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New quinolone-based thiol-reactive lanthanide luminescent probes

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Abstract

Luminescent lanthanide ion complexes are distinguished by unique light emitting properties that enable both highly sensitive detection of lanthanide labels attached to biomolecules and contrast imaging of various micro objects (cells, nanoparticles, etc.). Previously, we synthesized amine-reactive cs124-based luminescent lanthanide chelates with improved brightness and metal retention. Here we report the synthesis of new thiol-reactive derivatives of the same compounds including bromoacetamido-, and maleimido- forms of cs124 and cs124CF₃ fluorophores. Maleimido-compounds displayed exceptional reactivity instantaneously coupling to thiols at physiological conditions at micromolar probes concentrations. Surprisingly, they displayed strong quenching by adjacent maleimido-group, which was completely eliminated after reaction with thiols, thereby enabling their simple detection by monitoring the light emission of the reaction mixture. This reaction can be used for hyper-sensitive determination of biologically important sulphhydryl compounds (e.g. glutathione, co-enzyme A, etc.) in time-resolved mode.

Keywords

Lanthanide; luminescence; thiol; detection; reactive; labeling

1. Introduction

Luminescent lanthanide ion chelates are valuable alternatives to conventional fluorophores due to unusual properties of lanthanide ion emission (1–6). These chelates possess long luminescence lifetime, which suppresses the short-lived background fluorescence by acquisition the signal in time-gated mode. Large spectral distance between the excitation and emission light (Stokes shift) along with sharply spiked shape of the emission spectra further contribute to detection sensitivity, which is a few orders of magnitude higher than for regular fluorophores. Due to poor light absorption the lanthanide ion has to be sensitized. This is achieved by tethering of the ion (typically through a chelator) to a light-absorbing molecule, which after photon capture transfers the energy to the lanthanide ion, thereby

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acting as an antenna. Lanthanide is attached to the antenna through chelating group. Also lanthanide probes possess cross-linking group used for the labeling of the objects of interest.

Carbostyryl cs124 and cs124CF₃ (7–15) derivatives of oligoethyleneiminocarboxylate chelates represent attractive lanthanide probes for detection of biomolecules due to high quantum yield and high solubility in water. Previously, we described new amine-reactive isothiocyano-derivatives of cs124 and cs124CF₃-based lanthanide probes (16–19). Our current study was aimed at the design of simple synthetic protocols for corresponding thiol-reactive derivatives. Thiol-reactive light emitting probes are widely used for labeling proteins and nucleic acids in biochemical assays as well as for detection of biological thiols owing to their exceptional role in functioning of the living cells (20–21). These thiols include glutathione (GSH), cysteine (CYS), N-acetylcysteine (NAC), homocysteine (HCYS), γ -glutamyl cysteine (GGC) and co-enzyme A. Several luminescent probes of this class have been reported earlier (7, 11) including bromoacetamide, maleimide, pyridyldithio, iodoacetamides, and methanethiosulphonates. In these studies, the reactive group has been attached to a chelating moiety of a probe, which decreases Ln³⁺-coordination and brightness of the chelate allowing coordination of an extra water molecule. In the other study, coumarine-based chelates containing the reactive group in the fluorophore moiety were obtained (15). These probes are free of the above deficiencies, but they possess moderate quantum yield and do not support Tb³⁺ luminescence. In our probes (Fig. 1), the reactive groups are also attached to a fluorophore moiety, which preserves their high brightness and high metal retention. We developed convenient synthetic protocols for bromoacetamido and maleimido lanthanide probes in which all the reactions are carried out in the same tube. Fortuitous quenching exhibited by our maleimide-based probes (which disappears upon thiol conjugation to the reactive group) allows hyper-sensitive determination of sulfhydryl compounds in time-resolved detection mode. The obtained compounds were also validated by labeling of bacterial RNA polymerase.

2. MATERIALS AND METHODS

Diethylenetriaminepentaacetic acid dianhydride (DTPA), triethylamine; butylamine; 1,3-phenylenediamine; ethyl 4,4,4-trifluoroacetoacetate; ethylacetoacetate, 1,3-dicyclohexylcarbodiimide (DCC), ethylenedianime; methylbromacetate; anhydrous dimethylformamide and dimethylsulfoxide; 1-butanol, ethylacetate, chloroform; acetonitrile; ethanol; sodium and potassium hydroxide; TbCl₃ and EuCl₃; silicagel TLC plates on aluminum foil (200 μ m layer thick with a fluorescent indicator); distilled and deionized water (18 M Ω cm⁻¹) were purchased from Aldrich with a 99% pure and HPLC grade. All experiments including lanthanide complexes preparation and using thereof were performed either in glassware washed with mixed acid solution and rinsed with metal-free water, or in metal-free plasticware purchased from Biorad. Fluorescent intermediates used for the probes synthesis have been described in (17–19). Probe numbering (Fig. 1) continues from our previous publications (16, 19, and 22). Continuous excitation and emission fluorescence spectra were recorded using QuantaMaster 1 (Photon Technology International) digital fluorimeter at ambient temperature. UV absorption spectra were recorded on Cary 300 Bio UV-Visible spectrophotometer (Varian). Mass spectra were obtained at the Center for Advanced Proteomics Research (UMDNJ) using 4800 MALDI/TOF device (Applied Biosystems). NMR spectra were recorded by Process NMR Associates on Varian Mercury-300 superconducting NMR spectrometer operating at 300 MHz.

2.1. Synthesis

2.1.1. Probe 11 (Fig. 2)

2.1.1.1. 7-Amino-4-trifluoromethyl-4-carboxamido(6-aminohexyl)methyl-2-(1H) quinolone. Improved protocol

2.1.1.1.1. 7-Amino-4-trifluoromethyl-3-carbomethoxymethyl-2-(1H) quinolone

(**compound II**): The solution of 94 mg of 3-carboxymethyl-cs124-CF₃ (compound **I**, obtained as described³) in 1 ml of DMSO was treated by two equivalents (84 ml) of methyl iodide in the presence of one equivalent of diisopropylethylamine at room temperature for 30 min. TLC analysis in ethyl acetate showed complete conversion to alkylation product **II**. The reaction mixture was poured into a 15 ml tube containing 10 ml of ether and 2 ml of water. After partitioning, the organic layer was extracted with water (2 × 2 ml), dried over anhydrous sodium sulfate and evaporated in *vacuo*. The residue was dissolved in 5 ml of acetonitrile followed by evaporation to dryness under reduced pressure. Yield – 110 mg.

2.1.1.1.2. 7-Amino-4-trifluoromethyl-4-carboxamido(6-aminohexyl)methyl-2-(1H) quinolone (**compound III**)

(**compound III**): Solution of 100 mg of compound **II** in 10 molar excess of premelted 1,6-diaminohexane was heated at 100 °C. After 2 h TLC analysis in ethyl acetate revealed complete conversion to reaction product ($R_f = 0$). The product (compound **III**) was precipitated by 10 ml of ether, washed by water (3 × 10 ml), dried in *vacuo*, re-suspended in 0.5 ml of hot acetone, placed at –10 °C for 30 min and centrifuged. The precipitate was washed with 10 ml of ether and dried in *vacuo*. Yield – 150 mg.

2.1.1.1.3. Probe 11: Solutions of 30 mg (0.1 mmol) of compound **III** in 0.2 ml of DMSO and 30 mg (0.1 mmol) of 4-nitrophenylbromoacetate in 0.05 ml of DMSO were mixed and the solution incubated for 5 min at 40 °C. TLC in chloroform – ethanol 4:1 developing system revealed nearly complete conversion to the product (compound **IV**) migrated with $R_f = 0.5$. The reaction mixture was supplemented with the solution of 0.3 mmol of DTPA dianhydride in 0.5 ml of DMSO and incubation continued for additional 50 min at the same temperature. The mixture was poured drop-wise into 5 ml of ice-cold 0.1 M lithium citrate pH 4.0. Subsequently pH was adjusted to 5.0 by 1 M LiOH and the mixture was extracted by 5 ml of ethyl acetate followed by centrifugation at 6000 rpm for 5 min at 4 °C. The product from supernatant was purified by preparative HPLC (20 × 250 mm) column in linear gradient (700 ml) of methanol in water (0 – 40% MeOH) at the elution rate 2 ml/min. Fractions corresponding to desired product (total volume 150 ml) were combined and methanol removed by evaporation in *vacuo*. The resulting solution was applied to the same HPLC column and the product eluted in 15 ml of 80% methanol. The solution was titrated with 0.2 M aqueous europium chloride and the fluorescence of the solution was monitored at 615 nm with excitation at 350 nm. The titration was continued until fluorescence emission stopped increasing. The solution was evaporated in *vacuo* to ca. 2.5 ml affording 7.5 mM probe 1. Yield – 18%. ¹H NMR chemical shifts (d) for compound **IV** in DMSO were as follows: 1.248 (m, 4H), 1.390 (m, 4H), 3.03 (m, 4H, J 5.7), 3.680 (s, 2H, CH₂Br), 3.815 (s, 2H, 3CH₂-), 6.01 (s, 2H, broad, 7 amine), 6.441 (s, 1H, 8H), 6.5 (2 d, 1 H, 6H, J 2.9), 7.3 (d, 1H, 5H, J 6.6), 7.796 (t, 1H), 8.243 (t, 1H), 11.8 (s, 1H, quinolone amide). UV for probe **11**: $\lambda_{max} = 347$ nm ($\epsilon = 14300$ M⁻¹cm⁻¹). MS: Eu³⁺DTPA-cs124-CF₃-CH₂C(O)-NH(CH₂)₆NHC(O)CH₂Br (–H⁺) 1027.29 (found) 1027.62 (calculated). Eu³⁺DTPA-cs124-CF₃-CH₂C(O)-NH(CH₂)₆NHC(O)CH₂Br (–Eu³⁺), (+4H⁺) 880.23 (found) 880.67 (calculated).

2.1.2. Probe 12 (Fig. 2)—Solution of 39 mg (~100 μmol) of compound **III** was dissolved into 400 μl of DMSO and supplemented with 31 mg (~100 μmol) of N-succinimidyl-3-

maleimidiopropionate (Mlpr-OSu). After 15 min incubation at room temperature TLC analysis in chloroform- ethanol (4:1) developing system revealed nearly complete conversion of original compound ($R_f = 0$) to reaction product with $R_f = 0.53$. The mixture was supplemented with ~300 μmol of DTPA dissolved in 400 μL of DMSO at 70°C and cooled to room temperature before addition. After 40 min incubation at 40 °C the mixture was poured drop-wise into 5 ml of ice-cold 0.1 M lithium citrate pH 4.0. Subsequently, pH was adjusted to 5.0 by 1 M LiOH and the mixture was extracted by 5 ml of ethyl acetate followed by centrifugation at 6,000 rpm for 5 min at 4 °C. The product from the supernatant was purified by preparative HPLC and converted to Eu^{3+} complex as described above. Yield - 11 μmol s (11%). Cysteine reaction product was obtained from the probe as described below. UV: $\lambda_{\text{max}} = 347 \text{ nm}$ ($\epsilon = 14300 \text{ M}^{-1}\text{cm}^{-1}$). MS: $\text{Eu}^{3+}\text{DTPA-cs124-CF}_3\text{-CH}_2\text{C(O)-NH(CH}_2)_6\text{NHC(O)3MI-Cysteine (-H}^+)$ 1177.411 (found) 1178.988 (calculated). DTPA-cs124-CF₃-CH₂C(O)-NH(CH₂)₆NHC(O)3MI-cysteine 1032.358 (found) 1032.028 (calculated). Eu^{3+} DTPA-cs124-CF₃-CH₂C(O)-NH(CH₂)₆NHC(O)3MI 1059.838 (found) 1060.3092 (calculated).

2.1.3. Probe 13 (Fig. 3)—Solutions of 32 mg (0.1 mmol) of 7-amino-4-carboxamido (6-aminohexyl) methyl-2(*1H*) quinolone (compound **VI**) in 0.25 ml of DMSO and 30 mg of 4-nitrophenylbromoacetate in 0.1 ml in the same solvent were cooled in ice and mixed. After 10 min incubation at 0 °C TLC analysis of the reaction mixture in chloroform-ethanol 4:1 developing system revealed complete conversion of the reactants to product **VII** with $R_f = 0.4$. DTPA acylation and preparation of the sample for HPLC was performed as described above for probe 12, except that acylation was carried out at room temperature for 20 min. HPLC was performed using linear gradient (800 ml) of methanol in water (0–30%). The elution rate was 2 ml/min. The major product (probe **13**, 540 optical units at 347 nm, 30 μmol) was collected and mixed with equivalent amount of 0.1 M TbCl_3 . The solution was concentrated without heating by evaporation under reduced pressure to final volume 7.5 ml with addition of acetonitrile to facilitate evaporation. Yield – 30 μmol (30%). UV: $\lambda_{\text{max}} = 332 \text{ nm}$ ($\epsilon = 18900 \text{ M}^{-1}\text{cm}^{-1}$). MS: $\text{Tb}^{3+}\text{DTPA-cs124-CH}_2\text{C(O)-NH(CH}_2)_6\text{NHC(O)CH}_2\text{Br}$ 967.2170 (found) 967.536 (calculated). DTPA-cs124-CH₂C(O)-NH(CH₂)₆NHC(O)CH₂Br 812.2607 (found) 812.668 (calculated).

2.1.4. Probe 14 (Fig. 3)—Solution of 39 mg (0.1 mmol) of 7-amino-4-carboxamido (6-aminohexyl) methyl-2(*1H*) quinolone (compound **VI**) in 0.4 ml of DMSO was supplemented with 30 mg (0.11 mmol) of Mlpr-OSu and the mixture kept at room temperature. After 15 min incubation TLC analysis in chloroform- ethanol (4:1) developing system revealed nearly complete conversion of original compound ($R_f = 0$) to reaction product **VIII** with $R_f = 0.3$. DTPA acylation step (producing probe **14**) and subsequent preparation of the sample for HPLC purification was performed as described above for probe **13**. The sample was applied to HPLC column and the product eluted using linear gradient (560 ml) of methanol in water (0 – 40%) at the elution rate 2 ml/min. The fractions corresponding to desired product eluted between 140 and 210 min were combined and methanol evaporated under reduced pressure. After titration by TbCl_3 as described in previous section the resulting solution was freeze dried. Yield – 20 μmol . LC/MS samples were prepared with final product and cysteine, the reaction mixture was purified in 2.5:1 acetonitrile H₂O with $R_f = 0.2$ of reactant and $R_f = 0.3$ of Probe 14. UV: $\lambda_{\text{max}} = 332 \text{ nm}$ ($\epsilon = 18900 \text{ M}^{-1}\text{cm}^{-1}$). MS: $\text{Tb}^{3+}\text{DTPA-cs124-CH}_2\text{C(O)-NH(CH}_2)_6\text{NHC(O)CH}_2\text{CH}_2\text{-3MI-Cysteine (+H)}$ 1120.2 (found) 1119.9 (calculated). DTPA-cs124-CH₂C(O)-NH(CH₂)₆NHC(O)CH₂CH₂-3MI-Cysteine (+4H) 964.3356 (found) 964.026 (calculated).

2.1.5. Probe 15 (Fig. 4)—5.2 mg (14 μmol) of compound **IX** was dissolved in 200 μl of DMSO at 50 °C, cooled to room temperature and mixed with the solution of 23 μmol (1.5

equivalent) of 4-nitrophenylbromoacetate. After 10 min reaction at room temperature TLC analysis in 9:1 acetonitrile-water developing system revealed complete conversion of the starting fluorophore ($R_f = 0.4$) to the reaction product **X** ($R_f = 0.7$). The mixture was supplemented with the solution of 56 μmol (4 fold excess) of DTPA anhydride **e** in 200 μl of DMSO. After 20 min incubation the reaction mixture was supplemented with 100 μl of water and left for another 10 min at room temperature. Five milliliters of BuOH and 2 ml of water were added and after the extraction the organic layer was collected. Extraction was repeated one more time, organic layers combined and the amount of the DTPA acylation product in butanol solution was determined by UV absorption. The solution was supplemented with 1.5 equivalents of 0.1 M TbCl_3 . After rigorous agitation the organic layer was separated and evaporated in *vacuo* to 2–3 ml. The product was collected by centrifugation and purified by preparative TLC in acetonitrile-water (4:1) developing system. The product ($R_f = 0.24$) was recovered by elution with 50 % aqueous MeOH. Yield: 1.35 μmol (10 %). MS: $\text{Tb}^{3+}\text{DTPA-cs124-CH}_2\text{-BiPh-CH}_2\text{NHC(O)CH}_2\text{Br (+H}^+)$ 1022.1 (found) 1021.7 (calculated). $\text{DTPA-cs124-CH}_2\text{-BiPh-CH}_2\text{NHC(O)CH}_2\text{Br (+H}^+)$ 867.2 (found) 866.7 (calculated).

2.1.6—Probe 16 (Fig. 4) was obtained and purified essentially as described for probe **15** (except that a maleimide activated ester was used to produce intermediate **XI**). MS: $\text{Tb}^{3+}\text{DTPA-cs124-CH}_2\text{-BiPh-CH}_2\text{NHC(O)CH}_2\text{CH}_2\text{MI (+H}^+)$ 1052.2 (found) 1051.8 (calculated). $\text{DTPA-cs124-CH}_2\text{-BiPh-CH}_2\text{NHC(O)CH}_2\text{CH}_2\text{MI (+H}^+)$ 896.3 (found) 895.8 (calculated).

2.2. Reaction with cysteine

For qualitative TLC analysis the reaction mixture (10 μl) contained 50 mM sodium-borate pH 9.0, 10 mM cysteine and 0.05 – 1 mM reactive luminescent probe. Control mixture was the same but did not include cysteine. The mixtures were incubated at room temperature for 5 min and analyzed by TLC in acetonitrile – water 2.5:1 developing system. The reaction was monitored by appearance of the product with reduced mobility. In another version the reactions were performed in the measuring cell at concentrations of cysteine 0.1–10 μM and the probes concentration 50 nM in 10 mM sodium borate buffer pH 9.0. The emission spectra were recorded before and after cysteine addition at time intervals indicated in Fig. 7. Control measurements were performed in the same mixture but in the absence of cysteine. Excitation 331 nm was used for probe **14** and 328 nm for probe **16**. The emission was scanned from 350 to 650 nm.

2.3. Labeling of RNA polymerase

RNAP sample (2 mg/ml) in 20 mM tris-HCl pH 8.0 was supplemented with 10 mM DTT and kept at 40 °C for 40 min followed by gel filtration on sephadex G-50 column (0.5 $\times$ 10 cm) in 50 mM sodium borate pH 9.0. Thirty microliters of RNAP solution containing 1 μM enzyme was supplemented with equimolar amount of a cysteine-reactive Ln probe. After 1 h incubation at ambient temperature the mixture was subjected to size-exclusion chromatography at the same column. The fractions were collected and their fluorescence was determined (Fig. 8).

2.3. Physical methods

2.3.1. Continuous Excitation and Time Resolved Fluorescence Measurements

—Excitation and emission fluorescence spectra in the steady-state mode (displayed in Figs 6–7 and annotated in Table 2) were recorded at ambient temperature at the concentration of the compounds 0.1 μM , or 10 nM in H_2O and D_2O . The emission spectra for the compounds were recorded at maximum excitation wavelength (which coincided with the corresponding

light absorption maxima for the compounds in the region 320–380 nm) which were: 347 nm for probes **11** and **12**; 332 nm for probes **13** and **14**; 328 nm for probes **15** and **16**. Time-resolved emission measurements were executed as described in (16). Lifetime measurements for thiol-reactive probes **11** - **16** and reference compounds were performed in a solution containing 1 mM Na-borate buffer at pH 9.0 and 100 nM Ln chelate. Cysteine was added to final concentration 10 μ M and the measurements repeated after 5 min incubation at room temperature.

3. RESULTS

3.1. Synthesis of the Reactive Luminescent Probes

For the synthesis we used fluorophores amino-derivatives (compounds **III** of Fig. 2, **VI** of Fig. 3, and **IX** of Fig. 4) obtained in our previous studies (16, 19). In the present research we were able to significantly improve the yield for compound **III**. In this way we took advantage of the base-catalyzed reaction leading to compound **I** discovered in our previous study (17). Then we used quantitative methylation reaction to obtain compound **II**. Subsequent treatment of compound **II** with 1,6-diaminohexane produced compound **III** with high yield. In the following reactions the fluorophores amino derivatives were modified with 4-nitrophenylbromoacetate, of with N-hydroxysuccinimide ester of 3-maleimidopropionic acid to obtain compounds **IV** and **V** of Fig. 2, **VII** and **VIII** of Fig. 3, and **X** and **XI** of Fig. 4. TLC analysis revealed nearly quantitative conversion of the original fluorophores to the desired products. Since these reactive products were unstable we performed following DTPA acylation reaction in the same mixture without purification of the reactive intermediates. Acylation products were purified by preparative HPLC or TLC to yield the desired compounds (probes **11**–**16** of Fig. 1). These probes were converted to corresponding chelates by addition of equivalent amount of LnCl_3 . The yields of the final products were 10–30%. Generally, the yields were higher for cs124 derivatives and for bromoacetyl compounds due to shorter incubation times with DTPA and higher stability of bromoacetyl compounds.

3.2. Light Absorption Spectra of the Lanthanide Chelates

UV absorption spectra of the synthesized probes are shown in Fig. 5. They all exhibit absorption in the range 300–380 nm, typical for cs124 chromophores. Probes **15** and **16** also possess strong absorption in the range 250–300 nm due to biphenyl group. The presence of maleimide group in probes **12**, **14**, and **16** can account for their additional absorption in the range 220–240 nm. Notably, modification of cs124 fluorophore at N1 used to produce probes **15** and **16** causes a small bathochromic shift (ca. 5 nm) of the spectrum in the region 280–360 nm.

3.3. Light Emitting Properties of the Lanthanide Chelates

As seen from Table 2, bromoacetamido- compounds display nearly the same brightness compared to the corresponding isothiocyano-derivatives synthesized by us earlier (16). On the contrary, light emission of maleimido-compounds was significantly reduced. Thus 10–12 fold quenching was observed for probes **14** and **16**. This might be due to the contact quenching of the fluorophores by the maleimido group (favored by the proximation effect), since FRET quenching is unlikely, because of the absence of the spectral overlap between the fluorophores emission and the maleimido group absorption. In accord with this proposition, the reaction with cysteine (which eliminates a double bond in the maleimido group, and therefore the group's absorption) caused dramatic increase in the lanthanide light emission (see Table 2 and Fig. 6). Similar, but smaller (2.4 fold) quenching effect was observed for the corresponding Eu^{3+} -DTPA-cs124CF₃ maleimide chelate. Lifetime measurements detected short- and long-lived components for maleimide cs124-based probes

with the corresponding ratios 4-to-1 for probe **14** and 6-to-1 for probe **16**. We believe that long-lived components represented trace amounts of impurities, while short-lived components belonged to quenched maleimide derivatives. As seen from Table 2 the lifetime for probes **14** and **16** was strongly reduced compared to corresponding bromoacetamido probes, which suggests the quenching mechanism through reverse energy transfer from excited lanthanide ion to antenna fluorophore sensitized by maleimido group. The later is supported by the increase of the lanthanide emission lifetime back to that observed for reference isothiocyano, or bromoacetamido compounds after reaction of probes **14** and **16** with cysteine. At the same time, the presence of maleimido group in probe **12** did not decrease the life time for Eu^{3+} emission, suggesting different quenching mechanism (Fig. 6A and Table 2).

As expected, the light emission of the compounds in heavy water significantly increased due to the reduction of the non-radiative dissipation of the energy of the excited state through the vibrational excitation of O-H bonds (14). This process with heavy water is less efficient due to the lower frequency of the O-D bond vibration. Interestingly, the anomalous quenching reversal has been detected in praseodymium luminescent chelates containing C-D and C-H bonds (23).

3.4. Reactivity of the Probes 11-16 with cysteine

In our preliminary studies the reactivity of the synthesized probes was tested with cysteine using TLC analysis. It turned out that incubation with cysteine in a sodium-borate buffer at pH 9.0 resulted in quantitative conversion of the probes to the products with lower chromatographic mobility. MS analysis confirmed the expected structure of the reaction products. Apparently, coupling to cysteine strongly increased the brightness of the maleimide compounds. In the following tests we monitored the same reaction with various cysteine concentrations in the measuring cell by recording the light emission. The results are presented in Table 2 as well as in Figs. 6–7. It appeared that the reaction with maleimido-compounds was virtually instantaneous with cysteine concentrations up to [1 μM]. At sub-micromolar concentrations the reaction proceeded slower. Thus, 30 s and 60 s half-reaction times were observed for 200 nM and 100 nM [cys] correspondingly (not shown).

3.5. Modification of Bacterial RNA Polymerase by Maleimide-based Probes 12, 14 and 16

The suitability of the probes for protein labeling was demonstrated with *E.coli* RNA polymerase. The reaction proceeded with high rate at sub-micromolar concentrations of the enzyme and reactive probes. As in the reaction with cysteine, incubation of the maleimide probes with the enzyme resulted in progressive enhancement of light emission. Subsequent size-exclusion chromatography of the samples confirmed conjugation to RNA polymerase. The highest coupling efficiency of the fluorescent material (ca. 50 %) was observed for probe **16** (Fig. 8), while probes **12** and **14** provided 15–20 % efficiency. Notably, after the attachment to RNA polymerase the emission of Eu^{3+} -based probe **12** was quenched, as evidenced by increased antenna-to-lanthanide emission ratio compared to the same probe reacted with cysteine. This effect was less pronounced with the Tb^{3+} -based probes **14** and **16**. The same quenching effect we observed upon attachment of corresponding isothiocyano-reactive probes to avidin (19).

4. DISCUSSION

In our previous studies (16–19) we described the synthesis of cs124 and cs124CF₃ luminescent lanthanide ion chelates with enhanced lanthanide ion retention containing amine-reactive isothiocyano groups. Here we present synthetic protocols for thiol-reactive derivatives of the same chelates. They were obtained from amino-derivatives of the

fluorophores by selective acylation first, at aliphatic aminogroup (to introduce thiol-reactive function) and then, at aromatic aminogroup (to introduce a chelating moiety). This was possible due to different reactivity of the amino groups. Because the intermediate products were unstable, we performed both reactions in the same tube without purification of the intermediates. The resulting products were purified and complexed with lanthanide ions to yield the desired probes. Maleimide probes displayed exceptionally high reactivity with thiols, reacting instantaneously at micromolar thiol concentrations. Surprisingly, the maleimide group exerted strong quenching effect on the Tb³⁺ chelates, which was due to reverse energy transfer from excited lanthanide ion to antenna. The light emission was restored after coupling to a thiol, which converted maleimido group to non-absorbing succinimido-derivative. Enhancing of light emission of the probes upon reaction with thiols enables simple detection of sulfhydryl compounds. Thus, in our testing we were able to reliably detect cysteine at 100 nM concentration.

5. CONCLUSION

We synthesized highly reactive thiol-specific luminescent lanthanide probes of enhanced brightness and metal retention. The probes display high coupling efficiency to cysteine-containing proteins. Light emission of maleimide-based Tb³⁺ chelates strongly increases after conjugation with thiols, which can be used for ultra-sensitive determination of biologically important sulfhydryl compounds in time-resolved mode in the cases where the amount of the material is limited.

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Highlights

- Thiol-reactive luminescent Eu^{3+} and Tb^{3+} chelates with bromoacetamido- and maleimido-groups were synthesized.
- The maleimide chelates possessed exceptionally high reactivity instantaneously coupling to thiols at micromolar concentrations of the reactants.
- Tb^{3+} maleimide chelates were quenched due to stacking interaction of the maleimido-group with antenna fluorophore. Reaction with thiols eliminated the light absorbance of maleimido group, thereby restoring the energy transfer and strongly increasing the lanthanide luminescence.
- The above effect can be used for hyper-sensitive detection of thiols in time-resolved mode.

Fig. 1.
Structure of thiol reactive luminescent probes.

Fig. 2.
The Synthesis of Probes **11** and **12**.

Fig. 3.
The Synthesis of Probes **13** and **14**.

Fig. 4.
The Synthesis of Probes 15 and 16.

Fig. 5.

Light absorption spectra for probes **11-16**. The spectra were recorded at following probe concentrations: probe **11** – 13.5 μM , probe **12** – 12.8 μM , probes **13** and **14** – 8.5 μM , probes **15** and **16** – 7.4 μM .

Fig. 6.

Light emission spectra for probes **12** (A), **14** (B), and **16** (C) and their reaction products with cysteine. The spectra were recorded in water and deuterium oxide-based solutions.

Fig. 7. Time-course for the reaction of probe **14** with cysteine. Cysteine concentrations are indicated. Control curve represents the monitoring of the light emission of the mixture lacking cysteine.

Fig. 8.

Labeling of RNA polymerase with thiol reactive probes **12**, **14**, and **16**. The bars show the light emission of the material covalently bound to RNA polymerase and unbound material as determined after size-exclusion chromatography of the reaction mixtures.

Table 1R_f values for probes **11-16** and reference compounds on TLC in acetonitrile-water 2.5:1 developing system

Compound	R _f of cysteine adduct	R _f of original compound
PrI-Eu ³⁺ *		
PrII-Tb ³⁺ *	0.26	0.46
PrIV-NCS-Tb ³⁺ ^	0.61	0.65
cs124-DTPA-Eu ³⁺	NA	NA
cs124-DTPA-Tb ³⁺ *	NA	NA
PrI-AcBr-Eu ³⁺	0.32	0.50
PrII-AcBr-Tb ³⁺	0.28	0.48
PrI-3MI-Eu ³⁺	0.30	0.42
PrII-3MI-Tb ³⁺	0.32	0.48
PrIV-AcBr-Tb ³⁺	0.34	0.59
PrIV-3MI-Tb ³⁺	0.50	0.77

* Reference isothiocyanate compounds

Table 2
Light emitting properties of lanthanide probes and reference compounds (at [10 nM]) in water and oxide solutions

Luminescent chelates	Excitation wavelength, nm	Emission in H ₂ O		Emission of cys adducts in H ₂ O		Quenching factor	Emission in D ₂ O, counts	Cysteine adducts in D ₂ O, counts	Relative brightness H ₂ O/D ₂ O	D ₂ O/H ₂ O cysteine adducts
		counts**	lifetime, ms	counts**	lifetime, ms					
cs124-DTPA-Tb ³⁺ *	341	5,050 *	0.6	NA	NA	NA	22,000 *	NA	4.36	NA
Probe 1 *	347	9,450 *	0.5				35,000 *		3.70	
Probe 11 -Eu ³⁺	347	8,600	0.56	8,700	0.55	1.0	34,000	34,100	3.9	3.92
Probe 12 -Eu ³⁺	347	2,300	0.57	5,500	0.56	2.4	9,700	20,800	4.2	3.78
cs124-DTPA-Tb ³⁺ *	328	169,000 *	1.5	NA	NA	NA	260,000 *	NA	1.54	NA
Probe 2 *	332	137,000 *	1.2	115,000	1.12	0.84	190,000 *	193,700	1.39	1.68
Probe 13 -Tb ³⁺	332	112,000	1.2	104,800	1.05	0.94	174,000	160,000	1.55	1.53
Probe 14 -Tb ³⁺	332	4,300	0.09 ***	50,000	1.11	11.6	7,400	66,000	1.72	1.32
Probe 15 -Tb ³⁺	328	90,258	1.2	88,000	1.14	0.98	119,800	119,000	1.33	1.35
Probe 16 -Tb ³⁺	328	3,300	0.22 ***	39,000	1.07	11.8	6,000	65,500	1.81	1.68

* Reference compounds described in (16, 19).

** Counts are the reading for maximum wavelength emission (547 nm for Tb³⁺, or 615 nm for Eu³⁺ chelates)

*** Short-lived component