-MS $(ESI)^-$ calc for the Eu-DNA conjugate **9** ($[M\text{-}H]^-$): m/z 10938.7; found: 10938.6. See Supporting Information Figure S4 for the LC-MS and ESI-MS of the Eu-DNA conjugate probe **9**.

Eu-AptaSwitch (1). Eu-DNA conjugate (**9**) was heated with a molar equivalent of the complementary strand (100 μM) in PBS buffer (pH 7.0) at 95 °C for 15 min and allowed to cool to room temperature over 15 h.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectrum of the ligand DO2A-Phen-Amine (**7**) and the complex $[EuDO2A\text{-}Phen\text{-}Amine]^+$ (**8**), HPLC-ESI^--MS spectrum of Eu-DNA Conjugate (**9**). 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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ABBREVIATIONS

DNA, deoxyribonucleic acid; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; GTP, guanosine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; CTP, cytosine triphosphate; PeT, photoelectron transfer; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; SD, standard deviation; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; ESI, electrospray ionization; HPLC, high pressure liquid chromatography; DMF, dimethylformamide; UV−vis, ultraviolet−visible light; PBS, phosphate-buffered saline

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