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Stable and Highly Fluorescent Europium(III) Chelates for Time-Resolved Immunoassays

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ABSTRACT: Derivatives of 4-[2-(4-isothiocyanatophenyl)-ethynyl]-2,6,-bis{[*N*,*N*-bis(carboxymethyl)-amino]methyl}-pyridine europium(III) (**1**) bearing one (**6**) or two (**7**) additional iminodiacetate coordinating arms have been synthesized. **6** and **7** were significantly more stable than **1** as evidenced by competition experiments with ethylenediaminetetraacetic acid (EDTA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). While the luminescence quantum yield of **1** remained modest, the other two complexes displayed substantial luminescence efficiency. The introduction of a supplementary iminodiacetate arm in **6** brought important improvements to both the stability and the luminescence properties of the Eu complex. In contrast, although **7** is more luminescent than **1**, the introduction of a second iminodiacetate coordinating arm brings no further benefit on the photophysical properties. The most promising results were obtained with the nine-dentate chelate **6** and its Eu complex, which was conjugated to biotin and applied within the frame of a bioaffinity immunoassay of human C-reactive protein.

INTRODUCTION

Luminescent lanthanide(III) chelates have several special properties that make them excellent tools in homogeneous bioaffinity assays.[1−4] Their large Stokes' shift has a decreasing effect on scattering phenomena. The long fluorescence decay after excitation of these molecules allow time-resolved signal detection, which eliminates completely the background luminescence originating, for example, from buffer components, plastics, and biomaterials. The very narrow emission lines allow the use of effective filters which diminish the background. Furthermore, since the lanthanide(III) chelates do not suffer from concentration quenching it is possible to have several chelates in close proximity enabling multilabeling. This phenomenon allows also the development of chelates bearing several light absorbing moieties.

However, the use of stable chelates in bioaffinity assays demands optimization of the chelate structure. The optimal luminescent lanthanide chelate must be stable in the presence of additional chelators even at low pH and high temperature. The chelate has optimal emission profile, high hydrophilicity, small size, good biocompatibility, little effect on biomolecules, and good energy transfer properties. In addition, the chelate should be feasible for different energy acceptors, and when possible, the synthesis of the molecule should be simple, cheap, and scalable.

The commercially available europium(III) chelate of 4-[2-(4-isothiocyanatophenyl)ethynyl]-2,6,-bis{[*N*,*N*-bis-(carboxymethyl)-amino]methyl}pyridine[5] (**1a**; Figure 1) is one of the most commonly used biomolecule labeling reagents, since it fulfills almost all of the above-mentioned requirements. The most serious drawback of **1** as well as other 7-dentate lanthanide(III) chelates is that they exhibit rather low chelate stability limiting their use in applications involving treatments at elevated temperature, low pH, and in the presence of additional chelating agents such as ethylenediaminetetraacetic acid (EDTA). The chelate stability has been increased by using cyclic chelating moieties,[6−8] but neutralization of the net charge decreases the water solubility.

We report here the synthesis of stabilized derivatives of **1**. The synthetic routes are simple, and the chelates are significantly more stable and luminescent than **1**. The applicability of the biotin conjugated chelates was successfully demonstrated in a bioaffinity immunoassay of human C-reactive protein (hCRP). hCRP is a pentameric protein found in blood with a monomer molar mass of 25 kDa, which is an important biomarker for inflammation and cardiovascular disease.

EXPERIMENTAL PROCEDURES

General Procedures. All reagents and solvents used were of reagent grade. The ^{1}H and ^{13}C NMR spectra were recorded on a

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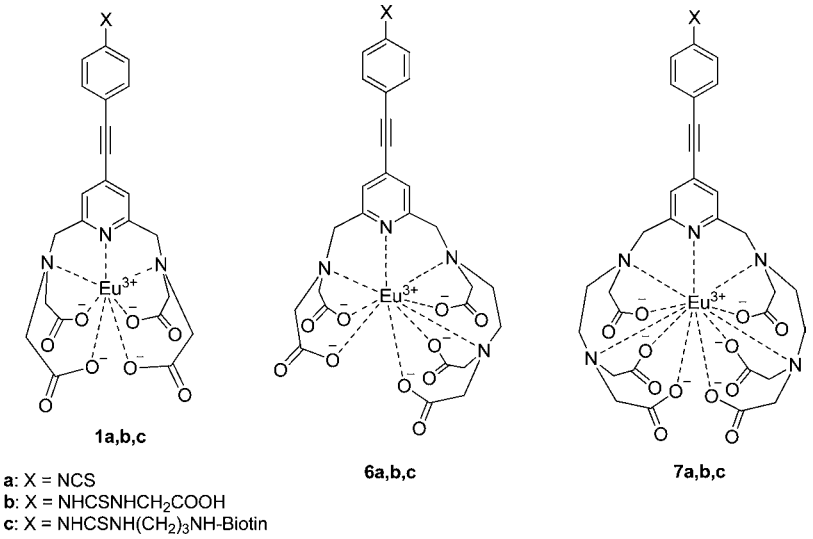

Figure 1. Structures of Eu(III) chelates and their conjugates.

Bruker Avance 600 MHz NMR spectrometer operating at 600.1337 and 150.9179 MHz for ^{1}H and ^{13}C, respectively. HR mass spectra were recorded on a Bruker micrOTOF-Q mass spectrometer. UV–visible absorption spectra were recorded on a Specord 205 (Analytik Jena) spectrometer. Steady state emission and excitation spectra were recorded on a Horiba Jobin Yvon Fluorolog 3 spectrometer working with a continuous 450 W Xe lamp. Detection was performed with a Hamamatsu R928 photomultiplier. All spectra were corrected for the instrumental functions. When necessary, a 399 nm cutoff filter was used to eliminate the second order artifacts. Phosphorescence lifetimes were measured on the same instrument working in the phosphorescence mode, with 50 μs delay time and a 100 ms integration window.

Emission decay profiles were fitted to monoexponential and biexponential functions using the FAST program from Edinburgh Instrument or with the Datastation software from Jobin Yvon. Hydrations numbers, q, were obtained using eq 1, were τ_{H2O} and τ_{D2O} respectively refer to the measured luminescence decay lifetimes (in ms) in water and deuterated water, using A_{Eu}= 1.2 and a_{Eu}= 0.25 for Eu^{III}[9]

$$q = A_{Ln}(1/\tau_{H_2O} - 1/\tau_{D_2O} - a_{Ln}) \quad (1)$$

Luminescence quantum yields were measured according to conventional procedures, with diluted solutions (optical density <0.05), using $[Ru(bipy)_3]Cl_2$ in nondegassed water (Φ = 4.0%) as reference.[10] The estimated relative error is ±15%.

Compound **2** was synthesized according to literature procedures.[5]

Tetra-*tert*-butyl 2,2′,2″,2‴-{{{[(4-bromopyridine-2,6-diyl)bis(methylene)]bis([2-(*tert*-butoxy)-2-oxoethyl]azanediyl}}bis(ethane-2,1-diyl)}bis(azanetriyl)}tetraacetate (4a). A mixture of 4-bromo-2,6-bis(bromomethyl)pyridine (**2**;[5] 344 mg, 1.0 mmol), *tert*-butyl{[bis(*tert*-butoxycarbonyl methyl) aminoethyl]amino}tris-(acetate)[11] (843 mg, 2.10 mmol) and dry potassium carbonate (1.38 g, 10 mmol) in dry acetonitrile (50 mL) were stirred overnight at 55 °C. The solid was removed by filtration. The solvent was evaporated in vacuo, and the product was purified on a silica gel column using 1% (v/v) triethylamine in dichloromethane as the eluent. Yield was 0.56 g (57%). ^{1}H NMR ($CDCl_3$): δ 7.59 (s, 2H), 3.79 (br, 4H), 3.39 (s, 8H), 3.33 (br, 4H), 2.84 (br, 8H), 1.40 (s, 18H), 1.38 (s, 36H). ^{13}C NMR ($CDCl_3$): δ 170.48, 160.79, 134.32, 124.09, 80.88, 59.79, 56.05, 53.40, 52.63, 52.06, 28.12, 28.10. HR-MS for $C_{47}H_{81}BrN_5O_{12}^+$: required 986.5060 and 988.5040, found 986.4993 and 988.4985.

Di-*tert*-butyl 2,2′-{{{6-{{{2-(bis[2-(*tert*-butoxy)-2-oxoethyl]-amino}ethyl}[2-(*tert*-butoxy)-2-oxoethyl]amino}methyl}-4-bromopyridin-2-yl}methyl}azanediyl}diacetate (4b). Di-*tert*-butyl 2,2′-{[4-bromo-6-(bromomethyl)pyridin-2-yl]methylenenitrilo}bis-(acetate) (**3**)[8] (385 mg, 0.76 mmol), *tert*-butyl{[bis(*tert*-butoxycarbonylmethyl)aminoethyl]amino}acetate[11] (319 mg, 0.80 mmol), and potassium carbonate (dry) (524 mg, 3.80 mmol) were stirred overnight at 55 °C. The solid was removed by filtration. The solvent was evaporated in vacuo, and the product was purified on a silica gel column using 20% (v/v) of ethyl acetate in petroleum ether as the eluent. Yield was 562 mg (89%). ^{1}H NMR ($CDCl_3$): δ 7.69 (s, 1H), 7.59 (s, 1H), 3.94 (s, 4H), 3.41 and 3.40 (2s, 10H), 2.86 (br s, 4H), 1.41 (s, 27H), 1.38 (s, 18H). ^{13}C NMR ($CDCl_3$): δ 170.45, 170.29, 160.60, 134.49, 124.39, 124.26, 81.10, 81.00, 59.65, 59.42, 56.01, 55.81, 52.64, 51.88, 28.13, 28.11. HR-MS for $C_{39}H_{66}BrN_4O_{10}^+$: required 829.3957 and 831.3937, found 829.4050 and 831.4038.

Tetra-*tert*-butyl 2,2′,2″,2‴-(((((4-((4-aminophenyl)ethynyl)-pyridine-2,6-diyl) bis(methylene))bis((2-(*tert*-butoxy)-2-oxoethyl)azanediyl))bis(ethane-2,1-diyl))bis(azanetriyl))-tetraacetate (5a). Compound **4a** (364 mg, 0.37 mmol) and 4-ethynylaniline (52 mg, 0.44 mmol) in the mixture of tetrahydrofuran (THF, 10 mL) and *N*,*N*-diisopropylethylamine (DIPEA, 10 mL) was deaerated with nitrogen for 5 min. $Pd(PPh_3)_2Cl_2$ (10.4 mg, 0.015 mmol) and CuI (2.9 mg, 0.015 mmol) were added as the catalysts, and the reaction mixture was stirred overnight under nitrogen at 60 °C. The solvents were removed in vacuo, and the product was purified on a silica gel column using a mixture of 20–30% (v/v) of ethyl acetate in petroleum ether containing 1% (v/v) triethylamine as the eluent. Yield was 290 mg (76%). ^{1}H NMR ($CDCl_3$, 50 °C): δ 7.44 (s, 2H), 7.30 (d, J = 8.45 Hz, 2H), 6.61 (d, J = 8.50 Hz, 2H), 3.96 (br s, 4H), 3.43 (s, 8H), 3.40 (br s, 4H), 2.90 (b, 8H), 1.46 (s, 18H), 1.42 (s, 36H). ^{13}C NMR ($CDCl_3$, 50 °C): δ 170.55, 158.78, 147.56, 133.32, 132.57, 122.66, 114.54, 111.47, 85.87, 80.83, 80.75, 60.07, 56.24, 52.88, 52.47, 28.17, 28.13. HR-MS for $C_{55}H_{87}N_6O_{12}^+$: required 1023.6376, found 1023.6308.

Di-*tert*-butyl 2,2′-(((4-((4-aminophenyl)ethynyl)-6-(((2-(bis(2-(*tert*-butoxy)-2-oxoethyl)amino)-ethyl)(2-(*tert*-butoxy)-2-oxoethyl)amino)methyl)pyridin-2-yl)methyl)azanediyl)-diacetate (5b). The synthesis was performed as above for compound **5a** but using **4b** (562 mg, 0.68 mmol) as the starting material. Yield was 421 mg (72%). ^{1}H NMR ($CDCl_3$): δ 7.45 (s, 1H), 7.35 (s, 1H), 7.18 (d, J = 8.46 Hz, 2H), 6.51 (d, J = 8.52 Hz, 2H), 4.17 (br s, 2H), 3.91 (s, 2H), 3.81 (s, 2H), 3.38 (s, 4H), 3.35 (s, 4H), 3.27 (s, 2H), 2.77 (t, d, J = 6.54, 25.21 Hz, 4H), 1.37 (s, 27H), 1.33 (s, 18H). ^{13}C NMR ($CDCl_3$): δ 170.68, 170.55, 170.38, 159.06, 158.64, 147.93, 133.18, 133.15, 122.47, 122.38, 114.38, 110.70, 94.80, 85.66, 80.89, 80.72, 80.70, 60.21, 60.08, 59.63, 56.14, 56.09, 55.71, 52.55, 52.16, 28.09, 28.07, 28.04. HR-MS for $C_{47}H_{72}N_5O_{10}^+$: required 866.5274, found 866.5294.

Preparation of Complex 6a. Compound **5b** (220 mg, 0.215 mmol) was dissolved in TFA (2 mL) and the mixture was stirred in a water bath at 25 °C for 2 h. All volatiles were removed in vacuo. The

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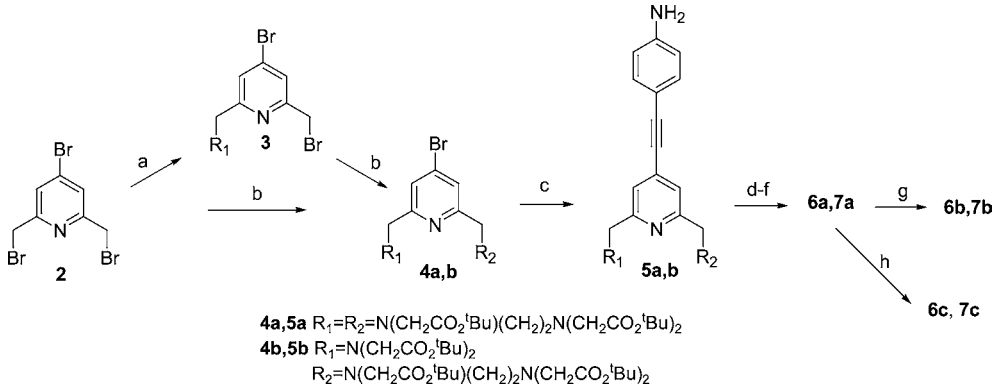
Scheme 1[a]



[a](a). $HN(CH_2CO_2{}^tBu)_2$, K_2CO_3, acetonitrile, 55 °C. (b). $HN(CH_2CO_2{}^tBu)(CH_2)_2N(CH_2CO_2{}^tBu)_2$, K_2CO_3, acetonitrile, 55 °C. (c). 4-Ethynylaniline, $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, THF, 60 °C. (d). TFA, r.t., 2 h. (e). $EuCl_3$, pH 7.0. (f). $SCCl_2$, water-$CHCl_3$, pH 7.0 (g) glycine. (h). N-Biotinyl-3-aminopropylamine.

residue was dissolved in water (2 mL), and a $EuCl_3$ solution (0.236 mmol in 0.5 mL water) was added and stirred for 10 min. The pH was adjusted to 7.0 with triethylamine, and the mixture was stirred for another 10 min. The pH was adjusted to 9.0 with sat. Na_2CO_3. The precipitate formed was removed by centrifugation. The pH of the solution was adjusted to 7.0 with acetic acid. Acetone (45 mL) was added, and the mixture was shaken for 1 min. The precipitate was collected by centrifugation, washed with acetone (50 mL), and dried with airflow. HR-MS for $C_{27}H_{27}EuN_5O_{10}^-$: required 732.0961 and 734.0975, found 732.1024 and 734.1032. The precipitate was dissolved in water (1 mL). Chloroform (1 mL) and thiophosgene (0.33 mL, 4.3 mmol) were added, and the mixture was stirred vigorously for 5 min. The pH was monitored and kept at 7.0 with 15% of $NaHCO_3$ solution. Chloroform was removed followed by addition of acetone (50 mL) with stirring. The precipitate formed was isolated by centrifugation, washed with acetone (50 mL) and dried. HR-MS for $C_{28}H_{25}EuN_5O_{10}S^-$: required 774.0526 and 776.0539, found 774.0649 and 776.0694.

Preparation of Complex 7a. The title compound was prepared as described above for compound **6a** but using compound **5a** (234 mg, 0.27 mmol) as the starting material. HR-MS for the amino form $C_{31}H_{33}EuN_6O_{12}^{2-}$: required 416.0683 and 417.0690, found 416.0721 and 417.0740. HR-MS for the isothiocyanato form $C_{32}H_{31}EuN_6O_{12}S^{2-}$: required 437.0465 and 438.0472, found 437.0521 and 438.0512.

Preparation of Glycine-Complexes 1b, 6b, and 7b. The isothiocyanates (**1a**, **6a**, **7a**; 20 mg each) were allowed to react with glycine (300 mg) at pH of about 7. The product was purified by HPLC (column: Supelco Ascentis RP-Amide, 21.2 mm × 25 cm. Particle 5 μm, flow rate 8.0 mL/min; eluent 20 mM TEAA buffer in 2−25% acetonitrile, v/v). The fractions were collected and concentrated. The salts were removed on HPLC by using the above-mentioned system by omitting the buffer component from the eluent.

HR-MS for **1b** $C_{26}H_{22}EuN_5O_{10}S^{2-}$: required 373.5148 and 374.5155, found 373.5163 and 374.5173.

HR-MS for **6b** $C_{30}H_{29}EuN_6O_{12}S^{2-}$: required 424.0387 and 425.0393, found 424.0398 and 425.0380.

HR-MS for **7b** $C_{34}H_{36}EuN_7O_{14}S^{2-}$: required 474.5625 and 475.5632, found 474.5676 and 475.5690.

Preparation of Biotin-Complexes of 1c, 6c, and 7c. The isothiocyanates (**1a**, **6a**, **7a**; 1 mg each) were allowed to react with N-Biotinyl-3-aminopropylamine (TFA salt, 5 mg each) at pH of about 8.0. The product was purified by HPLC (column: Supelco Ascentis RP-Amide, 4.6 mm × 15 cm, particle 5 μm, flow rate 1.0 mL/min; eluent 20 mM TEAA buffer at pH 7.0 with 2−60% methanol, v/v). The fractions were collected and dried in vacuum.

HR-MS for **1c** $C_{37}H_{41}EuN_8O_{10}S_2^{2-}$: required 486.0798 and 487.0805, found 486.0764 and 487.0780.

HR-MS for **6c** $C_{41}H_{48}EuN_9O_{12}S_2^{2-}$: required 536.6037 and 537.6044, found 536.6014 and 537.6023.

HR-MS for **7c** $C_{45}H_{55}EuN_{10}O_{14}S_2^{2-}$: required 587.1275 and 588.1282, found 587.1251 and 588.1260.

Conditional Stability Constants Determination. In a typical experiment,[12] batches of 2 mL of about 2×10^{-5} M solutions of the europium complexes in TRIS buffer at pH = 7.4 were mixed with various quantities of a stock solution of 5×10^{-2} M EDTA (or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)) in the same buffer, so that the ratio of added EDTA per Eu complex varies from 0 to 450000. For each solution, the emission spectrum was measured and the decrease in intensity was fitted to eq 2[13] using the nonlinear regression analysis of the SPECFIT software:[14]

$$[EuL] + EDTA \leftrightarrow [Eu(EDTA)] + L \quad \text{with}$$
$$K = \frac{[Eu(EDTA)] \times [L]}{[EDTA] \times [EuL]} = \frac{K_{condEDTA}}{K_{condL}} \qquad (2)$$

The conditional stability constant could be determined using values of $K_{condEDTA}$ calculated from literature data.[15]

hCRP Immunoassay. Human C-reactive protein (hCRP) was obtained from Orion Diagnostica (Espoo, Finland) and monoclonal anti-CRP antibody 6404 from Medix Biochemica (Kauniainen, Finland). Nunc C12 low fluor maxi wells (Thermo Scientific, Roskilde, Denmark) were coated with 150 ng of 6404 antibody in 40 μL of 50 mM phosphate buffer (pH 7.4) for 16 h at 4 °C. The wells were washed twice using wash buffer from Kaivogen Oy (Turku, Finland). Final blocking of the well surface was carried out with 200 μL of 0.1% BSA in the phosphate buffer for 2 h at 25 °C. The same 6404 antibody was conjugated with *d*-biotin NHS ester (Sigma-Aldrich, St. Louis, U.S.A.) using 20-fold excess in 50 mM phosphate buffer (pH 7.8). After conjugation the antibody was purified with NAP-5 column (GE Healthcare, Uppsala, Sweden) using TBS-buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl). Three replicates of 0−10 mg/L hCRP were incubated for 60 min in 50 μL of TBS-buffer in prewashed 6404 coated wells. The wells were washed once, and 50 ng of biotinylated 6404 antibody was added to the wells in 50 μL of TBS and incubated for 60 min. The wells were washed once, and 50 ng of streptavidin (BioSpa, Milan, Italy) in 50 μL of TBS was added, incubated for 10 min and washed once. Thereafter, 50 μL of 40 nM biotinylated europium chelates were added and incubated for 10 min. The wells were washed twice and measured with a Victor^2 1420 multilabel counter (PerkinElmer, Wallac OY, Turku, Finland) in time-resolved luminescence mode using an excitation wavelength of 340 nm and an emission wavelength of 615 nm, a 400 μs delay, and 400 μs integration times.

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Table 1. Spectroscopic Data for the Eu Complexes in 0.01 M TRIS/HCl Buffer at pH 7.4

compound	λ_{max}/ nm	ε/M^{-1} cm^{-1}	τ_{H2O} /ms	τ_{D2O} /ms	q	Φ_{Ov}	η_{eff}	Φ_{Eu}
1b	319	16350	0.39	2.10	2.2	0.06	0.54	0.11
6b	317	24350	1.15	1.96	0.1	0.14	0.45	0.31
7b	318	20900	1.07	1.69	0.1	0.12	0.41	0.29

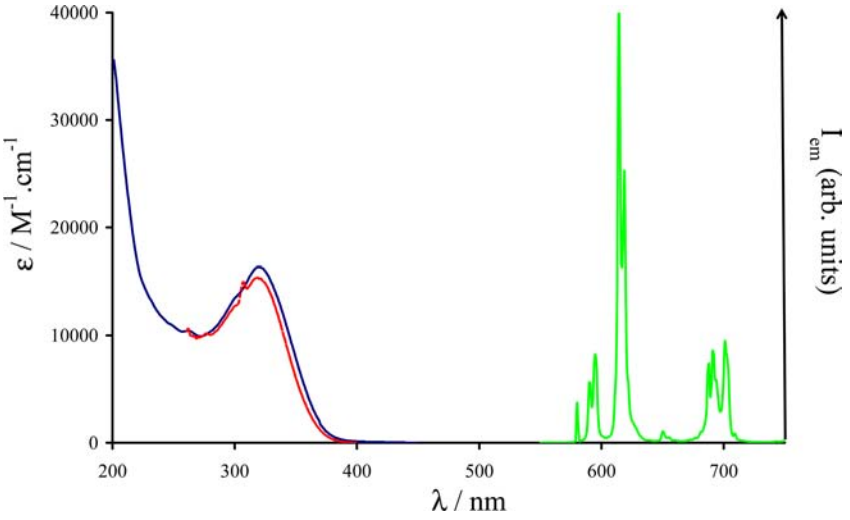

Figure 2. UV−vis absorption (blue), excitation (λ_{em} = 615 nm, red) and emission (λ_{exc} = 319 nm, green) spectra of **1b** in 0.01 M TRIS/HCl buffer at pH 7.4.

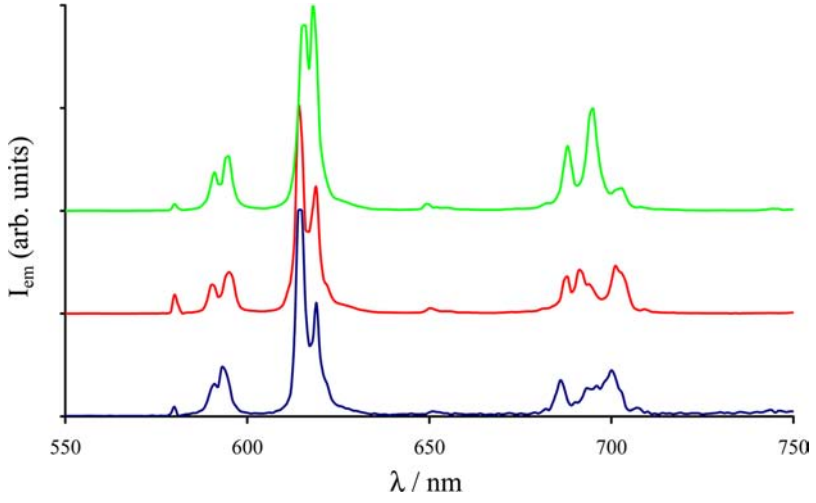

Figure 3. Emission spectra of the **1b**, **6b**, and **7b** complexes (from bottom to top) in TRIS/HCl buffer (0.01 M, pH 7.4; λ_{exc} = 318 nm).

RESULTS AND DISCUSSION

Syntheses of the Chelates. Synthesis of the europium(III) chelates is depicted in Scheme 1. Accordingly, treatment of 4-bromo-2,6-bis(bromomethyl)pyridine (**2**)[5] and di-*tert*-butyl 2,2′-(((4-bromo-6-(bromomethyl)pyridin-2-yl)methyl)azanediyl)diacetate (**3**)[8] with (*tert*-butyl{[bis(*tert*-butoxycarbonylmethyl)aminoethyl]amino}-acetate,[11] gave rise to **4a,b**. They were subsequently converted to the corresponding ethynyl derivatives (**5a,b**) by Sonogashira coupling reaction with 4-ethynylaniline. Removal of the *tert*-butyl groups by acidolysis followed by treatment with europium(III) chloride afforded the corresponding europium(III) chelates. Finally, treatment with thiophosgene[16] gave the desired activated chelates **6a** and **7a**.

Spectroscopic Properties of the Chelates. The spectroscopic properties of the complexes were measured in 0.01 M TRIS/HCl buffer at pH 7.4 on the glycine functionalized complexes (**1b**, **6b**, **7b**), and the most important parameters are gathered in Table 1. The UV−vis absorption spectra of the three Eu complexes are very similar, displaying a strong absorption band centered at about 318 nm, corresponding to $\pi \rightarrow \pi^*$ transitions on the pyridyl rings (see Figure 2 for **1b**). The presence of the *para*-(thiourea)-toluyl substitution resulted in a strong bathochromic shift of this absorption band, when compared to nonsubstituted pyridines for which the maximum of absorption can be found at 265−267 nm.[17,18] Upon excitation into the $\pi \rightarrow \pi^*$ absorption bands, all complexes display well resolved emission bands between 575 and 730 nm associated with f-f transitions on the europium ion.[19] These emission bands correspond to the $^5D_0 \rightarrow {}^7F_J$

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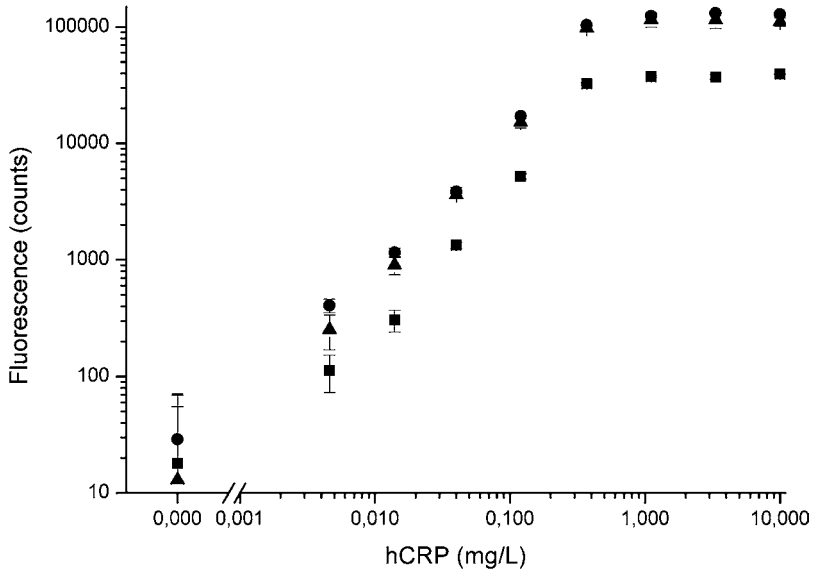

Figure 4. hCRP immunoassays with biotin-conjugated europium chelates: **1c** (■), **6c** (●), and **7c** (▲). The lower limit of detections for the biotin chelates were 6.5, 1.5, and 1.9 μg/L, respectively. The excitation wavelength is 340 nm, and the emission wavelength is 615 nm.

transitions with $J = 0$ (single band at 575 nm), $J = 1$ (between 578 and 600 nm), $J = 2$ (strong, 605 to 625 nm), $J = 3$ (weak around 650 nm) and $J = 4$ (690 to 715 nm). The corresponding excitation spectra are perfectly superimposable with the absorption spectra, evidencing an efficient ligand to metal energy transfer process that is a good antenna effect.[20] The luminescence decay profiles measured at the maximum of emission were all perfectly fitted with monoexponential functions, pointing to the presence of single species in solution. As expected, the complex obtained from the heptadentate ligand of **1b** displayed the shorter lifetime (0.39 ms), the coordination sphere of the europium being probably unsaturated. This was confirmed by the calculation of the hydration numbers of the complexes according to the method developed by Horrocks,[21] using Beeby's coefficients[9] (Table 1). While the heptadentate ligand of **1b** releases the place for two inner sphere water molecules, the replacement of one or two acetate functions by iminodiacetate ones resulted in the fulfilment of the coordination sphere and the removal of solvent molecules from the first coordination sphere.

The europium ion centered emission spectra of the complexes are presented in Figure 3. As expected for low symmetry complexes,[22] the $^5D_0 \rightarrow {}^7F_2$ transition represents the most intense emission band. The main variations in the series are observed on this transition and on the pattern of the $^5D_0 \rightarrow {}^7F_4$ transition, this transition becoming more intense (compared those with $J = 0$ to 3) for **7b**.

Based on the emission spectra of the three complexes (Figure 3), it was possible to determine the europium centered quantum yield, Φ_{Eu}, using the methodology developed by Werts and co-workers,[23,24] from which one can calculate the sensitization efficiency, η_{eff}, that reflects the capacity of the ligand to transfer the absorbed energy to the europium, using eq 3:

$$\Phi_{Ov} = \eta_{eff} \times \Phi_{Eu} \quad (3)$$

Where Φ_{Ov} is the overall luminescence quantum yield measured by the direct method (Table 1).

The calculated values of η_{eff} and Φ_{Eu} evidenced that the sensitization is almost the same for all compounds ranging from 41 to 54%, as expected for a similar antenna unit. In contrast, the metal centered quantum yield of **1b** is largely affected by the presence of the inner sphere water molecules, losing two-thirds of the value obtained when the coordination sphere is saturated. Finally, the introduction of a second iminodiacetate coordinating arm in **7b** has no significant further impact on the photophysical properties, but it can provide extra stability to the chelate.

Determination of the Conditional Stability Constants. The determination of the conditional stability constants of the different complexes was addressed by means of competition experiments with EDTA and DOTA. Only in the case of **1b** was it possible to displace the equilibrium in the presence of EDTA (from 1 to 5000 equiv). Considering a conditional stability constant of 14.53 Log units for EDTA at this pH,[15] the fitting resulted in a conditional stability constant of 16.7[2] Log units for **1b** at pH = 7.4. This value is very similar to reported values in the literature for similar coordination environments such as in the case of pyridyl chelates with H or OMe functions in the *para* position (Log K_{cond} = 16.47 and 16.67, as calculated from refs 17 and 18). For both **6b** and **7b**, even in the presence of large excess of EDTA (up to 1000 equiv in both cases), it was not possible to displace the equilibrium, and to extract the Eu atom from its coordinating ligand. We thus turned our attention toward DOTA, known to form very stable complexes with Ln cations,[25] as competing ligand, but even after 3 days at 80 °C in the presence of 50 equiv, it was not possible to observe demetalation. Although this last situation may be due to kinetic inertness of the complexes, the experiments with EDTA and DOTA allowed us to estimate a lower limit of 21 Log units for the values of the conditional stability constants of **6b** and **7b** with europium. It is believed that **7b** can be more stable than **6b** although the results are the same in our experimental conditions.

Conjugation to Bioactive Molecules and hCRP Immunoassay. The applicability of the chelates were demonstrated in a sandwich-type hCRP immunoassay. Therefore, the chelates **1b**, **6b**, and **7b** were conjugated with N-Biotinyl-3-aminopropylamine. The synthesized chelates **6c** and **7c** performed equally well in the hCRP assay (Figure 4). The

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biotin-chelate **1c** resulted in a lower luminescence signal than the **6c** and **7c** which is in accordance with the measured quantum yields and emission lifetimes. The analytical detection limits for **1c**, **6c**, and **7c** were 6.5, 1.5, and 1.9 μg/L, respectively, as calculated from 3 SD above the mean of the zero hCRP concentration and from the following equations, **1c**: $y = 32515x - 34$, $R^2 = 0.96$; **6c**: $y = 87730x + 16$, $R^2 = 0{,}99$; **7c**: $y = 90760x - 31$, $R^2 = 0.99$.

In summary, the stability of 4-[2-(4-isothiocyanatophenyl)-ethynyl]-2,6,-bis{[*N*,*N*-bis(carboxymethyl)-amino]methyl}-pyridine europium(III) can be dramatically enhanced by addition of an iminodiacetate coordinating arm to the chelate structure. This modification has also a positive effect on the luminescence quantum yield. The synthetic protocol is straightforward and applicable to other chelates bearing various substituents at 4-position of pyridine moiety.[26] The bio-conjugated chelates were also demonstrated to be highly suitable for diagnostic assay purposes as the immunoassays using the biotin labels cover the clinical range of the hCRP requirements.[27]

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Notes

The authors declare no competing financial interest.

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