Luminescent Probes for Ultrasensitive Detection of Nucleic Acids

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Novel amino-reactive derivatives of lanthanide-based luminescent labels of enhanced brightness and metal retention were synthesized and used for the detection of cDNA oligonucleotides by molecular beacons. Time-resolved acquisition of the luminescent signal that occurs upon hybridization of the probe to the target enabled the avoidance of short-lived background fluorescence, markedly enhancing the sensitivity of detection, which was less than 1 pM. This value is about 50 to 100 times more sensitive than the level achieved with conventional fluorescence-based molecular beacons, and is 10 to 60 times more sensitive than previously reported for other lanthanide-based hybridization probes. These novel luminescent labels should significantly enhance the sensitivity of all type of nucleic acid hybridization probes, and could dramatically improve the detection limit of other biopolymers and small compounds that are used in a variety of biological applications.

INTRODUCTION

Some lanthanide chelates possess the unique spectral property known as luminescence (*1*−*9*). The principle features of luminescence are long-lived excited states (in the microsecond to millisecond range) and narrow emission spectra. Temporal and spectral gating enables unusually sensitive detection of lanthanide emission, even in samples that exhibit significant, short-lived autofluorescence (e.g., biological specimens or tissues). These compounds are therefore potentially useful in a wide variety of technical and biological tasks, such as tracing analysis, immunoanalysis, tissue-specific imaging, and detection of single molecules in living cells. Despite the benefits offered by this technique, its widespread use is impeded by the limited choice of instrumentation and the extremely high price of commercial probes. Yet, existing lanthanide-based probes could be improved to meet the requirements of new challenging applications.

Due to their very low absorbance ($\varepsilon < 1$ M^{-1} cm^{-1}), lanthanide ions have to be "pumped", which is achieved by tethering the lanthanide ion (through chelation) to an appropriate organic fluorophore (antenna). In such constructs, the energy absorbed by the antenna is transferred radiationlessly to the lanthanide, which finally emits the light. The chelating group also shields the lanthanide ion from the quenching effects of the medium, increasing the quantum yield of the probe. Development of new luminescent probes is challenging, since the transfer of energy from the antenna to the lanthanide is complex (a process not yet well understood) and very sensitive to subtle structural variations in the fluorophore. Another challenge is the necessity of combining three functional units within the same reporting probe: an antenna, a chelated lanthanide, and a cross-linking group (for attachment to the biomolecule of interest). This requires a complex synthetic strategy, eventually leading to compounds whose size often exceeds 1000 Da.

Two commonly used classes of lanthanide chelates are diethylenetriaminepentaacetic acid (DTPA) and tetraethylene-tetraminohexaacetic acid (TTHA). These chelates attached to 7-animo quinolones (*10*−*17*) are known as DTPA and/or TTHA-cs124 derivatives. The advantage of these classes of compounds is their high quantum yield, high solubility in water, and the possibility of introducing chemical modifications in the fluorophore to spectrally optimize the transfer of energy to the lanthanide and to enable the attachment of a cross-linking group. A number of methods for the conjugation of these chelates to biomolecules have been suggested. One of them is to use the dianhydride form of DTPA, in which one of the anhydrides modifies the amino group of the chromophore, while the other anhydride reacts with amino group of the biomolecule (*17*). Even though this approach is technically simple, it raises concerns about the side reactions (modification of other nucleophilic groups) due to the high reactivity of anhydrides. The second approach takes advantage of the conjugation of one of the DTPA anhydride groups with the cs124 moiety, followed by reaction of the remaining anhydride with the diamine. The unmodified amino group of the resulted adduct can then be converted to an amino-reactive isothiocyano or thiol-reactive groups (*12*). This mode of attachment of the cross-linking group weakens the retention of the lanthanide within the chelate by eliminating one ligating carboxylate, and it also reduces the brightness of the lanthanide (*14*) (30% to 1000%) due to the quenching effect of the additional coordinated water molecule. These factors restrict *in vivo* applications where high concentration of metal scavengers is an issue (e.g., intracellular imaging). Analogous derivatives of the fluorophore coumarine have been suggested and used in biophysical studies (*18*). However, compared to their quinolone counterparts, they are less bright and do not support terbium (Tb) luminescence.

To further perfect lanthanide probes, we now report the development of two novel approaches for the introduction of cross-linking groups into DTPA and/or TTHA-cs124 chelates by modification of the chromophore moiety. We demonstrate that the synthesized luminescent probes (Chart 1) are more resistant to EDTA challenge than previously described DTPA-cs124-based probes, and we demonstrate that these new probes are highly luminescent. These compounds were validated as

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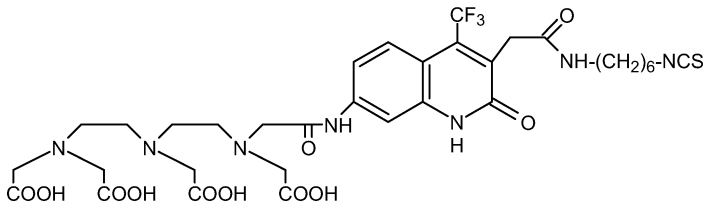
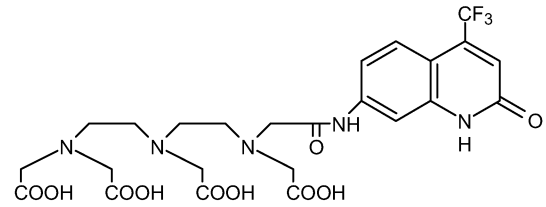
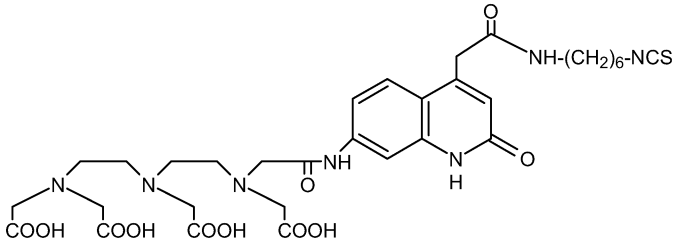
Chart 1. Structure of Reactive Luminescent Probes and Reference Compounds Used in This Study



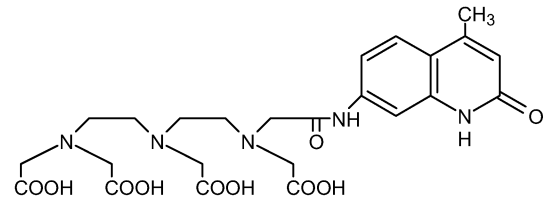
DTPA-cs124-CF_3-NCS (Probe 1)



DTPA-cs124-CF_3



DTPA-cs124-NCS (Probe 2)



DTPA-cs124

luminescent labels by including them in molecular beacon probes, which are widely used for DNA and RNA detection (*19*). The resulting sensitivity of these molecular beacons was between 0.5 and 1 pM, which is the most sensitive level of detection ever reported for nonamplified DNA detection systems.

EXPERIMENTAL PROCEDURES

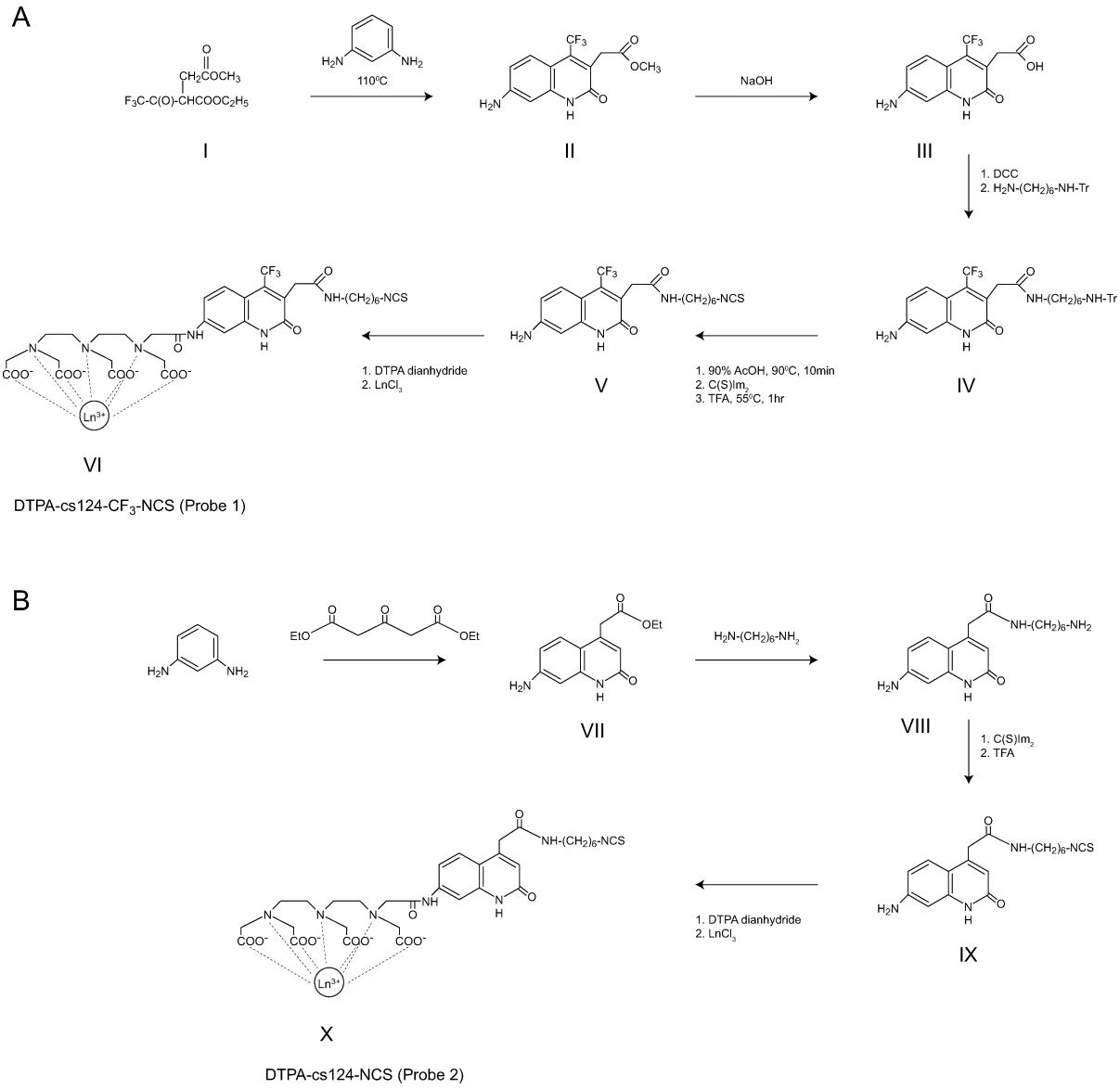
Synthesis. The following reagents were purchased from Aldrich: diethylenetriaminepentaacetic acid dianhydride (DTPA), triethylamine, 1,3-phenylenediamine, ethyl 4,4,4-trifluoroacetoacetate, ethylacetoacetate, 1,3-dicyclohexylcarbodiimide (DCC), ethylenedianime, *N*-trityl-1,6-diaminohexane, methylbromacetate, anhydrous dimethylformamide and dimethylsulfoxide, 1-butanol, ethylacetate, chloroform, acetonitrile, ethanol, sodium and potassium hydroxide, $ZnCl_2$, Na_2SO_4, Na_2CO_3, acetic acid, citric acid, thiocarbonyldiimadazole, $TbCl_3$, $EuCl_3$, $SmCl_3$ and $DyCl_3$, silica gel TLC plates on aluminum foil (200 μm layer thick with a fluorescent indicator). Only distilled and deionized water (18 MΩ cm^{-1}) was used. All experiments, including the preparation and use of lanthanide complexes, were performed either in glassware washed with mixed acid solution and rinsed with metal-free water or in metal-free plasticware purchased from Bio-Rad. All chemicals were the purest grade available.

Probe 1 (Scheme 1A). *1. 7-Amino-4-trifluoromethyl-3-carbomethoxymethyl-2(1H) quinolone (**II**) (cs124CF_3−CH_2-$COOCH_3$).* 4,4,4-Trifluoroacetoacetate (2.2 mL, 15 mmol) and KOH (0.86 g, 15 mmol) were mixed in 7 mL of dimethylformamide and stirred at 40 °C until dissolved. To this mixture, 1.5 mL of methylbromoacetate was added and the solution was incubated overnight at room temperature. Three hundred milligrams (5.2 mmol) of KOH was then added, and incubation continued at 60 °C for 1 h. This mixture was diluted by the addition of 20 mL of water and was then extracted with chloroform. The organic layer was collected, dried over anhydrous sodium sulfate, and then evaporated in vacuo, first at 30 °C, and then at 70 °C for 30 min. The residue (1.9 g, product **I**) was dissolved in 3.5 mL of DMSO, and then 0.76 g (7 mmol) of 1,3-phenylenediamine was added, followed by incubation at 110 °C for 6 h. Under these conditions, four fluorescent products were detected by thin layer chromatography in ethyl acetate as developing solvent: $R_f = 0.90$ (green−blue fluorescence); $R_f = 0.45$ (blue fluorescence); $R_f = 0.30$ (blue fluorescence); and $R_f = 0.03$ (green−blue fluorescence). This mixture was diluted with 30 mL of 0.05 M aqueous NaOH and extracted with ether (2 × 40 mL). The aqueous layer was separated and treated as described below (section 2). The organic layer was extracted with 1 vol 0.1 M citric acid, and then collected, dried over anhydrous sodium sulfate, and evaporated in vacuo. The residue was subjected to silica gel chromatography on a 40 mL column, using a hexane/acetone mixture (3:1) as eluent. Fractions corresponding to the products migrating with $R_f = 0.45$ (product **II**) were collected and evaporated to dryness. The residue was then washed with chloroform and dried. Yield ~ 130 mg. UV: $\lambda_{max} = 365$ nm ($\varepsilon = 14\,300$ M^{-1} cm^{-1}), $\lambda_{min} = 295$ nm ($\varepsilon = 2800$ M^{-1} cm^{-1}). ^{1}H NMR chemical shifts (d) in DMF were as follows: 3.65 (3H, methyl), 3.94 (q, 2H, 3-methylene, $J = 3.6$), 6.24 (broad, 7 amine), 6.72 (m, 1H, 6H), 6.72 (m, 1H, 8H), 7.48 (1H, 5H), and 11.9 (broad, amide).

*2. 7-Amino-4-trifluoromethyl-3-carboxy-2(1H) quinolone (**III**) (cs124CF_3-CH_2COOH).* One milliliter of 1 M aqueous NaOH was added to 100 mg of product **II** dissolved in 2 mL dioxane. After 4 h incubation at 50 °C, the mixture was diluted by the addition of 15 mL water and extracted with ether. The product (**III**) was precipitated from the aqueous phase by the addition of citric acid to pH 3−3.5, collected by centrifugation, washed a few times with water until neutral reaction, and dried in vacuo. Yield ~ 70 mg.

The aqueous phase obtained after ether extraction (see previous section), containing product **III**, was acidified by the addition of citric acid to pH 3−3.5; the precipitate was collected, washed a few times with water, dried, and then combined with the above. Recrystallization from ethyl acetate resulted in the isolation of pure product **III** (total yield ~ 200 mg). ^{1}H NMR chemical shifts (*d*) in DMF were as follows: 3.93 (2H, 4

Scheme 1. Schemes for the Synthesis of Probe 1 (A) and Probe 2 (B)



methylene), 6.19 (2H broad, 7 amine), 6.71 (1H, 6H), 6.71 (1H, 8H), 7.48 (1H, 5H), 11.85 (broad, amide), and 12.75 (broad, carboxyl).

*3. cs124CF$_3$-CH$_2$C(O)-NH(CH$_2$)$_6$NH-Tr (**IV**).* 100 mg (~0.3 mmol) of product **III** were dissolved in 8 mL of THF and supplemented with 210 mg (1 mmol) of DCC. After 1 h incubation, thin layer chromatographic analysis in ethyl acetate/ethanol (10:1) revealed a single product ($R_f = 0.80$) with intense blue fluorescence. 450 μmol of *N*-trityl-1,6-diaminohexane were added and incubation continued for another 30 min at 20 °C. Thin layer chromatography in ethylacetate/ethanol (10:1) revealed the main reaction product ($R_f = 0.70$). This mixture was diluted with 20 mL of 0.1 M aqueous Na_2CO_3 and extracted with an equal volume of chloroform. The organic phase was then collected and rinsed with 1 volume 0.2 M citric acid, dried over anhydrous sodium sulfate, and the solvent removed by evaporation under reduced pressure. The product was purified by silica gel chromatography, using ethylacetate/ethanol (10:1) mixture as eluent. Yield ~ 160 mg. ^{1}H NMR chemical shifts (*d*) in DMF were as follows: 1.45 (m, 8H), 1.65 (m, 2H), 1.78 (m, 2H), 2.03 (q, 2H, $J = 7.25$), 2.41 (t, 1H, $J = 7.2$), 3.08 (q, 2H, $J = 7.2$), 3.82 (q, 2H, 4CH$_2$-, $J = 4.0$), 6.14 (s, 2H, broad, 7 amine), 6.7 (1H, 8H), 6.7 (1H, 6H), 7.18 (t, 3H, p-ArH, $J =$ 7.2), 7.30 (t, 6H, m-ArH, $J = 7.4$), 7.48 (d, 6H, o-ArH, $J =$ 7.4), 7.48 (1H, 5H), and 11.75 (1H, broad, amide).

*4. cs124CF$_3$-CH$_2$C(O)-NH(CH$_2$)$_6$N═C═S (**V**).* 140 mg of compound **IV** were dissolved in 2 mL of 90% acetic acid and incubated at 90 °C for 15 min. After evaporation in vacuo, the resulting residue was suspended in water and extracted with ether to remove triphenylcarbinol. The aqueous phase was then evaporated to dryness. The resulting residue was dissolved in 2 mL of methanol, and 80 mg of thiocarbonyldiimadazole was then slowly added to this solution under rigorous agitation. After 10 min incubation at room temperature, the mixture was supplemented with 100 μL trifluoroacetic acid and kept at 50 °C. Thin layer chromatographic analysis in ethylacetate/ethanol (12:1) revealed nearly quantitative conversion of the original compound ($R_f = 0.05$) to an isothiocyanate ($R_f = 0.40$). This product was purified by column chromatography using the same eluent. Yield ~ 60 mg. ^{1}H NMR chemical shifts (*d*) in DMF were as follows: 1.35 (m, 4H), 1.46 (m, 2H), 1.65 (m, 2H), 3.13 (m, 2H, $J = 7.25$), 3.69 (t, 1H, $J = 7.2$), 3.08 (q, 2H, $J =$ 7.2), 3.82 (q, 2H, 3CH$_2$-, $J = 3.7$), 6.14 (s, 2H, broad, 7 amine), 6.7 (1H, 8H), 6.7 (1H, 6H), 7.48 (m, 1H, 5H), 7.76 (t, 1H, $J =$ 5.5), and 11.77 (1H, broad, amide).

5. Lanthanide Complexes of DTPA-cs124-CF_3-NCS (probe 1). Thirty milligrams (0.1 mmol) of compound **V** were added to a solution of 80 mg (0.3 mmol) of DTPA dianhydride in 0.8 mL of DMSO. After incubation (45 min at 50 °C), the mixture was supplemented with 10 mL of ether, and the resulting precipitate was spun down, washed with ether, air-dried, dissolved in 1 mL of DMF, and mixed with 0.3 mL of water. After incubation for 10 min at 45 °C, the mixture was diluted with 5 mL of water and extracted with 40 mL of butanol. The organic phase was separated and divided into four equal parts. Each portion was mixed with 0.3 mL of a 0.1 M solution of a lanthanide trichloride (Tb^{3+}, europium (Eu^{3+}), dysprosium (Dy^{3+}), and samarium (Sm^{3+})). After vigorous agitation, the organic phase was collected and concentrated by co-evaporation with water in vacuo at 30 °C. Analytical thin layer chromatography, using an acetonitrile/water system (3:1) as the developing solvent, revealed two main Ln^{3+} products ($R_f = 0.25$ and 0.50). The products with $R_f = 0.50$ (desired compound) were purified using preparative thin layer chromatography under the same conditions. The fluorescent material was eluted with 50% aqueous ethanol and was recovered as a colorless powder after evaporation in vacuo. UV: $\lambda_{max} = 347$ nm ($\varepsilon = 14\,800$ M^{-1} cm^{-1}), $\lambda_{min} = 270$ nm ($\varepsilon = 4700$ M^{-1} cm^{-1}). MS: Eu^{3+}DTPA-cs124-CF_3-CH_2C (O)-NH$(CH_2)_6$N═C═S (-H^+) 950.1 (found), 950.0 (calculated). Ln^{3+} complexes of DTPA-cs124-CF_3 were obtained using the same protocol.

Probe 2 (Scheme 1B). *1. 7-Amino-4-carboethoxymethyl-2(1H) quinolone (**VII**).* A suspension of 1.36 g (10 mmol) of $ZnCl_2$ in 5 mL of DMSO was supplemented with 1.08 g (10 mmol) of 1,3-phelylenediamine and 2.02 g (10 mmol) of diethyl-1,3-acetonedicarboxylate. The mixture was kept at 95 to 100 °C for 24 h. Thin layer chromatography in chloroform/ethanol (10:1) detected one main fluorescent product ($R_f = 0.35$). This mixture was diluted with 8 mL of ethanol, poured into 150 mL of ice-cold 0.1 M citric acid, and left for 3 h at 4 °C. The residue was filtered and successively washed with water (2 × 10 mL), and with hot acetonitrile (2 × 5 mL), and then dried in vacuo. Yield ~ 1.4 g (60%). ^{1}H NMR chemical shifts (*d*) in DMSO were as follows: 1.17 (t, 3H, -OCH_2CH_3, $J = 7.2$), 3.76 (s, 2H, 3-methylene), 4.06 (q, 2H, -OCH_2CH_3, $J = 7.2$), 5.81 (2H, broad, 7 amino), 6.01 (s, 1H, 3H), 6.37 (d, 1H, 8H, $J = 2.4$), 6.43 (dd, 1H, 6H, $J_1 = 7.2$, $J_2 = 2.4$), 7.25 (d, 1H, 5H, $J = 7.2$), and 11.28 (1H, broad, amide).

*2. 7-Amino-4-carboxamido(6-aminohexyl)methyl-2(1H) quinolone (**VIII**).* Premelted 1,6-diaminohexane (2 g, 17 mmol) was mixed with 7-amino-4-carboethoxymethyl-2(1*H*) quinolone (0.5 g, 2 mmol). After incubation (15 h, 90 °C), the mixture was poured into 30 mL of water. The precipitate was washed with water (3 × 30 mL) and ethyl acetate (3 × 20 mL), and then stirred with hot methanol (50 mL), filtered, and the filtrate evaporated to dryness in vacuo. The product appeared as light-brown crystals. Yield ~ 0.5 g. ^{1}H NMR chemical shifts (*d*) in DMSO were as follows: 1.2−1.4 (m, 10H), 3.04 (q, 2H, α-CH_2, $J = 7.2$), 3.49 (s, 2H, 4-methylene), 5.75 (2H, broad, 7 amino), 5.98 (s, 1H, 3H), 6.36 (d, 1H, 8H, $J = 2.4$), 6.43 (dd, 1H, 6H, $J_1 = 7.2$, $J_2 = 2.4$), 7.38 (d, 1H, 5H, $J = 7.2$), 8.07 (t, 1H, amide, $J = 7.2$), and 11.3 (1H, broad, amide quinolone).

*3. 7-Amino-4-carboxamido (6-isothiocyanohexyl) methyl-2 (1H) quinolone (**IX**).* 44 mg (0.22 mmol) of 1,1′-thiocarbonyldiimidazole was added to a solution of 63 mg (0.2 mmol) of product **VIII** dissolved in 4 mL methanol. After 5 min, this mixture was supplemented with 10 μL of TFA and incubated for 40 min at 50 °C. The solvent was removed by evaporation in vacuo, and the product was then washed with water and purified by column chromatography on silica gel using a chloroform/ethanol mixture (4:1) as eluent. Yield ~ 40 mg. ^{1}H NMR chemical shifts (*d*) in DMSO were as follows: 1.2−1.5 (m, 6H), 1.60 (m, 2H, e-CH_2, $J = 7.2$), 3.04 (m, 2H, α-CH_2, $J = 7.2$), 3.49 (s, 2H, 4-methylene), 3.64 (t, 2H, ζ-CH_2, $J = 7.2$), 5.75 (s, 2H, broad, 7 amine), 5.98 (s, 1H, 3H), 6.36 (d, 1H, 8H, $J = 2.4$), 6.43 (dd, 1H, 6H, $J_1 = 7.2$, $J_2 = 2.4$), 7.38 (d, 1H, 5H, $J = 7.2$), 8.07 (t, 1H, amide, $J = 7.2$), and 11.20 (1H, broad, amide quinolone).

4. Lanthanide Complexes of DTPA-cs124 and DTPA-cs124-NCS (probe 2). These products were obtained and purified essentially as described for the synthesis of analogous probe 1 compounds in section 5 above; however, the incubation time of the corresponding isothiocyano compound (**IX**) with DTPA dianhydride was 15 min at 20 °C. UV: $\lambda_{max} = 341$ nm ($\varepsilon = 18\,900$ M^{-1} cm^{-1}), $\lambda_{min} = 308$ nm ($\varepsilon = 10\,000$ M^{-1} cm^{-1}). MS: Tb-DTPA-cs124-NCS (−1) 888.3 (found), 888.0 (calcd); and Eu-DTPA-cs124-NCS (−1) 882.3 (found), 882.0 (calcd).

Synthesis of Luminescent Hybridization Probes. A 10 μL water solution containing 3 to 7 nmol of an oligonucleotide (5′ amino - CTTCGTCCACAAACACAACTCCTGAAG - 3′ Black-hole Quencher 2), prepared according to protocols described previously (*20*), was supplemented with 5 μL of $Na_2B_4O_7$ (pH 10.0), and 15 μL of a 10 to 20 mM aqueous solution of luminescent probe 1 or probe 2. After incubation for 3.5 h at 56 °C, the probe conjugated oligonucleotide was precipitated by the addition of 200 μL of ethanol, and then collected by centrifugation after cooling at −80 °C for 15 min. This procedure was repeated 3 to 4 times. Finally, the residue was dissolved in water and purified by HPLC chromatography, as described in Supporting Information. Yield 40−80%.

Physical Methods. Excitation and emission fluorescence spectra in a steady-state mode were recorded using a QuantaMaster 1 (Photon Technology International) digital fluorometer at ambient temperature. Time-resolved and gated luminescence measurements were performed using a home-built experimental setup (Supporting Information Figure S1). A Suprasil fluorescence cell filled with sample solutions was irradiated by pulsed (ca. 15 ns) UV light from an excimer laser (351 nm, XeF). Before passing through the cell, the laser beam was formed by a rectangular aperture 0.5 cm × 1.0 cm (width × height). Fluorescence from the cell collected at an angle of 90 degrees was focused onto the entrance slit of a grating spectrograph (SpectraPro-300i, Acton Research Corporation, diffraction grating 150 grooves/mm blazed at 500 nm) using a fused silica lens with a focal distance of 2.5 cm. The spectrograph was equipped with a gated intensified CCD Camera (ICCD-MAX, Princeton Instruments) to record transient spectra. A slit width of 0.5 mm was used for time-resolved luminescence measurements, which corresponds to a spectral resolution of 5 nm. Time-gated spectra were recorded with a spectral resolution of 0.3 nm (a slit width of 0.01 mm in combination with a pixel size on the ICCD camera of 0.026 mm). ICCD gating, with a delay after the laser pulse, was used to determine the temporal behavior of the transient fluorescence. For measurements of luminescence lifetimes, the light was diverted to a photomultiplier tube mounted on the exit slit of the spectrograph. The PMT signal was preamplified and averaged, using a digital storage oscilloscope (LeCroy 9310A). High-resolution spectra were recorded with a time delay of 1 μs and a gate width of 1 ms for probe 1 chelated with Eu and Sm and for probe 2 chelated with Tb and Dy.

Steady-State Fluorescence Measurements. Hybridization experiments of the lanthanide-based molecular beacon with its complementary target DNA (5′ TTAGGAGTTGTGTTTGTG-GACTT 3′) were performed in a measuring cell (150 μL) in a hybridization buffer containing 50 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.0). The concentrations of the molecular beacon and the cDNA oligonucleotide (target) were 300 nM

and 1000 nM, respectively. Water-based or deuterium oxide-based solutions were used.

Time-Resolved Luminescence Measurements. *Protocol A.* Ten microliters of various concentrations (0−100 nM) of target DNA (see previous section) was added to 3 mL of a 1 nM solution of molecular beacons in hybridization buffer (see previous section), and was then transferred to a measuring cell. The luminescence of the sample was measured at different time intervals.

Protocol B. Different concentrations (0−3 nM) of DNA target were added to 3 mL of 10 pM molecular beacon hybridization buffer in a glass tube, and this solution was left for 3 days at room temperature. The solution was then transferred to the measuring cell. The glass tube was washed with 1 mL of the same hybridization buffer containing 30% ethanol, and this solution was also transferred to the measuring cell. The resulting luminescent signal was then measured as described above.

RESULTS

Synthesis and Properties of Cross-Linkable Lanthanide Chelates. The structure of the synthesized luminescent lanthanide probes is shown schematically in Chart 1. These probes are derivatives of previously described cs124 and cs124-CF_3 fluorophores (*10*−*17*). For the synthesis of these compounds, we used an approach based on the condensation of 1,3-phenylenediamine with an ester of an acetoacetic acid derivative. Scheme 1A presents the synthetic strategy developed for probe 1. Alkylation of trifluoroacetoacetate by methylbromoacetate in the presence of a proton acceptor produced trifluoroacetylmethylethylsuccinate (compound **I**). Reaction of the latter with 1,3-phenylenediamine resulted in fluorescent quinolone derivative **II**, which was converted to the corresponding carboxyl derivative **III** by saponification. We intended to introduce the amino function in this compound by treatment with DCC in the presence of 4-nitrophenol and subsequent reaction of nitrophenyl ester with aliphatic diamine. Surprisingly, incubation with DCC and 4-nitrophenol yielded a product that did not contain 4-nitrophenol. The ultraviolet spectrum of this product differed significantly from that of compound **III**. In aqueous medium, this product converted into **III** with a half-time of about 30 min at 20 °C. Incubation of this product with aliphatic amines resulted in acylation of the amino groups. Most likely, this reactive intermediate emerged as a result of intramolecular acylation of the amide oxygen of **III** by activated carboxylate. Incubation of this compound with monotritylated 1,6-diaminohexane yielded the desired compound **IV**. After deprotection, the resulting aminoalkyl derivative was converted to isothiocyanate (compound **V**) by treatment with thiocarbonyldiimidazole and trifluoroacetic acid. Under these conditions, the aromatic amino group of the compound remains intact. Acylation of compound **V** by DTPA anhydride in anhydrous medium, followed by hydrolysis of the second anhydride group, resulted in compound **VI** (probe 1), which was separated from the excess of DTPA by partitioning in butanol/water. Further addition of aqueous lanthanide trichloride to the butanol extract led to complexes between the lanthanides and the probes. The lanthanide complexes were analyzed and purified by thin layer chromatography in acetonitrile:water mixtures that proved to be highly efficient (see Figure 1).

For the synthesis of probe 2, we developed the strategy shown in Scheme 1B. The first step of this synthesis was based on a published protocol (*13*) that includes incubation of *m*-phenylenediamine with diethyl 1,3-acetonedicarboxylate at high temperature to yield quinolone derivative **VII**. Compared to published protocols, a dramatic improvement in the yield of the final product (from <1% to ca. 60%) was achieved by using a mild Lewis acid ($ZnCl_2$) as a catalyst, which allowed lowering

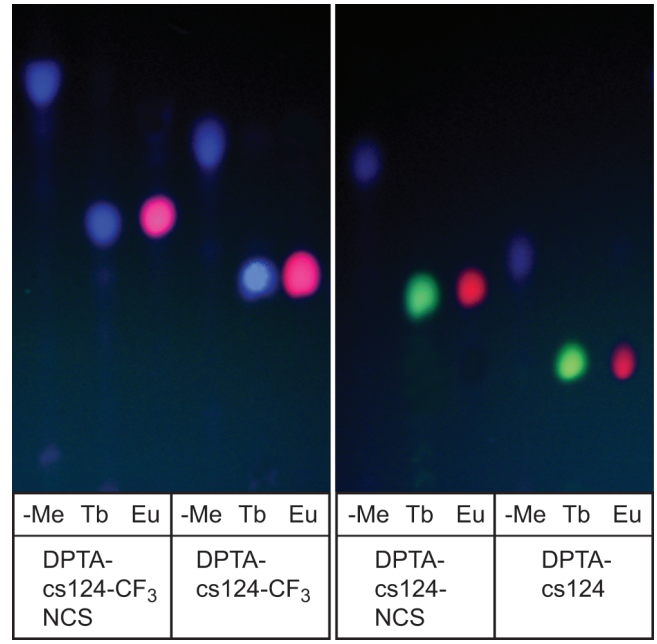


Figure 1. Image recorded by ultraviolet excitation of ligand antenna compounds (−Me) and their lanthanide complexes (Tb and Eu), after separation by thin layer chromatography.

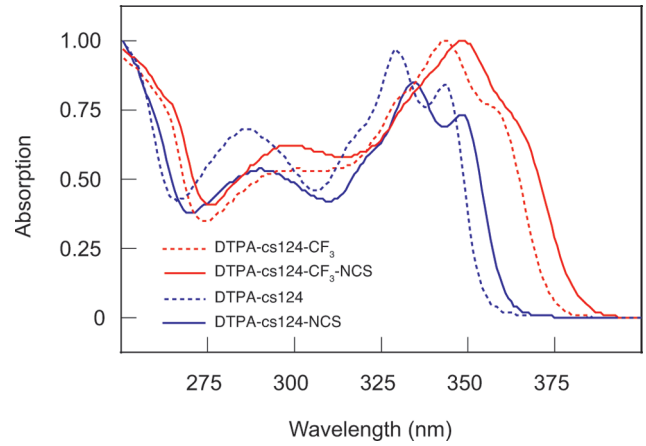


Figure 2. UV absorption spectra of synthesized reactive luminescent probes and reference compounds used in this study.

of the reaction temperature. Reaction of this product with a diamine (added in a few-fold excess to avoid cross-linked products) at high temperature resulted in the formation of aminoalkyl derivative **VIII**, with nearly quantitative yield. Cross-linkable DTPA derivative (**X**, probe 2) was obtained in the same manner as probe 1.

Absorption Spectra. Absorption spectra of the synthesized cs124 and cs124-CF_3 derivatives are shown in Figure 2. They were nearly identical to published spectra for analogous compounds (*10*−*17*). A small red shift of 6 nm was observed for probe 1 (comparing to the reference compound cs124-CF_3) and probe 2 (comparing to the reference compound cs124). The molar extinction coefficients for DTPA-cs124 and DTPA-cs124-CF_3 were determined by direct comparison of the absorption spectra of the original compounds (ε_{max} = 18 900 M^{-1} cm^{-1} at 341 nm for cs124 and ε_{max} = 14 500 M^{-1} cm^{-1} at 360 nm for cs124-CF_3) to their acylated derivatives, which was achieved by monitoring spectral changes in the reaction mixture during the course of the reaction (see Supporting Information Figures S2A and S2B). The presence of isosbestic points in both cases was indicative of the conversion of the original compounds to

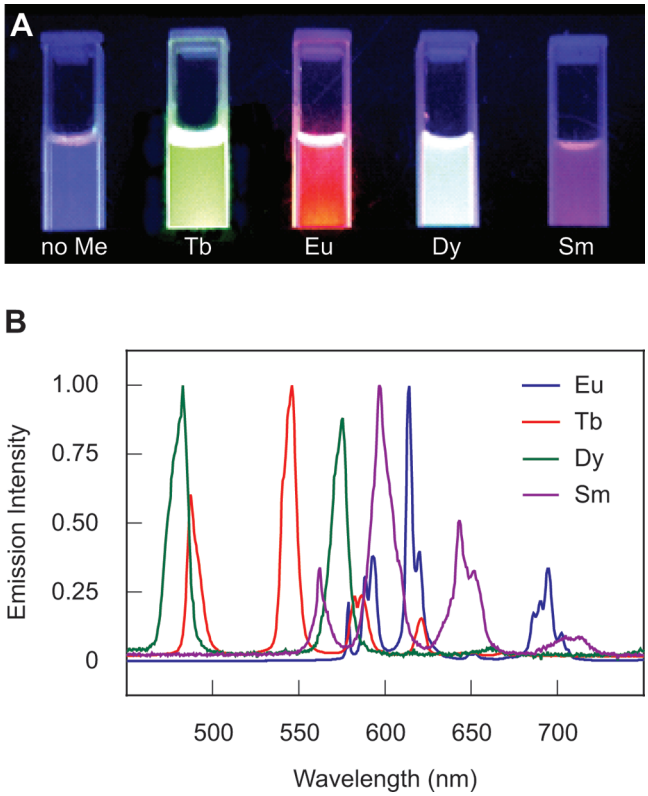


Figure 3. (A) Luminescence of 50 μM solutions of probe 2 (no Me) and its lanthanide complexes: terbium (Tb), europium (Eu), dysprosium (Dy), samarium (Sm); in heavy water, excited at 308 nm. (B) Normalized time-resolved emission of probe 1 Eu and Sm chelates and of probe 2 Tb and Dy chelates.

a single reaction product. Indeed, chromatographic analysis confirmed the formation of single acylation products in both cases. Thus, values of $\varepsilon_{max} = 18\ 200\ M^{-1}\ cm^{-1}$ at 328 nm and $\varepsilon_{max} = 14\ 800\ M^{-1}\ cm^{-1}$ at 341 nm were obtained for DTPA-acylated derivatives of cs124 and cs124-CF_3, respectively. Essentially the same values were obtained for probes 1 and 2 (data not shown). A significant difference in the absorption spectra of probes 1 and 2 (18 nm) allows selective excitation by common sources (at 351 nm by excimer XeF laser for probe 2, and at 365 nm by mercury UV lamp for probe 2). Such selective excitation is important for applications relying on simultaneous monitoring of two independent processes in the same sample.

Emission Spectroscopy of Lanthanide Complexes. The ease of observing the luminescence of these lanthanide complexes is illustrated in Figure 3A for probe 2. All complexes displayed the narrow emission spectrum typical of luminescent lanthanide chelates (Figure 3B). Table 1 shows the luminescence intensities of the synthesized reactive lanthanide chelates, as well as for the intensities obtained for the reference compounds, cs124 and cs124-CF_3 described earlier (*11, 17*). The process of antenna-mediated lanthanide emission includes the transfer of energy from the antenna fluorophore to the coordinated metal and the subsequent emission of photons by the excited lanthanide. The first step is the most crucial part of the process, because even slight modifications of chromophore-antenna structure dramatically affect lanthanide luminescence (*13*). In this work, we explored a synthetic approach that allows the introduction of a cross-linking group in position 3 (probe 1) or position 4 (probe 2) of quinolone-based antenna fluorophores. Comparison with reference fluorophores (with a non-substituted quinolone at position 3 or a methyl-substituted quinolone at position 4) demonstrates that the structural modification affected the brightness of the lanthanide chelates in different ways. For cs124-CF_3-based antennae, a significant decrease in brightness was observed for Tb^{3+} chelates (ca. 20-fold) and for Dy^{3+} chelates (>30-fold), while the emission of Eu^{3+} and Sm^{3+} chelates was not significantly affected. A similar effect of substitution was previously observed for the analogous Tb-cs124 chelates (*13*). In the case of probe 2, the substitution of a cross-linking group for a methyl group in position 4 did not significantly alter the brightness of all lanthanide complexes. Moreover, a detectable increase in the brightness for Eu (1.5-fold), Dy (1.7-fold), and Sm (1.6-fold) complexes was observed. This is consistent with the results (*13*) previously obtained for the analogous Tb and Eu derivatives of cs124 containing a carboxymethyl group at position 4. Surprisingly, significant luminescence was detected for Tb^{3+}-DTPA-cs124-CF_3 chelates, which were previously reported to be nonluminescent. We do not know the reason for this discrepancy. As seen in Table 1, comparing the emission of probe 1 and probe 2, probe 1 gives brighter complexes with Eu and Sm, while probe 2 is optimal for Tb and Dy. Time-resolved measurements indicated that there is a single-exponential decay mode for the luminescent signal from probe 1 chelates and from probe 2 chelates (not shown), which is indicative of the homogeneity of the complexes.

Effect of Heavy Water on Lanthanide Chelate Emission. The quantum yield of the excited lanthanide ion (defined as the probability of the excited state emitting a photon) in the antenna−chelate complex depends strongly on the number of coordinated water molecules (*21*), due to nonradiative dissipation of the energy of the excited state through the vibration of O−H bonds. This process does not occur with heavy water due to the different frequency of O−D bond vibration. This effect accounts for the enhanced brightness of lanthanide luminescence in heavy water. Indeed, as seen from Table 1 for DTPA ligands in D_2O, the brightness of the Tb^{3+} chelates was 1.3- to 1.5-fold higher than in H_2O-based solutions. As expected, the effect was more pronounced for DTPA-Eu^{3+} chelates (~3- to 3.8-fold), as well as for Dy^{3+} and Sm^{3+} complexes (~ 4- to 6-fold). The number of coordinated water molecules in Tb^{3+} and Eu^{3+} complexes can be calculated from the luminescence lifetime in water and in deuterium oxide-based solutions (*22*). For our probes, the number of coordinated water molecules was close to unity (see Table 1), which is in agreement with the results reported for similar compounds. The same is expected for Dy^{3+} and Sm^{3+} chelates, since they have analogous coordination chemistry.

Effect of EDTA on the Rate of Lanthanide Chelate Decay. The ability to retain the chelated metal ion is an important characteristic of lanthanide probes. This property is especially crucial for intracellular applications, due to the abundance of metal scavengers in living cells (such as free amino acids, amino acid residues in proteins, nucleoside triphosphates, nucleic acids, etc.). To this end, we investigated the decay rates of the lanthanide chelates used in our probes and in those probes described previously (*15*). Reaction of cs124 with excess of DTPA dianhydride yields a primary adduct possessing a preserved acylation function. In previous studies, this function was used to attach the cross-linking group to the chelate construct by subsequent treatment with diamine, which yielded aminoalkylamide derivatives whose amino group was converted to amine reactive isothiocyanates. This modification of the chelating group is likely to weaken the retention of metal. To this end, as a model, we synthesized analogous, but nonreactive, compounds containing a butylamide group. In accordance with expectations, the lanthanide complexes were about 10 times less stable (comparing to those possessing nonmodified chelating groups) when challenged with EDTA (see Supporting Information Figure S3).

Table 1. Emission and Relative Brightness of Lanthanide Chelates under Various Conditions

compound	emission in H_2O, counts	emission in D_2O, counts	relative brightness D_2O/H_2O	relative brightness in H_2O (%)	life time in H_2O (ms)	life time in D_2O (ms)	number of coordinated H_2O molecules
			Dysprosium (Dy^{3+}) Complexes (emission at 482 nm)				
DTPA-cs124-CF_3[b]	555	917	1.75	20	0.0023	0.0045	
DTPA-cs124-CF_3-NCS (probe 1)	-	-	-	-	-	-	
DTPA-cs124[b]	2720	10522	3.87	100	0.011	0.033	-
DTPA-cs124-NCS (probe 2)	4550	13000	2.86	167	0.009	0.027	-
			Terbium (Tb^{3+}) Complexes (emission at 545 nm)				
DTPA-cs124-CF_3[b]	12,200	14170	1.16	7.2	0.2	0.2	-
DTPA-cs124-CF_3-NCS (probe 1)	833	790	0.95	0.5	-	-	-
DTPA-cs124[b]	169000	260000	1.53	100	1.5 (1.55[a])	2.3 (2.63[a])	0.97 (1.1[a])
DTPA-cs124-NCS (probe 2)	137000	190000	1.40	81	1.2	1.7	1.03
			Samarium (Sm^{3+}) Complexes (emission at 598 nm)				
DTPA-cs124-CF_3[b]	192	764	3.98	168	0.0080	0.036	
DTPA-cs124-CF_3-NCS (probe 1)	230	900	3.91	200	0.0092	0.042	
DTPA-cs124[b]	114	513	4.50	100	0.0082	0.023	-
DTPA-cs124-NCS (probe 2)	180	1010	6.60	158	0.0082	0.034	-
			Europium (Eu^{3+}) Complexes (emission at 615 nm)				
DTPA-cs124-CF_3[b]	10350	41000	3.96	205	0.5	1.9	1.54
DTPA-cs124-CF_3-NCS (probe 1)	9450	35000	3.70	187	0.5	1.7	1.47
DTPA-cs124[b]	5050	22000	4.36	100	0.6 (0.62[a])	1.6 (2.42[a])	1.1 (1.26[a])
DTPA-cs124-NCS (probe 2)	7300	25000	3.42	145	0.6	2.0	1.19

[a] Determined in ref *13*. [b] Model compounds.

Chemical Reactivity of Synthesized Luminescent Probes. The chemical reactivity of the synthesized probes was evaluated first in reactions with aliphatic diamines and with cysteine, because the resulting reaction products (corresponding thioureas and thiocarbamates) can easily be identified by thin layer chromatography, due to the strong retardation effect. Under the incubation conditions that were used, a nearly quantitative conversion of the probes to the corresponding reaction products was observed, either immediately upon mixing (with 0.1 M cysteine) or after 2 to 3 h at 56 °C (with 10 mM diamine), suggesting that the isothiocyanate groups in the probes survived purification. At the same time, nonreactive control chelates (Ln^{3+}-DTPA-cs124 and Ln^{3+}-DTPA-cs124-CF_3) did not change their mobility after incubation under the same conditions (see Supporting Information).

Synthesis of Luminescent-Based Molecular Beacon Probes. With conventional strategies, the nucleic acids are first coupled with metal-free probe derivatives, followed by the addition of a lanthanide to the probe conjugated products, which is not convenient. To this end, we obtained metal-chelated, ready-to-use luminescent tags. This was especially important for probe 1, since a metal-free compound is not stable.

For the attachment of the luminescent tags to the oligonucleotides, we obtained aliphatic isothiocyanate-reactive probes, as opposed to their more reactive aromatic counterparts that are typically used for modification of biopolymers. Partially, this choice was due to a synthetic strategy that required survival of the reactive cross-linking group during acylation by DTPA dianhydride (see Scheme 1) and during subsequent purification. Also, a closely positioned aromatic moiety of isothiocyanate could affect the energy transferred to the lanthanide by the occurrence of stacking interactions with antenna fluorophores. Under optimized reaction conditions, we were able to achieve a 70% coupling efficiency with amine-containing oligonucleotide constructs. The resulting hybridization probes were purified by ethanol precipitation, followed by size-exclusion chromatography and HPLC. Comparison of the absorption spectra of 3′ BHQ-2 labeled oligonucleotides, with and without probe 2 attached to their 5′ terminal (Figure 4B), revealed additional absorption in the range 300−350 nm due to the presence of the luminescent label. The survival of the lanthanide chelates during all of these operations is indicative of the extraordinarily high chemical stability of these probes.

Steady-State Fluorescence Measurements of Molecular Beacon Probes. Molecular beacon probes are nucleic acid hybridization probes that fluoresce when they bind to a target DNA or RNA sequence (*19*). When they are free in solution and not hybridized to a target nucleic acid, they remain nonfluorescent. Figure 4A illustrates the principle of molecular beacon probes. The probes are single-stranded oligonucleotides that form a stem-and-loop structure. The loop portion of the oligonucleotide is a probe sequence that is complementary to a target sequence in a nucleic acid. The probe sequence is embedded between two "arm" sequences, which are complementary to each other. Under assay conditions, the arms bind to each other to form a double-helical stem hybrid that encloses the probe sequence, forming a hairpin structure. A reporter fluorophore is attached to one end of the oligonucleotide and a nonfluorescent quencher is attached to the other end of the oligonucleotide. The stem hybrid brings the fluorophore and quencher in close proximity, allowing energy from the fluorophore to be transferred directly to the quencher through contact quenching. At assay temperatures, when the probe encounters a target nucleic acid, it forms a relatively rigid probe−target hybrid that is longer and more stable than the stem hybrid. Consequently, the molecular beacon probe undergoes a conformational reorganization that forces the stem hybrid to dissociate, and results in the separation of the fluorophore and the quencher, restoring fluorescence.

Currently, most molecular beacon probes are labeled with organic fluorophore labels (*23*). To increase the sensitivity of detection, we explored the performance of lanthanide-based molecular beacons by using the novel luminescent probes described in this study, instead of probes possessing conventional fluorophores. An example is shown in Figure 4C. In this case, we used a europium complex of probe 1 as the luminophore for molecular beacon. As expected, the addition of a cDNA target resulted in the development of a signal that is distinguished by the narrow emission peaks that occur in lanthanide luminescence. Remarkably, the ratio of the intensity of the luminescence signal to the intensity of the background fluorescence of the molecular beacon (which is due to the fluorescence of the molecular beacon after subtraction of the background fluorescence of the medium) was >400, which is significantly higher than the signal-to-background ratio typically obtained for fluorophore-based hybridization probes, including molecular beacons (*23*). This higher signal-to-background ratio

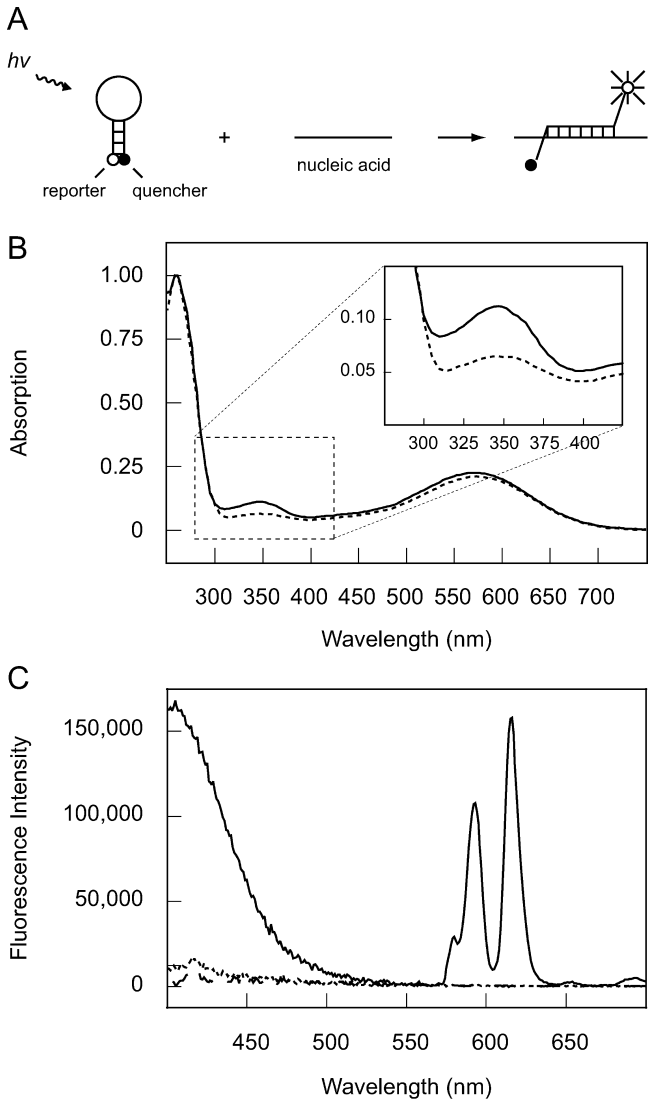


Figure 4. Detection of cDNA oligonucleotide targets using luminescence-based molecular beacons labeled by probe 1 Eu chelate in a steady-state mode. (A) Principle of detection. (B) Normalized absorption spectra of 3′ BHQ-2 labeled oligonucleotides, with (solid line) and without (dotted line) probe 2 conjugated to their 5′ terminal. (C) Luminescence emission spectra of the hybridization buffer (dashed line), the molecular beacon alone (dotted line), and the molecular beacon after hybridization to a complementary oligonucleotide (solid line).

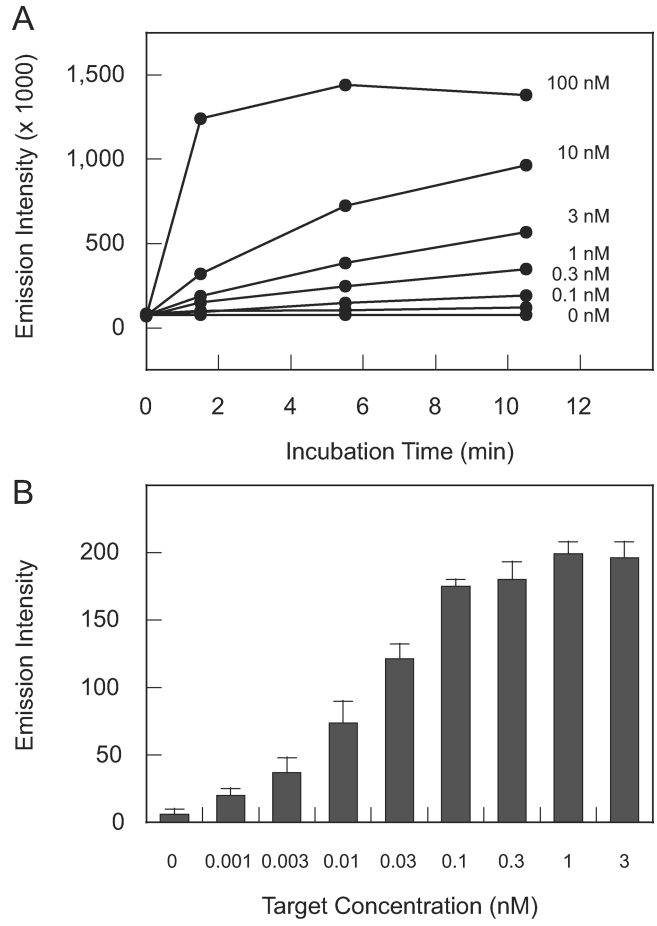


Figure 5. Time-resolved detection of a cDNA oligonucleotide target using a Eu-probe 1-based luminescent molecular beacon. (A) Emission intensity of a molecular beacon (1 nM) in the presence of different concentrations of target. (B) End-point emission detection of an oligonucleotide target at different concentrations in the presence of a 10 pM molecular beacon.

is due to both the suppression of the fluorescence of the antenna and the luminescence of the lanthanide in the "closed" form of the molecular beacon by the quencher. As expected, the signal that occurs upon hybridization of the probe to its target is significantly brighter in heavy water-based solutions.

Time-Resolved Luminescence Measurement of Molecular Beacons Probes. Figure 5A shows the time-course for the development of the luminescence signal that is detected in the time-resolved mode from hybridization mixtures that contain probe 1-based molecular beacons and various concentrations of complementary target DNA. The results demonstrate that, when 1 nM hybridization probe is present, subnanomolar concentrations of the target can be detected after only 10 min of incubation at ambient temperature. As seen from the time-course curves at low concentrations of the target, the hybridization rate decreases significantly, suggesting that the sensitivity of the detection can be improved by increasing the incubation time. Indeed, by lowering the concentration of the hybridization probe to 10 pM (to reduce background emission), and by prolonging the incubation time, we were able to achieve detection limits as low as 0.5−1 pM (Figure 5B), which is about 50- to 100-fold more sensitive than the results that are obtained in the same system using conventional fluorescein-based molecular beacons. Moreover, these detection limits are better than those reported previously for other lanthanide-based hybridization probes.

DISCUSSION

The unique photon emission properties of lanthanide-based probes render them suitable for a wide variety of applications that require ultrasensitive detection of biomolecules. Progress in this field depends on the availability of efficient probes. The mechanism of energy pathways in luminescent lanthanide chelates is not fully understood, leaving much room for improvements in their applications as labels for probes. Even though the first probes of this class were synthesized more than three decades ago and are not very efficient, many of them continue to be used for commercial applications. The development of more efficient probes is highly desirable, because new, more challenging applications have arisen (e.g., for the detection of rare pathogens in environmental samples, and for the detection of single molecules in cells).

In the present studies, we have taken advantage of new synthetic strategies that enable the preparation of highly luminescent probes with high yield. The resulting probes are ready-to-use, since they contain prebound lanthanides, unlike the lanthanide-labeled probes utilized in earlier applications, in

which the biomolecule of interest was first modified with metal-free chelates, followed by the addition of a lanthanide (*10, 15*). The new compounds that we describe were tested in the form of molecular beacons, which are widely utilized nucleic acid hybridization probes. Our experiments revealed a higher sensitivity of detection than can be achieved with conventional fluorescence-based molecular beacons. Moreover, the detection sensitivity was 10 to 60 times better than previously reported for other lanthanide-based hybridization probes (*24, 25*). Also, the brightness of the probes is significantly increased in heavy water, suggesting the use of this medium to increase the sensitivity of detection. Because of the superior properties, these new compounds could also be used as luminescent labels in other biopolymers, such as proteins and polysaccharides, as well as labels for small compounds, such as drugs and cellular metabolites.

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Supporting Information Available: Detailed protocols, including protocols for determining the luminescent and optical properties of the synthesized compounds, studies of their chemical reactivity and stability, as well as a description for purification of the molecular beacon hybridization probes.This material is available free of charge via the Internet at http://pubs.acs.org.

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