The Synthesis and Characterization of New Cross-Linkable Quinolone and Quinoline-Based Luminescent Lanthanide Chelates

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***Abstract*-Luminescent lanthanide probes possess long emission lifetime, which enables their hypersensitive detection in time-resolved mode that avoids short-lived background fluorescence. Sharply spiked emission spectra of lanthanide probes and large Stokes shifts further enhance detection sensitivity, which is about 1000 fold higher than that for regular fluorescent probes. The wide spread of this promising technology is limited by high cost of commercially available compounds, which is mainly due to their complex structure and laborious synthetic procedure. In this study, new efficient strategies to simplify the synthesis of the probes were explored. New lanthanide chelates, containing click-, and amine-reactive cross-linking groups, which are highly bright and can be produced with high yield, are synthesized and characterized.**

Keywords- Quinoline; Quinolone; Lanthanide; Luminescence; Cross-Linking; Probes

I. INTRODUCTION

Lanthanide luminescent chelates possess unique light-emitting properties that allow wide application of the compounds for technical and biological tasks [1-10]. The probes can be used for highly sensitive assays for detection of biological macromolecules as well as for imaging of micro objects. Technical applications of lanthanide complexes include design of organic light emitting diodes [11-13], sensors [14-18] and waveguide amplifiers for lasers [19-22].

Emission of the excited lanthanide ion is long lived (from microsecond to millisecond ranges), which enables time-gated detection. Long luminescence life-time is due to the fact that the transitions from the excited to the ground states are formally forbidden by the Laporte rule. Another remarkable property of the lanthanide emission is the narrow emission bands. The emission lines are relatively narrow, because the electron transitions occur within the inner electron orbits, which are shielded from the medium by outer electrons. Such protection prevents broadening of the emission bands due to the interactions of the excited electron with a solvent. As a result, lanthanide emission exhibits fine structured, fingerprint-like spectrum, which facilitates its discrimination from background fluorescence. Also, lanthanide probes have a large (up to 270 nm) spectral separation (Stokes shift) between the excitation and emission light, while for the majority of conventional fluorophores it is about 10-30 nm. All these factors contribute to the high detection sensitivity of the lanthanide probes, which is about 1000 times greater than for regular fluorophores.

Lanthanide probes represent nanostructures of complex design (Fig. 1). Since the light absorption by lanthanide ion is low, the probes contain an organic fluorophore, which absorbs light and transfers the energy to a tethered lanthanide ion, thereby acting as an “antenna”. The energy transfer occurs in radiationless manner, which is facilitated by proximity of the antenna to the lanthanide ion. Usually, the lanthanide ion is retained by chelating group fused to the antenna. The chelating groups also protect lanthanide ions from coordination with water that would otherwise reduce the luminescence brightness via dissipation of the excitation energy due to electronic-to-vibrational energy transfer on the O-H bonds vibrations. The lanthanide probe also contains a reactive group for coupling of the probe to an object of interest. All these structural features make the development of the probes difficult and require sophisticated synthetic strategies. High sensitivity of lanthanide emission efficiency to subtle substitutions in the antenna fluorophore (which are required for the cross-linking group attachment) further complicates design of the probes.

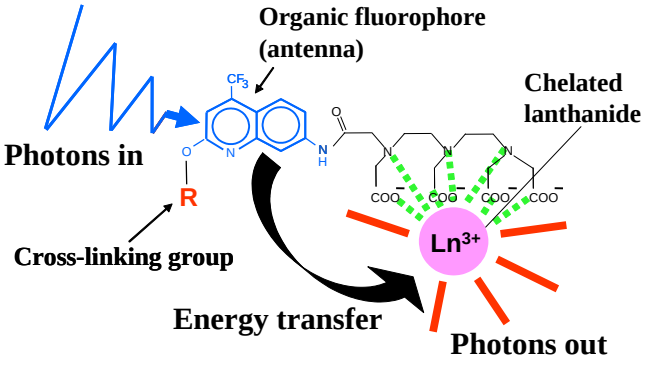


Fig. 1 The design and operational principle of a lanthanide luminescent probe

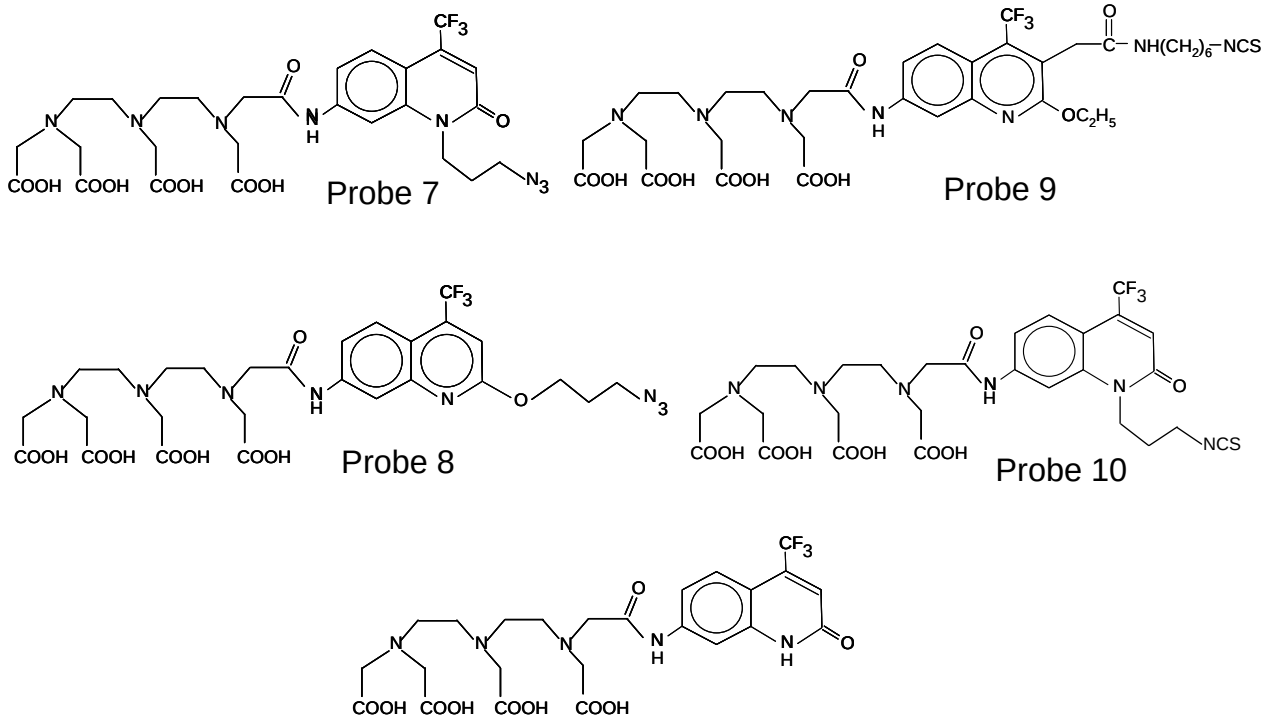


Fig. 2 The structure of luminescent lanthanide probes and the reference compound

Search for new simple efficient methods of the probes synthesis was the objective of this study. Carbostyril and coumarin-based lanthanide chelated are attractive candidates for probe development since they are highly soluble in water and possess high quantum yield [23]. In our previous studies, cross-linking lanthanide chelates of cs124 and cs124-CF_3 carbostyril fluorophores that possess desirable spectral properties and are highly soluble in water [23-34] were described. In the present study, novel strategies for the fast simple synthesis of lanthanide chelates of the same fluorophores (Fig. 2), which are highly bright and can be produced with high yields in 1-2 working days, are reported. The developed strategies ([32-33] and present study) allow synthesis of the reporter compounds with various reactive cross-linking groups as well as with other functional groups, thereby enabling the design of probes optimal for each particular application.

II. MATERIALS AND METHODS

A. Materials

The following reagents were purchased from Aldrich: Avidin, diethylenetriaminepentaacetic acid dianhydride (DTPA), triethylamine; 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; $EuCl_3$; silicagel TLC plates on aluminum foil (200 μm layer thick with a fluorescent indicator). Distilled and deionized water (18 MΩ cm^{-1}) was used. 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. All chemicals were the purest grade available. The fluorophores for the synthesis of Probes **7** to **10** were synthesized as described in our previous study [32, 33]. A 2'-O-methyl-RNA oligonucleotide containing the following sequence was used: 5' hexynyl – GCC UCG UCG CCG CAG CUA ACU AUC CGU GUG CGUC – NH_2 3'.

B. Synthesis

*Probes **7-10**.* DTPA dianhydride (25 mg) was dissolved in 0.2 ml of DMSO under heating (80°C). This solution was added to 10-12 mg of the corresponding fluorophore compound and the mixture was rigorously agitated. After incubation for 40 min at 45°C, the mixture was poured into 4 ml of ether. The residue was suspended in 0.3 ml of dioxane followed by addition of 0.2 ml of water. After 10 min incubation at room temperature, the solution was subsequently supplemented with 10 ml of butanol and 4 ml of water. After extraction, the organic layer was collected and supplemented with 1.2 equivalent of 0.1 M solution of $LnCl_3$. After rigorous agitation, the organic phase was collected and evaporated in *vacuo* to a volume of about 1 ml. The solution was left for 20 min at 4°C. The precipitate was collected, subsequently washed with butanol and ether and dried in *vacuo* to afford ca. 6 mg of the crude Probes **7 to 10**.

The probes were purified by preparative TLC on silicagel plates using acetonitrile-water (3:1) as developing system. MS (MALDI TOF). Probes **7, 8** (-1): -Eu, $+4H^+$ 687.2 (found), 687.0 (calculated). Probe **9** (-1); -Eu, $+4H^+$ 801.3 (found), 801.0 (calcd).

C. Click Reactions

Three microliters of 2 M triethylammonium acetate buffer, pH 7.0 and 15 µl of DMSO were mixed with 5 µl of 0.5 mM alkyne-modified oligonucleotide. One microliter of the 10 mM azido-fluorophore, 3 µl of 5mM ascorbic acid solution; and 3 µl of 10 mM Copper (II) – TBTA stock in 55% DMSO, were consequently added to the solution. After overnight incubation at room temperature, the mixture was supplemented with 3 µl of 3 M NaAc pH 5.5 and the oligonucleotide was precipitated by addition of 300 µl ethanol. The residue was additionally washed with 80% ethanol (2 x 0.3 ml), dissolved in water and subjected to reverse phase HPLC.

D. Physical Methods

Excitation and emission fluorescence spectra in the continuous mode were recorded using a Quanta Master 1 (Photon Technology International) digital fluorometer at ambient temperature. UV absorption spectra were recorded on a Cary 300 Bio UV-Visible spectrophotometer (Varian). Mass spectra were obtained at the Center for Advanced Proteomics Research (UMDNJ) using 4800 MALDI TOF/TOF device (Applied Biosystems).

III. RESULTS

A. The Synthesis of the Lanthanide Luminescent Probes

In our previous studies, [32, 33] strategies for derivatization of cs124 quinolone fluorophores, which produced the desired compounds with high yield have been developed. These methods utilize alkylation of the fluorophore at N1 and 2-oxo-groups yielding N-substituted quinolone and O-substituted quinoline derivatives. In the present study, the possibility to use these compounds as sensitizers for lanthanide emission was explored. To this end, the compounds were further derivatized by acylation with DTPA anhydride at the 7-amino group (as exemplified in Fig. 3 with amine-reactive quinolone fluorophore) followed by complexation with a lanthanide ion. The acylation reaction was monitored by recording the UV absorption spectrum of the reaction mixture aliquots. As shown in Fig. 4, acylation was accompanied by batochromic shift of the absorption maxima. Notably, acylation did not change the maximal absorption of the N-substituted quinolone compounds (Fig. 4A), while a significant increase in the absorption was observed for the O-substituted quinoline derivatives (Fig. 4B). The presence of isobestic points was indicative of formation of single reaction products, which was confirmed by chromatographic analysis of the reaction mixtures. The products were obtained with high yield and further purified by preparative TLC, or HPLC. Their identity was confirmed by MS. As follows from MS spectra at the desorbtion stage the compounds loose lanthanide ions. Most likely this is due to the highly acidic conditions resulting in protonation of the molecule, which converts the analyzed compounds to positively charged molecular ions amenable for the analysis.

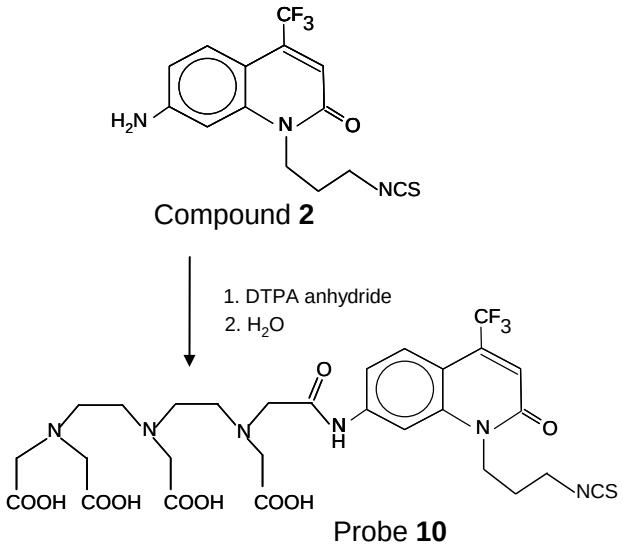
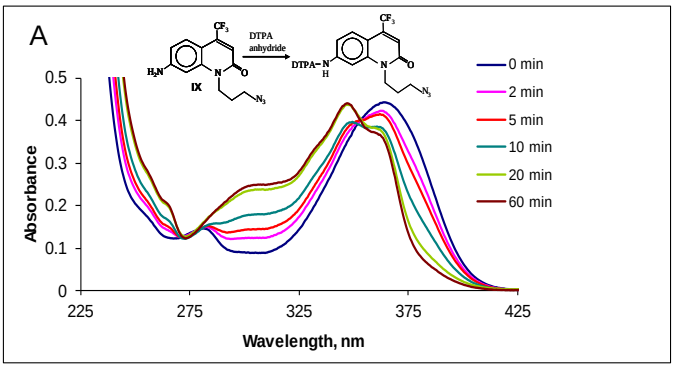


Fig. 3 The scheme for DTPA acylation reaction of reactive antenna fluorophore leading to luminescent lanthanide probe

B. Light Absorption Spectra of the Probes

As shown in Fig. 5, the probes possess distinctive UV absorption spectra in the spectral region of 280-400 nm. Probe **7** has the absorption maximum at 342 nm, while Probes **8** and **9** have two maxima (321 and 337 nm for Probe **8**; 328 and 342 nm for Probe **9**). The absorption spectrum of Probe **10** is the same as of Probe **7**.



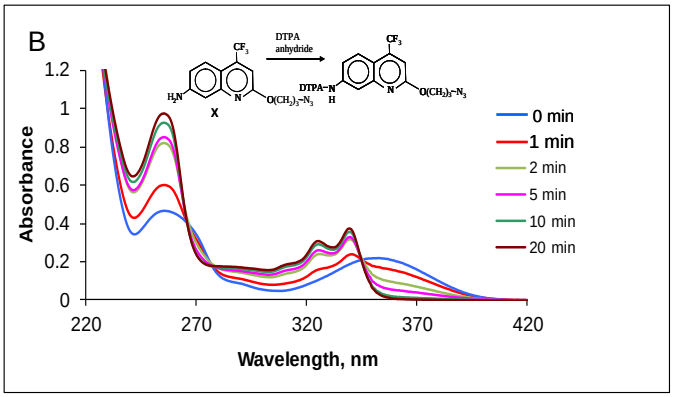


Fig. 4 UV absorption spectra of the reaction mixtures containing quinolone (A) and quinoline (B) reactive antenna fluorophore and DTPA anhydride recorded at indicated time intervals

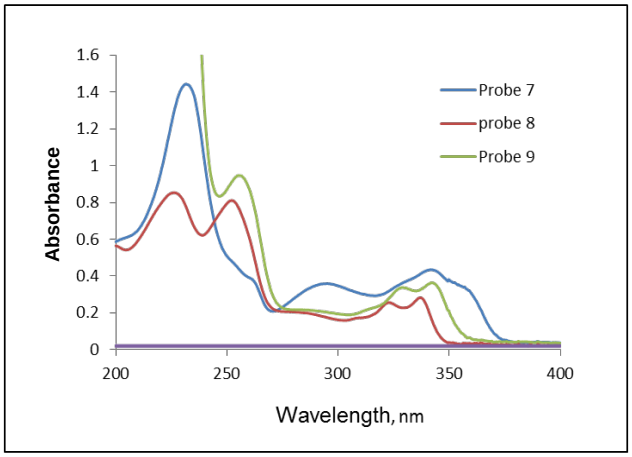


Fig. 5 UV absorption spectra of the Probes **7**, **8**, and **9**.

Notably, Probe **7** possesses significant absorption in the region 365-380 nm where Probes **8** and **9** do not absorb the light. Also, Probe **9** has absorption in the region 345-360 nm where Probe **8** does not absorb. This allows selective excitation of the probes using different light sources, which could be beneficial for multiplex assays carried in the same tube.

C. Light Emitting Properties of the Synthesized Compounds

The energy transfer from antenna to lanthanide is the most essential, complex process which is not yet understood in detail. The energy transfer process is highly sensitive to subtle variations in the antenna structure [31]. Given that our cross-linking groups are introduced directly in the antennae fluorophores, it is important to know how these modifications affect the brightness of the resulting lanthanide chelates. Fig. 6 shows the emission spectra of the synthesized probes. As expected, complexation of metal-free compounds with a lanthanide ion results in the decrease of the antenna fluorescence (in the region 400-450 nm) due to the energy transfer to the lanthanide ion and the appearance of sharply spiked emission typical for lanthanide ions (in the region 480-650 nm). Notably, in the case of Probe **8** the antenna emission spectrum profile does not change upon complexation (Fig. 6A), while a significant blue shift was observed for Probe **7** (Fig. 6B). The reason for such behavior is not yet recognized. As can be seen from Table 1, with Tb^{3+} the brightness of quinolone compound (Probe **7**) decreased ca. 3 fold compared to the model compound with a unmodified original fluorophore cs124CF_3, while the O-modified derivatives (Probes **8** and **9**) exhibit factor 1.3 to 1.5 increase in the light emission. The Eu^{3+} complexes behave differently. Probes **7** and **8** retained high brightness as compared to their reference complex, while a twofold decrease in the light emission intensity was observed for Probe **9**. The Dy^{3+} chelates of Probes **7** and **8** were significantly brighter (3.4 and 4.4 fold, respectively) than the corresponding complexes of the reference compound. In general, similar behavior was observed for the Sm^{3+} chelates.

The emision quantum yield of an excited lanthanide ion (defined as the probability of the excited state to emit a photon) in the antenna-chelate complex strongly depends on the number of coordinated water molecules due to the non-radiative dissipation of the energy of the excited state through vibration excitation of O-H bonds [35]. This process with heavy water is less efficient due to the lower frequency of the O-D bond vibration. This effect accounts for 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 ca. 1.05 to 1.20 fold higher than in H_2O-based solutions. As expected, the effect was more pronounced for DTPA-Eu^{3+} chelates (ca. 2.6 to 4 fold) as well as for Dy^{3+} and Sm^{3+} complexes (ca. 1.2 to 3.7 fold).

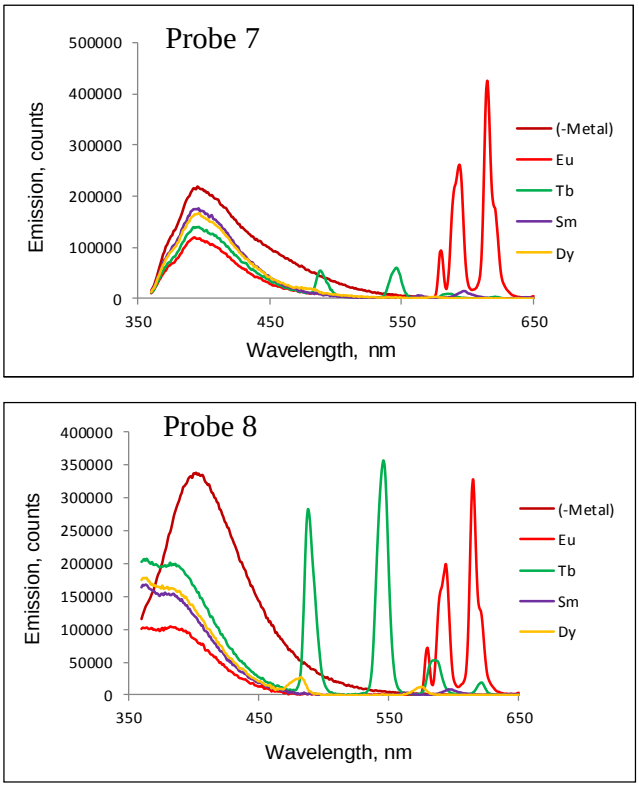


Fig. 6 Light emission spectra in heavy water for probes **7** and **8** complexed with different lanthanide ions

D. Coupling of the Probes to Oligonucleotides Using Click Reaction

The cycloaddition of azido compounds to acetylenic counterparts with the formation of triazoles known as 'click reaction' is widely used in biochemical studies for highly specific labeling of the biomolecules of interest [36, 37]. In the present study, the ability of our probes to couple 2'-O-methyl-RNA oligonucleotides through this reaction was tested. HPLC analysis of the reaction mixtures revealed nearly quantitative coupling of the fluorophores to the oligonucleotides. These results suggest suitability of the synthesized compounds for fluorescent DNA and RNA labeling. It should be mentioned that attachment of fluorophores to the oligonucleotide was accompanied by considerable quenching (more than 10 fold) as judged by the increased ratio of antenna-to-lanthanide emission (Fig. 7) and

general decrease of antennae fluorescence. Most likely this was due to the stacking interactions of the fluorophores with 2'-O-methyl-RNA bases, which should affect the energy transfer process. As seen from Fig. 7 this effect was more pronounced for the Eu-based probe.

Table 1. Emission (at [10 nM]) and relative brightness of lanthanide chelates in water and deuterium oxide solutions

Compound	Emission		Relative brightness	% Relative brightness	
	H_2O	D_2O	D_2O/H_2O	H_2O	D_2O
		Tb^{3+} complexes (emission at 545 nm,counts)			
DTPA-cs124CF$_3$[a]	12200	14170	1.16	100	100
DTPA-cs124CF$_3$-(N)-N$_3$ (probe 7)	4560	4700	1.03	37	33
DTPA-cs124CF$_3$-(O)-N$_3$ (probe 8)	18700	19680	1.05	153	140
DTPA-cs124CF$_3$-3NCS (probe 9)	15500	18700	1.21	130	132
		Eu^{3+} complexes (emission at 615 nm,counts)			
DTPA-cs124CF$_3$[a]	10350	41000	3.96	100	100
DTPA-cs124CF$_3$-(N)-N$_3$ (probe 7)	9770	36700	3.76	94	90
DTPA-cs124CF$_3$-(O)-N$_3$ (probe 8)	11760	47780	4.06	113	117
DTPA-cs124CF$_3$-3NCS (probe 9)	5300	13800	2.60	51	34
		Dy^{3+} complexes (emission at 482 nm,counts)			
DTPA-cs124CF$_3$[a]	567	1000	1.77	100	100
DTPA-cs124CF$_3$-(N)-N$_3$ (probe 7)	1930	2450	1.27	340	245
DTPA-cs124CF$_3$-(O)-N$_3$ (probe 8)	2540	2670	1.05	440	267
		Sm^{3+} complexes (emission at 598 nm,counts)			
DTPA-cs124CF$_3$[a]	200	760	3.71	100	100
DTPA-cs124CF$_3$-(N)-N$_3$ (probe 7)	460	1440	3.15	230	190
DTPA-cs124CF$_3$-(O)-N$_3$ (probe 8)	280	834	3.00	110	117

[a] Reference compound

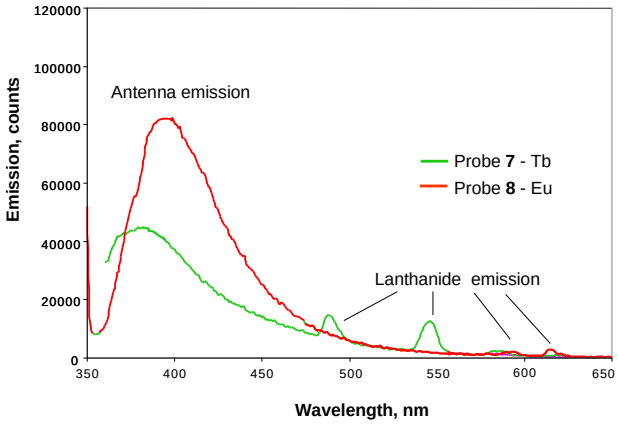


Fig. 7 Light emission spectra for Probes **7** and **8** attached to an oligonucleotide.

IV. DISCUSSION

The present study represents the closing step for the validation of a new simple and fast strategy for the synthesis of bright lanthanide probes. In our previous research, a new alkylation reaction, which allowed us to introduce reactive groups in commonly used carbostyril-based fluorophores at N1 and O2 positions with high yield was discovered. In this paper an attempt was made to use these compounds for design of luminescent lanthanide chelates. It turned out that in many cases the light emitting properties of the resulting probes were even superior to those of the original compound consisting of a non-modified antenna fluorophore and chelated lanthanide. Examples are: Tb^{3+}-chelated Probes **8** and **9** (1.4 and 1.3 fold increase), Eu^{3+}-chelated Probe **8** (13 % increase), Dy^{3+}-chelated Probes **7** and **8** (3.4 and 4.4 fold increase), as well as Sm^{3+}-chelated Probe **7** (2.3 fold increase). Emission of Eu^{3+} and Sm^{3+} chelates strongly increased in heavy water, therefore using this medium is recommended for more sensitive detection.

Probe **8** represents a chelate with a quinoline antenna fluorophore. This class of compounds has not been tested before as sensitizers for lanthanide emission. Remarkably, Probe **8** displayed high and comparable emission with both Tb^{3+} and Eu^{3+}, which makes this probe especially valuable for differential proteomic studies, whose goal is establishing the influence of the cellular environment or chemical agents on the expression of individual cellular proteins. These studies rely on using fluorophores with nearly identical size and electrophoretic mobility, but different emission spectra to label and separate protein samples from the cells that are grown at different conditions. After the labeling, the samples are mixed and the proteins are 2D separated according to their isoelectric points and size by using electrofocusing and denaturing SDS gel electrophoresis correspondingly. After the separation the light emission spectrum for each labeled separated protein spot is determined. For the proteins, whose expression is not

affected by a change in the growth condition, the ratio of the emission signals from the two labels is constant. Alteration in the ratio (which reveals the different ratio of the protein content at a particular growth condition) pinpoints the polypeptides, whose expression in the cell is sensitive to the growth conditions. Lanthanide chelates containing the same antenna fluorophore, but different lanthanide ions are ideal probes for these assays since they: i) possess distinctive, sharply spiked signature-like emission spectra, which can be easily deconvoluted; ii) can be sensitively detected in the time-resolved mode; iii) have nearly the same molecular mass (e.g. the difference between Tb- and Eu-based probes is only seven mass units, which is less than 1 % of their overall molecular weight).

In addition, suitability of these novel labeles for production of light-emitting oligonucleotide hybridization probes, which are widely used for sensitive determination of nucleic acids, was demonstrated. As follows from our previous study [31] the detection sensitivity of the hybridization probes labeled with amine-reactive lanthanide chalates was about 1 pM, which significantly exceeds the detection limit for regular fluorescent labels. These probes can be especially useful in the applications in which the detected signal cannot be additionally enhanced (e.g. using PCR amplification reaction). Such applications include intracellular imaging and detection of single molecules.

Compared to our previously developed luminescent probes [31] click-reactive labels (Probes **7** and **8**) are more avantageous due to their unusually high coupling efficiency at nearly equimolar oligonucleotide-to-label ratio. It was also demonstrated that the click-reactive probes obtained in this study possess high conjugation efficiency. However, coupling to an oligonucleotide (Fig. 7) significantly reduced the emission signal of the probes (ca. 10 fold). This drop was not due to the loss of a lanthanide during the cross-linking reaction since subsequent addition of the lanthanide to labeled oligonucleotide did not enhance the light emission. Previously we observed 2-7 fold quenching with cs124 fluorophore derivatives attached to oligonucleotides [32], which was most likely due to contact quenching facilitated by close proximity of the fluorophore to oligonucleotide nitrous bases. With the lanthanide probes the effect can be further aggravated by high sensitivity of antenna-to-lanthanide energy transfer to the stacking interactions with the bases [31]. The later conclusion is supported by enhanced ratio of antenna-to-lanthanide emission (compare Figs. 6 and 7), which is indicative for decreased energy transfer efficiency. The next challenge is to design lanthanide probes free of this shortcoming.

ACKNOWLEDGEMENT

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