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A New Quinoline Sensitizer-Centered Lanthanide Chelate and Its Use for Protein Labeling on Ni-NTA Beads for TR LRET Assays

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Abstract

A quinoline sensitizer-centered lanthanide chelate system of novel design with a thiol-reactive functional group was prepared by an efficient route and used to label the estrogen receptor α ligand binding domain (ER α -LBD) in a model system to demonstrate that labeling of his-tagged proteins can be done by a mixed metal chelate protocol, with the protein bound to nickel-NTA beads, to give material that functions well in time-resolved lanthanide resonance energy transfer (TR-LRET) protein-protein interaction assays.

Time-resolved lanthanide resonance energy transfer^{1, 2} (TR-LRET) assay systems are now widely used to develop convenient and sensitive high throughput assays for protein-protein,³ protein-ligand,^{4, 5} protein-gene,⁶ and gene-gene⁷ interactions. These assays have advantages over conventional fluorescence energy transfer (FRET) assays because of their large Stokes shift, low background, unique narrow emission bands, and long life time. Despite the high demand for TR-LRET assay systems for high-throughput screening, however, the supply of suitable UV-sensitized lanthanide chelates that can be used for protein labeling needs to be improved. Convenient methods for protein purification and direct labeling with such TR-LRET agents have also not been extensively studied. In fact, an indirect affinity labeling approach between a biotin-conjugated protein and a lanthanide chelate conjugated to streptavidin or related fusion-protein conjugates is typically used. The other labeling method uses a pre-purified protein followed by an inconvenient size-exclusion column chromatography.

The most commonly used lanthanide chelate systems for LRET are constituted of a DTPA chelate system to hold the metal, to which is attached to one side a UV sensitizer, such as Cs124 (7-amino-4-methyl-2-quinolone, carbostyryl 124) or a coumarin required for efficient excitation of the metal center, and to the other side a thiol- or amine-reactive functional group for protein conjugation (Figure 1, right). Such TR-LRET agents can be considered to have a “metal-centered design”, meaning that the metal chelate (e.g., DTPA) is at their core, and they are typically synthesized by the stepwise addition of the sensitizer unit and the protein conjugation unit to DTPA dianhydride.

The synthesis of metal-centered systems, however, inevitably produces product mixtures that require cumbersome and generally inefficient isolation and purification steps.^{4, 5, 8-10}

The lanthanide complex also has a net neutral charge that might decrease water solubility. To improve the availability of TR-LRET agents, we conceived of an alternate “sensitizer-centered design” (Figure 1, left) that could be synthesized more efficiently and purified easily, so that much larger quantities of these materials could be readily prepared.

To center the LRET agent on the sensitizer required that we convert the carbostyryl unit (**1**) into a bifunctional quinoline (**5**), to which the protein-conjugating group and DTPA could then be attached to give the chelate precursor **7**, without producing side products except for excess DTPA, from which **7** could readily be purified. In addition, the final lanthanide complexes (**9**) would have a net negative charge to improve solubility (Figure 1, left). We also developed a method by which purification and labeling of a his-tagged protein (e.g., the estrogen receptor alpha ligand binding domain, ER α -LBD), with the quinoline-sensitized lanthanide chelate system could be done simultaneously. Finally, to ascertain whether our new system was suitable for TR-LRET assays, we used the ER α -LBD labeled with our quinoline-sensitized Tb chelate in a protein-protein interaction assay with the nuclear receptor interaction domain of the steroid receptor coactivator 3 labeled with fluorescein (SRC3-Fl).¹¹

To prepare the key bifunctional quinoline intermediate **5**, carbostyryl Cs124 (**1**) was treated with POCl₃ (neat) under reflux, followed by Boc protection to generate the quinoline **2** quantitatively. Substitution of the chlorine in **2** with a diethylene glycol moiety was effected with 3 equiv sodium in diethylene glycol at 70 °C for 8 h in 83% yield after treatment with diBoc again. The tosylation of the terminal OH in **3** gave compound **4** in 98% yield. Tosylate displacement with sodium azide and subsequent hydrogenation over 10% Pd/C generated the key bifunctional quinoline compound **5** quantitatively.

The reaction of the free amine in **5** with maleimidopropionic acid N-hydroxysuccinimide (NHS) ester afforded the singly modified agent **6** in 87%. To attach the DTPA group, the N-Boc group in **6** was cleaved with 50% TFA, and the TFA salt of **7** was reacted with 3.0 eq. DTPA dianhydride in DMSO without base at rt for 2 h. Addition of EtOAc to the reaction mixture precipitated excess unreacted DTPA dianhydride and product **7**, together with less than 5% of a DTPA diadduct, all of which were collected by filtration. Unreacted DTPA dianhydride was removed by dissolution in DI water, and the remaining mixture of **7** and the diadduct, though insoluble in DI water, could be dissolved by adjusting the pH to 7.0 with sodium bicarbonate solution. Final purification was effected by C-18 reversed column chromatography, which could be done on a large scale. Remaining DTPA was eluted with water, and elution with 10% MeOH-water afforded **7** in 73% yield; none of the less polar diadduct was eluted under these conditions.

The europium and terbium complexes (**9**) were obtained as off-white powders after adding one equivalent of EuCl₃·6H₂O or TbCl₃·6H₂O to the chelate **7** and lyophilizing the solution. The identity of **9**-Tb and **9**-Eu was confirmed by ESI mass spectrometry, which showed the characteristic isotope patterns of Tb and Eu and the absence of free precursor.

We examined whether the quinoline lanthanide systems developed here (**9**-Eu³⁺ or **9**-Tb³⁺) were suitable as UV sensitizers to excite the metal center, as needed for LRET assays. Lifetime measurements of **9**-Eu³⁺ or **9**-Tb³⁺ in water exhibited single decays, as shown in Figure 2 (top), being 609 μ s for Eu³⁺ (Figure 2, upper right) and 1.24 ms for Tb³⁺ (Figure 2, upper left). The intensities of both **9**-Eu³⁺ (Figure 2, bottom right) and **9**-Tb³⁺ (Figure 2, bottom right) are ca. 60% relative to the well known Cs124-Tb³⁺ and Cs124-Eu³⁺ complexes (Figure 2, bottom). The λ_{max} of the quinoline in **9** is at 330 nm, making it suitable for excitation with a CW HeCd (325 nm) or N₂ laser (340 nm), again similar to Cs124 carbostyryl (λ_{ex} = 342 nm).

To demonstrate that the Tb or Eu complexes **9** could be used to label a protein, we labeled the ER α -LBD on a specific cysteine residue (only C417 is reactive in the construct we used; see SI), during purification of his-tagged protein over Ni-NTA beads, according to a previously described method.¹² The ER α -LBD-417, expressed in *E. coli*, was adsorbed onto Ni-NTA beads; a 30-fold excess of the Tb or Eu chelate of **9** was added, and gentle shaking was maintained for 1 h. After removal of unreacted **9** by washing the beads with 50 mM Tris buffer,

the labeled ER α was eluted from the Ni-NTA beads with 20 mM Tris buffer containing 100 mM imidazole and 10% glycerol.¹² ER α -LBD-417 labeled with **9** retained its full ligand binding activity. In addition, we observed that the lanthanide ion was not liberated from the chelate by exposure to 100 mM imidazole during elution from the Ni-NTA beads.

Tb³⁺ in the labeled ER α -LBD (**10**) reveals four main emission peaks that correspond to the four most significant transitions (⁵D₄ to ⁷F_{j (6~3)}) arising from the (4f) electron configuration of the Tb³⁺ ion (Figure 2).

The emission from ⁵D₄ $\rightarrow$ ⁷F₆ (488 nm) nicely matches the excitation of fluorescein. The europium chelate exhibits emission patterns derived from ⁵D₀ to ⁷F_{j (j=0~4)} transitions, among which the strong emission at 618 nm from the transition ⁵D₀ $\rightarrow$ ⁷F₂ nicely matches with the excitation of Cy5 (Figure 2). Thus, the emissions from both complexes are well positioned to act as energy donors for LRET type assays. Life time measurements for **10**-Tb³⁺ and **10**-Eu³⁺ show essentially monoexponential decays (1.17 ms and 0.75 ms, respectively), with less than ca. 5% of a shorter component.

We ascertained that no metal exchange occurred between the lanthanide (Ln) and Ni by quantification of the emission of the Ln, as shown in Figure 3 and MALDI-TOF MS analysis (SI). MS analysis showed that ca. 70% of ER α was labeled by this process, and the emission intensity of the labeled material was equivalent to 70% that of a standard solution of **9**-Tb³⁺ or **9**-Eu³⁺ prepared at the same concentration as that of ER α (Figure 3). It is remarkable that protein purification and labeling could be done at the same time, without affecting the lanthanide emission pattern and intensity. To the best of our knowledge, protein labeling with a lanthanide complex on a Ni-NTA affinity purification system has thus far not been reported.

We next examined whether the Tb- or Eu-labeled ER α could be used in LRET assays by demonstrating that the interaction of fluorescein-labeled steroid hormone response coactivator 3 NR box2 region (SRC3-FI) as an acceptor with **10** as a donor, which does not occur without a ligand (APO), could be induced to occur by addition of an agonist (E₂ estradiol) but not an antagonist (TOT, 4'-hydroxytamoxifen). Thus, the terbium-labeled ER was titrated with SRC3-FI, in the absence (APO) or presence of ligand (E₂ or TOT) (Ex 340 nm, Em 520 $\pm$ 25 nm, 50- μ s time delay). As expected, when SRC3-FI concentrations increased, interaction with ER α was only observed with E₂ (Figure 4). The EC₅₀ value of 9.2 nM corresponds to the known affinity of SRC3-FI to ER α .¹³

In conclusion, we have prepared a quinoline derivative (**7**) that links together a functional group for conjugation to protein thiols and a DTPA chelation system for lanthanides and functions as a UV sensitizer for the lanthanide complex.

In contrast to other protein-labeling, UV-sensitized lanthanide complexes of the metal-centered design, the sensitizer-centered quinoline agent **7** can be readily prepared and is easily purified. We showed that the lanthanide complex with Tb (**9**-Tb) could be used to label a his-tagged protein, the ER α LBD, while this protein was adsorbed onto Ni-NTA beads. The labeling was efficient, and no metal exchange occurred, indicating that both metal complexes were stable. The protein with both the Tb and Eu complexes showed the characteristic lanthanide emission patterns and could be used in a TR-LRET assay of protein-protein interaction. The facilitated synthesis and purification of TR-LRET systems of our novel sensitizer-centered design and our demonstration that his-tagged proteins can be labeled while bound to Ni-NTA beads without metal exchange should enable much wider use of the powerful TR-FRET assay methodology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Selvin PR. Annu Rev Biophys Biomol Struct 2002;31:275–302. [PubMed: 11988471]
2. Vogel KW, Vedvik KL. J Biomol Screen 2006;11(4):439–43. [PubMed: 16751339]
3. Bergendahl V, Heyduk T, Burgess RR. Appl Environ Microbiol 2003;69(3):1492–8. [PubMed: 12620834]
4. Riddle SM, Vedvik KL, Hanson GT, Vogel KW. Anal Biochem 2006;356(1):108–16. [PubMed: 16797477]
5. Terai T, Kikuchi K, Iwasawa SY, Kawabe T, Hirata Y, Urano Y, Nagano T. J Am Chem Soc 2006;128(26):8699–700.
6. Kapanidis AN, Ebright YW, Ludescher RD, Chan S, Ebright RH. J Mol Biol 2001;312(3):453–68. [PubMed: 11563909]
7. Li M, Selvin PR. Bioconjug Chem 1997;8(2):127–32. [PubMed: 9095352]
8. Xiao M, Selvin PR. J Am Chem Soc 2001;123(29):7067–73. [PubMed: 11459485]
9. Ge P, Selvin PR. Bioconjug Chem 2004;15(5):1088–94. [PubMed: 15366964]
10. Chen J, Selvin PR. Bioconjug Chem 1999;10(2):311–5. [PubMed: 10077482]
11. Tamrazi A, Carlson KE, Rodriguez AL, Katzenellenbogen JA. Mol Endocrinol 2005;19(6):1516–28. [PubMed: 15661830]
12. (a) Carlson KE, Choi I, Gee A, Katzenellenbogen BS, Katzenellenbogen JA. Biochemistry 1997;36(48):14897–905. [PubMed: 9398213] (b) Tamrazi A, Katzenellenbogen JA. Methods Enzymol 2003;364:37. [PubMed: 14631838]
13. Kim SH, Tamrazi A, Carlson KE, Katzenellenbogen JA. Molec Cell Proteom 2005;4(3):267–277.

Figure 1. Cartoon for the new “sensitizer-centered system” (net negative charge, left) and the typical “chelated-centered system” (net zero charge, right).

Figure 2. Lifetime measurements for the Tb^{3+} (top left) and Eu^{3+} (top right) chelates of **9**. Intensity comparison of **9**- Tb^{3+} (bottom left) and **9**- Eu^{3+} (bottom right) with Cs124- Tb^{3+} and Cs124- Eu^{3+} .

Figure 3.
Emission spectrum of **10** (red line) (10 nM ER α) and **9** (black line) (10 nM **9**). Ex. 340 nm. Tb emission (left), Eu emission (right).

Ligand-dependent Interaction of ER α -Tb with SRC3-FI **Figure 4.**

TR LRET Titration of FI-SRC3 NR BoxII to ER α -LBD-Tb in the presence of agonist (E₂, filled square box), antagonist (TOT, filled triangle), and in the absence of ligand (APO, reverse triangle). 5 nM ER α -LBD was used for this experiment.). (Ex. 340 nm, Em. 520 $\pm$ 25 nm, 50- μ s time delay)

Scheme 1.

Synthesis of bifunctional quinoline system sensitizer as a lanthanide complex and protein conjugation.

(i) POCl_3 , 110 °C, 98%; (ii) Pyr- CH_2Cl_2 , diBoc, rt. 100%; (iii) neat diethylene glycol, 3.0 eq. Na, 80 °C and then added 1 eq. diBoc, 83 %; (iv) 1.2 eq. TsCl, Pyr. -10 °C, 98%; (v) 4 eq. NaN_3 , DMF, 60 °C, 96%; (vi) H_2 , 10% Pd/C, MeOH, 100%; (vii) DMF, 2 eq. DIEA, 1.5 eq. N-hydroxysuccinimidyl male-imidopropionate, rt., 87%; (viii) CH_2Cl_2 -TFA (1:1) 30 min rt, 100%; (ix) 3eq. DTPA dianhydride, DMSO, 60 °C 10 min and then rt. for overnight, 71-75%.

Scheme 2.

Formation of the lanthanide complex and conjugation to a His-tagged protein on the Ni-NTA beads

(i) 1.05 eq. 1 mM $\text{TbCl}_3 \cdot 6 \text{H}_2\text{O}$ or $\text{EuCl}_3 \cdot 6 \text{H}_2\text{O}$ in MeOH and **7** in pH 7.0 ammonium acetate buffer, (ii) Incubation: 30:1 ratio of **9**- Tb^{3+} or **9**- Eu^{3+} and Ni^{2+} -NTA-His-ER α in pH 7.0 10% tris-glycerol buffer containing 0.1 mM TCEP for 1 hr at 4 °C; Dissociation: Tris-glycerol buffer containing 100 mM imidazole and 0.1 mM TCEP for 1 hr and dialysis with pH 8.0 Tris-glycerol buffer.