Anal. Chem. **2009,** *81,* 9449–9453

Highly Sensitive Protein Detection Based on Lanthanide Chelates with Antenna Ligands Providing a Linear Range of Five Orders of Magnitude

Thole Zuchner,*,† Frank Schumer,*,‡ Renate Berger-Hoffmann,† Katrin Müller,† Mathias Lukas,† Kornelia Zeckert,§ Jörg Marx,‡ Horst Hennig,§ and Ralf Hoffmann†

Institute of Bioanalytical Chemistry, Center of Biotechnology and Biomedicine, Faculty of Chemistry and Mineralogy, Leipzig University, Deutscher Platz 5, 04103 Leipzig, Germany, XynTec Chemie GmbH Wolfen, ChemiePark Bitterfeld Andresenstrasse 1a, D-06766 Wolfen, Germany, and Institute of Inorganic Chemistry, Faculty of Chemistry and Mineralogy, Leipzig University, Johannisallee 29, 04103 Leipzig, Germany

Protein detection is an important task for pharmaceutical and clinical research today. Numerous protein staining techniques exist but are limited regarding their sensitivity and often narrow linear quantification ranges. To the best of our knowledge, this is the first description of a novel class of lanthanide chelatators, which absorb in the lower energy region at 360 nm. The new compound (6,9-dicarboxymethyl-3-{4-([1,10]-phenanthrol-2-ylethinylphenyl-carbamoyl)-methyl}-3,6,9-triaza-)-undeca-1,11-dicarboxylic acid) was coupled to different proteins and showed highly sensitive protein detection limits in both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1.5 fmol of bovine serum albumin) as well as Dot Blot (100 amol of lysozyme). Furthermore, the novel compound shows an exceptionally broad linear quantification range over 5 orders of magnitude allowing applications that require the highest sensitivity alongside standard sensitivity. In addition, the new compound offers multiplexing capabilities.

A major task in pharmaceutical and clinical research today is the analysis of the proteome. The proteome is defined as the entire set of proteins expressed by the genome of a cell, tissue, or organism under well-defined conditions. As proteins are central to understanding most diseases, it is crucial that improvements are made in existing protein detection and analytical methods, with respect to sensitivity and dynamic range.

To the best of our knowledge, this is the first description of a novel class of lanthanide chelatators that absorb in the lower energy region at 360 nm. We also demonstrate its chemical coupling to proteins and its use for highly sensitive protein detection. The polydentate ligand diethylenetriaminepentaacetic acid (Scheme 1 DTPA, **1**) was linked via an amino phenylethinyl bridge with 1,10-phenanthroline to obtain (6,9-dicarboxymethyl-3-{4-([1,10]-phenanthrol-2-ylethinylphenyl-carbamoyl)-methyl}-3,6,9-triaza-)-undeca-1,11-dicarboxylic acid (LH_4, **3**).[11,12] The phenanthroline unit acts as an antenna group enabling light absorption in the lower energy region. The phenylethinyl bridge promotes an efficient intramolecular energy transfer from the electronically excited phenanthroline unit to coordinated Eu^{III}.

The antenna group allows for electronic excitation at lower energies when compared with corresponding Eu(III) chelates without antenna function. Low excitation energies are advantageous to avoid protein excitation that may lead to background noise in luminescence spectroscopic detection methods. Furthermore, the shift to a low energy excitation maximum of the Eu(III) complex (near-UV-spectrum, 360 nm) allows sensitive detection with the use of glass-optics instead of expensive fused-quartz optics (Figure S-5 and Table S-1 in the Supporting Information). These properties allow the use of this new lanthanide chelate for highly sensitive protein detection.

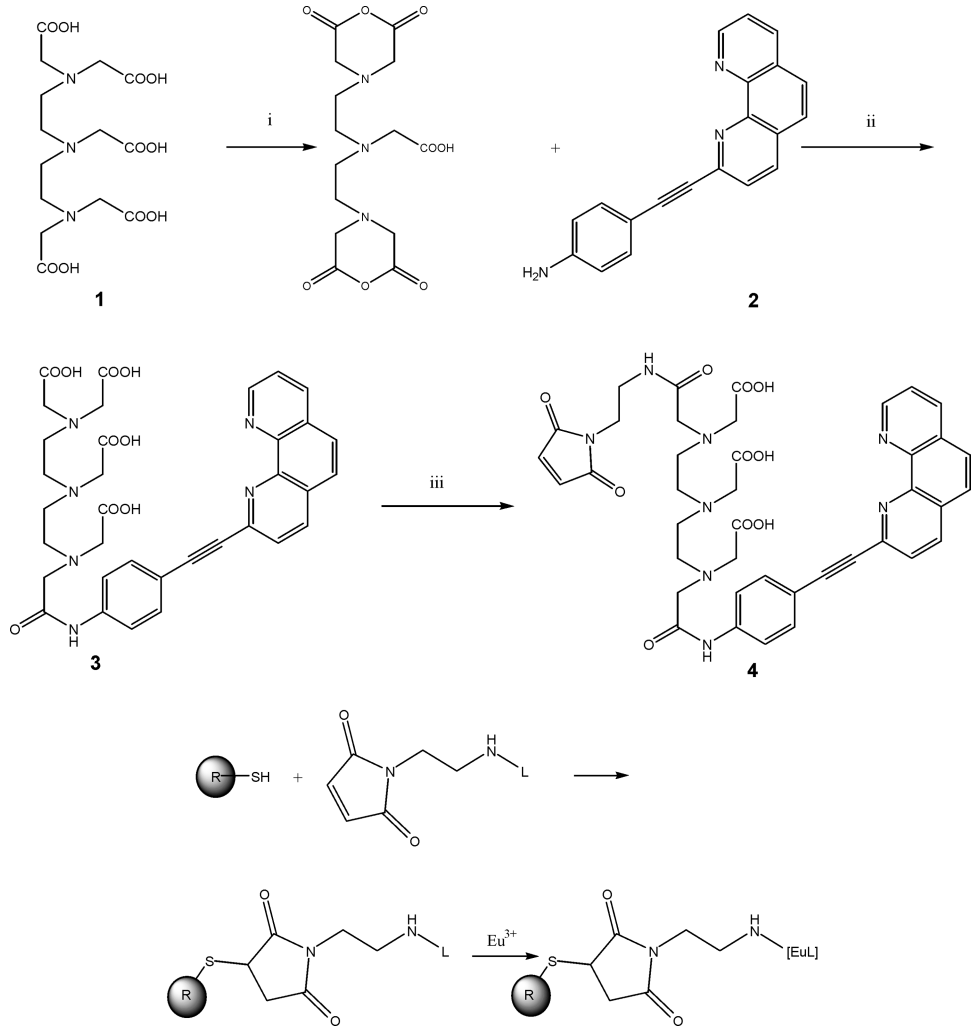
Most commonly applied methods for protein detection include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates protein mixtures according to their molecular masses, and 2D-gel electrophoresis, in which proteins are separated first by their isoelectric points and then by molecular mass. Both methods show limited protein detection limits, even when used with prelabeling techniques with fluorescence dyes (e.g., Cy3, Cy5). Current protein detection limits of common staining methods such as silver staining[7] or the more recent fluorescence dyes lie within the nanogram range, corresponding to picomolar to partly subpicomolar concentrations.[1−6] However, the number of a certain protein expressed can be as small as 10−100 molecules per cell, resulting in atto- or zeptomolar

(1) Wetzl, B. K.; Yarmoluk, S. M.; Craig, D. B.; Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2004**, *43*, 5400–5402.
(2) Suzuki, Y.; Yokoyama, K. *J. Am. Chem. Soc.* **2005**, *127*, 17799–17802.
(3) Heyduk, E.; Heyduk, T. *Anal. Chem.* **2005**, *77*, 1147–1156.
(4) Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*, 9th ed.; Molecular Probes: Eugene, OR, 2002.
(5) Jones, L. J.; Haugland, R. P.; Singer, V. L. *Biotechniques* **2003**, *34*, 850–861.
(6) Craig, D. B.; Dovichi, N. J. *Anal. Chem.* **1998**, *70*, 2493–2494.
(7) Switzer, R. C., 3rd; Merril, C. R.; Shifrin, S. *Anal. Biochem.* **1979**, *98*, 231–237.

* To whom correspondence should be addressed. E-mail: zuechner@rz.uni-leipzig.de (T.Z.); frank.schumer@xyntec.net (F.S.).
† Institute of Bioanalytical Chemistry, Center of Biotechnology and Biomedicine, Leipzig University.
‡ XynTec Chemie GmbH Wolfen.
§ Institute of Inorganic Chemistry, Leipzig University.

10.1021/ac902175g CCC: $40.75
Published on Web 10/09/2009

Scheme 1. Upper Part, Synthesis of the Maleimidyl-Derivative (4) of LH_4 (3),[a] and Lower Part, Coupling of LH_4 in Complex with Eu^{III} ([EuL]H) as Maleimide to Cys Residues of Arbitrary Protein[b]



[a] (i) acetic anhydride, 16 h, RT; (ii) Et_3N, DMF, 40 °C, 4 h then 18 h, RT; (iii) 2-aminoethylmaleimide hydrochloride, NHS, DIC, Et_3N, DMF, RT 24 h. [b] R = lysozyme or BSA (bovine serum albumin).

amounts of protein available for detection, depending on the available amount of sample material.[8,9]

Usual organic fluorophore dyes offer a great potential for improved sensitivities. However, the gels used in electrophoreses and even the membranes to which those proteins can be transferred show high background fluorescence, which dramatically reduces signal-to-noise ratios. Thus, these techniques are, in practice, limited by similar sensitivities as silver-based techniques, although their dynamic range for relative protein quantification is superior to them, spanning 3 to sometimes 4 orders of magnitude. The problem of background fluorescence can be overcome by using longer lived fluorescence dyes and time-resolved fluorescence spectroscopy (TRF). TRF relies on special dyes emitting light for much longer time periods (millisecond range) after excitation than the background fluorescence (nanosecond range). Data are recorded after the background fluorescence has decayed and thereby provide very high signal-to-noise-ratios (S/N ratios). Lanthanide ions are particularly interesting candidates for TRF due to their long lasting phosphorescence, large Stokes shifts, and narrow emission bands. However, very high excitation energies are needed for lanthanide ions and therefore existing lanthanide dyes like, e.g., Sypro Rose Plus or Bathophenanthroline disulfonate only give protein detection limits in the range of 2−16 ng.[10a−c] As an alternative, antenna ligands, which absorb in lower energy regions, are used to transfer the excitation energy to the lanthanide. Here, we describe such an antenna ligand and its use for protein detection.

EXPERIMENTAL SECTION

The ligand LH_4 (Xyntec LLC, Wolfen) was synthesized by coupling the anhydride of DTPA with 4-[1,10]phenanthrolin-2-ylethynyl-phenylamine **2** using the reaction conditions described in Scheme 1. It was converted into the maleimide derivative **4** for coupling to proteins. All products were chromatographically purified, and their identity was validated by matrix

(8) Leichtling, B. H.; Su, Y. F.; Wimalasena, J.; Harden, T. K.; Wolfe, B. B.; Wicks, W. D. *J. Cell. Physiol.* **1978**, *96*, 215–223.

(9) Stylianou, E.; O'Neill, L. A.; Rawlinson, L.; Edbrooke, M. R.; Woo, P.; Saklatvala, J. *J. Biol. Chem.* **1992**, *267*, 15836–15841.

(10) (a) Kemper, C.; Berggren, K.; Diwu, Z.; Patton, W. F. *Electrophoresis* **2001**, *22*, 881–889. (b) Lim, M. J.; Patton, W. F.; Lopez, M. F.; Spofford, K. H.; Shojaee, N.; Shepro, D. *Anal. Biochem.* **1997**, *245*, 184–195. (c) Diamandis, E. P. *Clin. Chem.* **1991**, *37*, 1486–1491.

(11) Zeckert, K. Ph.D. Thesis, Leipzig University, Leipzig, Germany, 2002.

(12) Marx, J. Sensient Imaging Technologies GmbH, WO 2005/108405, 2005.

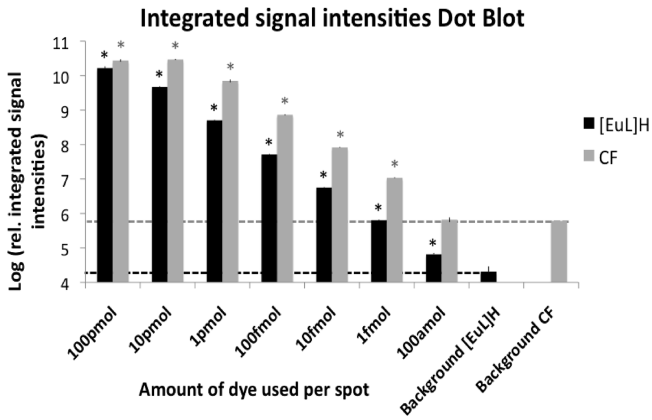


Figure 1. Average signal intensities of a dilution series of [EuL]H and carboxyfluorescein (CF) in Dot Blot. Peak volumes are shown. Only signals significantly higher than the corresponding background are given ($n = 3$; $p < 0.05$, paired two-sided t test). Error bars show standard deviation.

assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry and/or nuclear magnetic resonance (NMR) (Figure S-1−S-3 in the Supporting Information).

Emission and excitation spectra for [EuL]H and [TbL]H were recorded in imidazole buffer (50 mM, pH 8.0) on a Perkin Elmer LS 55. For Dot Blot experiments, a Hybond-LFP-PVDF membrane (Amersham Biosciences) was used. After activation of the membrane in methanol, samples were diluted in imidazole buffer (50 mM, pH 8.0) and spotted using a sample volume of 2 μL per spot.

LH_4 was coupled to the protein as a maleimide following standard protocols. For all experiments, Eu^{III} or Tb^{III} was complexed with samples containing coupled LH_3 in imidazole buffer for 30 min. BSA was labeled with [EuL]H, and SDS-PAGE was performed as standard SDS-PAGE minigels (Miniprotean; Bio-Rad Laboratories, Hercules, CA). After preincubation with Eu^{III} in imidazole buffer (50 mM, pH 8.0), labeled proteins were denatured in sample buffer for 5 min at 95 °C. Gels were directly scanned with a modified version of the "TRF Detection Cartridge" on the Paradigm Detection platform from Beckman Coulter (time delay 10 μs, excitation wavelength 360 nm). The same time delay was used for PVDF membranes. The modification of the Paradigm Detection platform was mainly based on changes of the TRF scanning cartridge. This cartridge was modified as follows: modification of the excitation source to a high-power UV-LED, improvement of the scan resolution to 100 μm edge length, and coordination of membrane transport time with measurement time to allow on-the-fly detection. The Multimode software was adapted to allow continuous scans of planar surfaces. For final imaging purposes, the software ImageJ 1.43 (http://rsbweb.nih.gov/ij) was used. BHHCT was obtained from Chemodex Ltd., Switzerland.

RESULTS AND DISCUSSION

After synthesis, purification, identification, and spectrophotometric characterization (see Supporting Information) of the novel dye LH_4, the compound was further characterized in terms of its applicability for sensitive time-resolved fluorescence (TRF) detection. As a first characterization, LH_4 in complex with Eu^{III}([EuL]H) was spotted on PVDF membranes. The signal intensity was compared to a commonly used fluorescence dye,

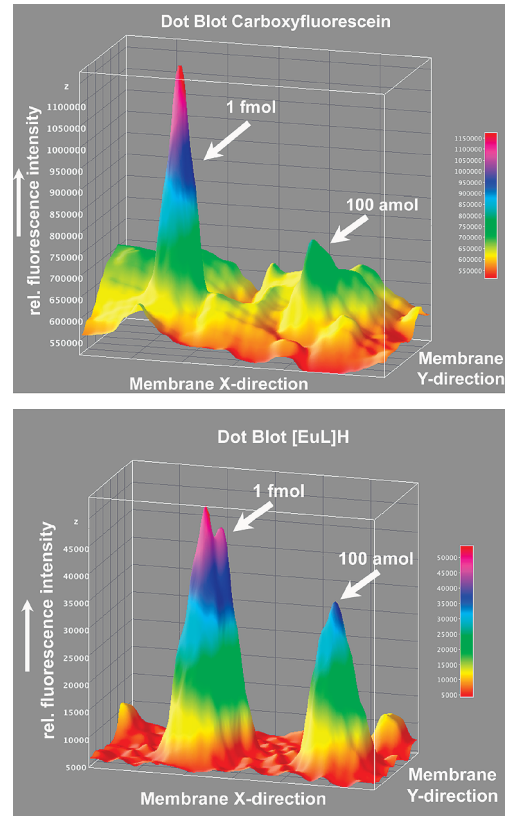


Figure 2. Example spots of 1 fmol and 100 amol of [EuL]H and 1 fmol and 100 amol of CF on Hybond-LFP-PVDF membrane. The background fluorescence masks the 100 amol of CF spot, whereas the 100 amol of [EuL]H can be clearly distinguished against the background.

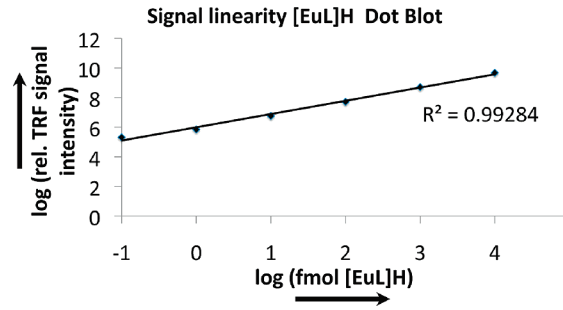


Figure 3. Signal linearity of TRF signal to [EuL]H concentration. Double logarithmic plot for practical reasons. See text for further explanations. Signal linearity given for 5 orders of magnitude with a regression coefficient of $R^2 = 0.992\,84$.

namely, fluorescein. The detection limit of LH_4 complexed with Eu^{III}([EuL]H) in this experiment was improved by a factor of 10 (Figure 1).

This improvement can be expained by the dramatically reduced background in TRF compared with normal fluorescence as can be seen from Figure 1. The high background fluorescence in the carboxyfluorescein (CF) scan masks the signal for 100 amol of CF. For all scans, only signals with a S/N ratio of higher than 5 were considered positive.

As an example for the spots quantified in Figure 1, a scan of spots corresponding to 1 fmol and 100 amol of [EuL]H and carboxyfluorescein (CF) in Dot Blot is shown in Figure 2. This demonstrates a much stronger signal for [EuL]H than for CF at

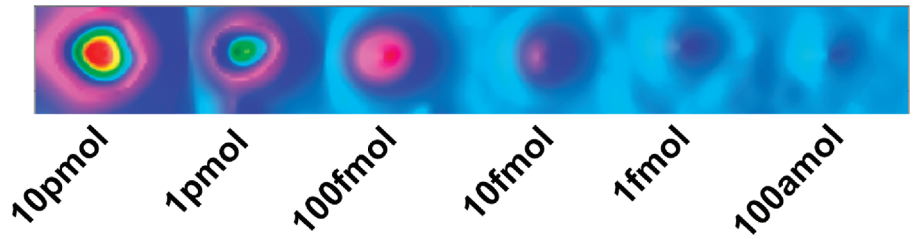


Figure 4. Scan of lysozyme labeled with [EuL] in Dot Blot. Colors correspond to increasing TRF signal intensities.

100 amol and most importantly demonstrates the improved signal-to-noise ratio in TRF measurements.

Apart from sensitive detection limits, another important feature of a suitable dye for protein detection is a broad range of signal linearity. Although some staining techniques like, e.g., silver staining, show good sensitivity (around 1 ng of protein), their usefulness for protein quantification is limited as their linear ranges only span a few orders of magnitude. In the case of silver staining, signal linearity only extends over 1 order of magnitude at the most. Even common fluorescence dyes, (Cy3, Cy5) or Coomassie staining exhibit signal linearities of only 2 to 3, or maximum 4, orders of magnitude.[7,14,15] In Figure 3, signal linearity is shown for [EuL]H. Signal linearity was given over a concentration range of 5 orders of magnitude (from 0.1 to 10 000 fmol of LH_4). For practical reasons, the linearity plot is shown as a double logarithmic plot. As the linearity of a curve is defined by demonstrating the same slope in every part of the curve, linearity was double-checked by plotting the slopes of every given concentration range against the concentration ranges. This revealed no significant differences in slopes in the range of 5 orders of magnitude (data not shown). To the best of our knowledge, a signal linearity for protein staining of 5 orders of magnitude has never been demonstrated before. This property allows for applications that require the highest sensitivity alongside standard sensitivity and offers great potential for significant savings in both cost and time for analytical procedures.

For protein detection, coupling of [EuL]H to the protein sample is required. We demonstrate coupling of [EuL]H to the following proteins: bovine serum albumin (BSA), lysozyme, phosphorylase B, and ovalbumin. The proteins were subsequently visualized using the TRF properties of the coupled [EuL]H. When [EuL]H was coupled to lysozyme as maleimide, Dot Blot techniques revealed a highly sensitive detection limit of 100 amol of protein (Figure 4). This demonstrates the signal stability of the antenna chelate when coupled to a protein as well as the high potential of [EuL]H in terms of improved protein detection limits.

Furthermore, the protein detection limit based on [EuL]H was tested in SDS-PAGE as SDS-PAGE is the most commonly used technique to analyze protein expression. As shown in Figure 5, the protein detection limit following SDS-PAGE was improved more than 10-fold compared to common detection methods, like silver staining, Coomassie staining, Krypton stain, or Sypro Ruby and equaled 1.5 fmol of BSA loaded onto the gel. A positive signal was defined as a S/N-ratio higher than 5. For other proteins apart from BSA (phosphorylase B, ovalbumin, Figure S4 in the Supporting Information), comparable detection limits were achieved.

In the field of other prestains, the Py dye shows a comparable detection limit (16 pg). However, Py dyes have a linear range from

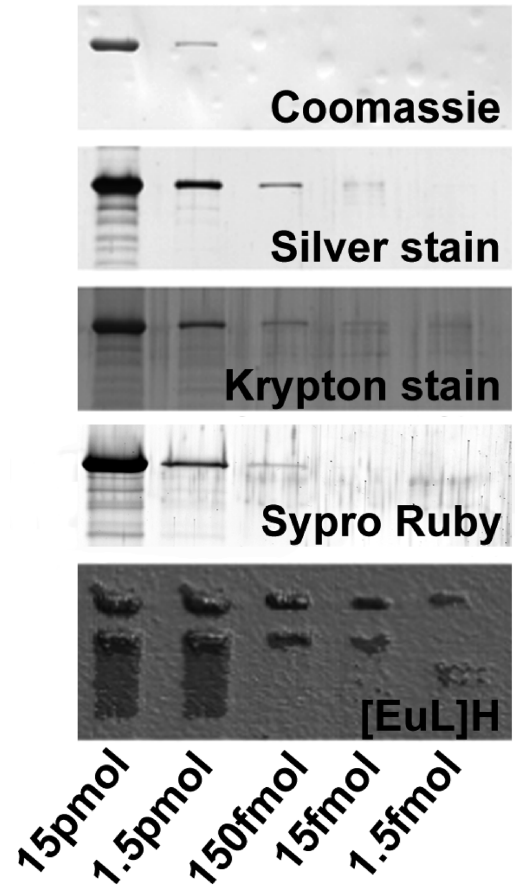


Figure 5. SDS-PAGE of BSA (bovine serum albumin) labeled with [EuL]. Identical aliquots were run on four different gels, and the gels were stained according to standard protocols.

0.8 to 100 ng only, i.e., quantitation in the range of the detection limit is not possible.[13]

The time-resolved fluorescence properties of the new compound LH_4 are based on the complexation of a lanthanide ion, in particular a europium ion. This leads to the characteristic pattern of sharp emission bands at 595 and 616 nm. However, as LH_4 can also complex other lanthanide ions aside from europium, it can be used in a more versatile way.

To study this potential, we complexed terbium instead of europium ions with LH_4. Figure 6 demonstrates that LH_4 can also activate terbium ions and that this results in a different emission spectrum with sharp emission bands at 495 and 548 nm. This property allows the use of LH_4 for multiplexing experiments. As the antenna and chelating part of [TbL]H are identical to those of [EuL]H, the shift in mass between the two when coupled to a protein is only caused by the mass difference of the lanthanide itself, in this case 7 Da and can therefore be

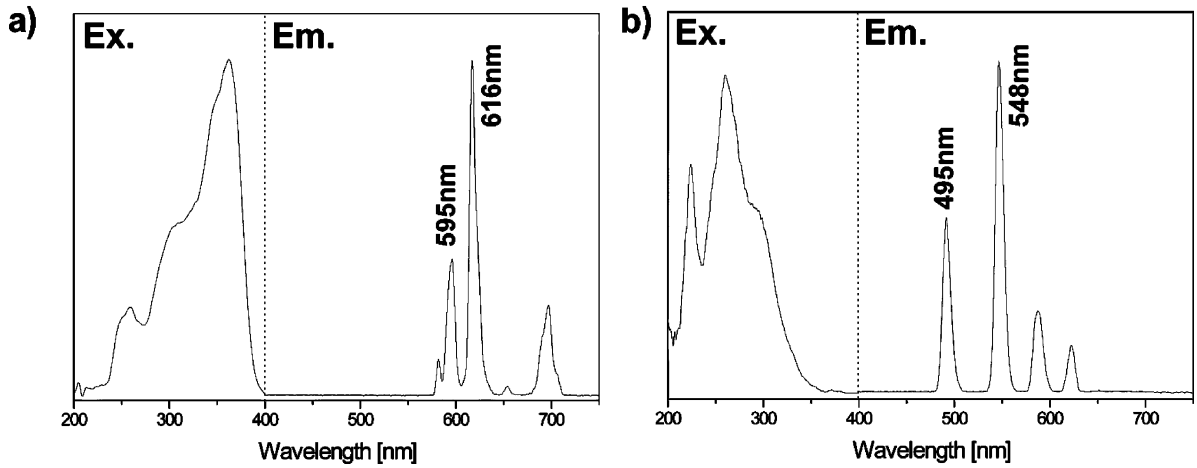


Figure 6. Emission (Em.) and excitation (Ex.) spectra of [EuL]H (a) and [TbL]H (b) (1×10^{-5} M in 0.05 M imidazole buffer pH 8).

neglected. Apart from its sharp emission bands, this is another advantage of the new compound when used for multiplexing experiments which involve protein separation techniques. LH_4 can also be used for other assay systems requiring high sensitivity and broad detection ranges, e.g., for FRET assays or peptide based ligand−receptor interaction studies.[16]

CONCLUSIONS

In conclusion, we have developed a new lanthanide chelate with antenna functionality and demonstrate its use for highly sensitive protein detection. The new compound described here shows a signal linearity of 5 orders of magnitude in concentration and shows improved sensitivity when used for protein detection. The fact that [EuL]H is suitable for quantifying very low amounts of proteins, as well as standard amounts, makes it both unique and ideal for protein analysis. Furthermore, [EuL]H is highly photostable and offers multiplexing capabilities.

(13) Meier, R. J.; Steiner, M. S.; Duerkop, A.; Wolfbeis, O. S. *Anal. Chem.* **2008**, *80*, 6274–6279.
(14) Nishihara, J. C.; Champion, K. M. *Electrophoresis* **2002**, *23*, 2203–2215.
(15) Johnson, J. A.; Lott, H. A. *Clin. Chem.* **1978**, *24*, 1931–1933.
(16) Shabanpoor, F.; Hughes, R. A.; Bathgate, R. A. D.; Zhang, S.; Scanlon, D. B.; Lin, F.; Hossain, M. A.; Separovic, F.; Wade, J. D. *Bioconjugate Chem.* **2008**, *19*, 1456–1463.

ACKNOWLEDGMENT

We gratefully acknowledge the expert technical assistance from Susen Joswiak. We would especially like to thank Beckman Coulter GmbH, Salzburg, for fruitful collaboration. We also thank Christina Nielsen-Marsh for proof-reading as well as Johannes Zeiser. This work was supported by BMBF Innoprofile, Project Number 03IP604, and BMBF, Project Number 03WKBI16p6.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 19, 2009. Accepted September 28, 2009.

AC902175G