

# Study of spectroscopic properties of Europium (III) Tris( $\beta$ -diketonate) complex and $\alpha$ -Cyclodextrin in aqueous medium

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Received: 2 September 2009 / Accepted: 12 November 2009 / Published online: 21 November 2009  
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**Abstract** The solubilization of an europium (III)  $\beta$ -diketonate chelate in aqueous medium and the changes in its photophysical properties upon its inclusion into an  $\alpha$ -cyclodextrin hydrophobic cavity are described. The complex  $[\text{Eu}(\text{tta})_3 \cdot (\text{H}_2\text{O})_2]$  ( $\text{tta} = 4,4,4$ -trifluoro-1-(thiophen-2-yl)butane-1,3-dione) was synthesized, characterized, and incorporated into the hydrophobic cavity by stirring in an  $\alpha$ -cyclodextrin aqueous solution. The inclusion was confirmed by  $^1\text{H}$  NMR, and the stoichiometry of association was obtained by the Job method. The maximum in the excitation spectrum of the  $\alpha$ -CD inclusion compound in aqueous solution was shifted 28 nm compared with the maximum of non  $\alpha$ -CD complex. The emission spectrum of the association is similar to that of the free solid complex and displays the characteristic  $^5\text{D}_0 \rightarrow ^7\text{F}_{0,4}$   $\text{Eu}^{3+}$  transitions.

**Keywords** Europium ·  $\beta$ -diketonate · Cyclodextrin · Inclusion compound

## Introduction

Cyclodextrins (CD) are an important class of cyclic oligosaccharides that are able to incorporate molecules of appropriate size in their cavity, forming the so-called inclusion compounds [1–3]. This inclusion generally

provides improved water solubility for the guest molecule and enables the use of luminescent europium (III) chelates in biological assays, among other applications [4–7].

Luminescent europium and terbium complexes are widely employed in investigations of the properties and functions of biochemical systems [8, 9]. Information on the composition, structure, and activity of these systems can be obtained with a luminescence revealed by the probe introduced into it [10, 11]. In this sense, lanthanide chelates offers important advantages compared with organic compounds, like better fluorescence features, longer life time, larger Stoke's shift, and sharper emission bands. Additionally, the europium chelates are luminescent in aqueous solution, exhibit multiple emissions in the UV-vis region (due to the several electronic transitions of  $\text{Eu}^{3+}$ ) and can be determined at in a very low concentration (picomolar) [12, 13].

Among all the lanthanides chelates, europium (III)  $\beta$ -diketonates complexes have been the most investigated, because of their high emission intensity, stability, hydrophilicity, and biocompatibility [14, 15]. However, their fluorescence in aqueous solution is too weak for a high analytical sensitivity, on account of the non-radiative losses to the medium [16, 17].

Here, the solubilization of an europium (III)  $\beta$ -diketonate chelate in aqueous solution and the changes in its photophysical properties upon its incorporation into a  $\alpha$ -cyclodextrin pocket are presented. Encapsulation can reduce the non-radiative losses by partial protection of the lanthanide coordination site from solvent molecules [18, 19].

First of all, the complex  $[\text{Eu}(\text{tta})_3 \cdot (\text{H}_2\text{O})_2]$  was synthesized and characterized. The inclusion in hydrophobic cavity of  $\alpha$ -cyclodextrin was achieved by stirring the solid in an aqueous solution of the oligosaccharide. The study of

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the properties of the materials was accomplished by luminescence spectroscopy, ultraviolet-visible absorption (UV-Vis), NMR, infrared (FTIR), and thermal analyses (TGA/DTA).

## Experimental procedure

### Europium complex

The  $[\text{Eu}(\text{tta})_3 \cdot (\text{H}_2\text{O})_2]$  complex was synthesized by addition of an  $\text{EuCl}_3$  aqueous solution to an aqueous/methanolic (1:1) solution of 4,4,4-trifluoro-1-(thiophen-2-yl)butane-1,3-dione (tta) under stirring overnight. The obtained solid complex was filtered and dried at room temperature, under reduced pressure [20]. Analytical data (C, H, Eu): Calculated: C: 33.85, H: 1.89, Eu: 17.85%. Found: C: 33.25, H: 2.10, Eu: 17.37 (termogravimetric analysis). IR spectra (KBr,  $\text{cm}^{-1}$ , selected): 1604 (C=O); 1300, 1550 (C=C); 550,750 (C–S–C); 1140 (C–F).  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ ):  $\delta$ 7.50 ppm, t, 1H, thiophenyl;  $\delta$ 7.90 ppm, dd, 1H, thiophenyl;  $\delta$ 8.05 ppm, dd, 1H, thiophenyl;  $\delta$ 4.10 ppm, s, 2H,  $-\text{CH}_2-$ .  $^{19}\text{F}$  NMR ( $\text{DMSO-d}_6$ ):  $\delta$ -11,60 ppm, s, 3H,  $-\text{CF}_3$ ; Solubility: methanol, DMSO, acetone, water (max 2.0 mg/100 g water).

### Inclusion compound

The  $\alpha$ -cyclodextrin was purchased from Aldrich. The inclusion compound was obtained by stirring 5.0 mg of the solid complex and approximately 20.0 mL of  $\alpha$ -cyclodextrin aqueous solution ( $\alpha\text{-CD} = 5.0 \text{ mmol L}^{-1}$ ; molar ratio complex: $\alpha\text{-CD} = 1:5$ ), for 1 week, at  $\sim 30^\circ\text{C}$ . The formation of the inclusion compound was confirmed by  $^1\text{H}$  NMR analysis and its photophysical properties were studied by luminescence and ultraviolet-visible absorption. To obtain the inclusion compound in the solid state, part of the solution was dried at  $\sim 40^\circ\text{C}$  (Scheme 1).

The stoichiometry of the  $\alpha\text{-CD}$  inclusion compound was obtained by means of the continuous variation method (Job's method) [21]. In this method, the total concentration of the species ( $[\text{S}]_0 + [\text{L}]_0 = \text{M}$ ) is kept constant, and the ratio ( $r = [\text{S}]_0 / \{[\text{S}]_0 + [\text{L}]_0\}$ ) between the europium

complex (S) and L ( $\alpha\text{-CD}$ ) species varies from 0 to 1. The maximum complex concentration is reached for  $r = (n + 1)^{-1}$  and does not depend on the concentration M or the binding constant ( $K_a$ ). In absorbance studies, the continuous variation method makes use of the difference between the maximum absorbance intensity of the guest in a pure solution and in each mixture with the host.

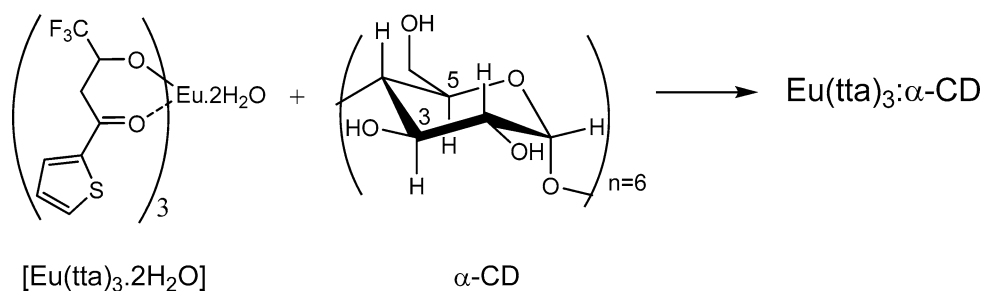
### Physical measurements

FTIR spectra were obtained for solid materials in KBr pellets (Perkin Elmer FT-IR 1600). TGA/DTA measurements were carried out (TA Instruments SDT 2960—Simultaneous DTA-TGA -Thermal Analyst 2100) in air flux, at a heating rate of  $10^\circ\text{C}/\text{min}$ , from 25 to  $1000^\circ\text{C}$ . NMR and 2D ROESY (Rotating-frame Overhauser Spectroscopy) experiments were recorded on a DRX 500 MHz Bruker spectrometer. The absorption spectra (UV-Vis) were recorded on an UV-Vis Spectrophotometer (Hewlett Packard 8452 Diode Array). The luminescence data were recorded in a Spectrofluorometer (SPEX Fluorolog III TRIAX 550 and 1934D phosphorescence device) at room temperature. Measurements of the emission quantum yields for the solid complexes and the inclusion complexes in aqueous solutions were done according to reported procedures, using MgO and quinine sulfate as standards for solids and aqueous solutions, respectively [22–25].

## Results and discussion

The formation and composition of the  $[\text{Eu}(\text{tta})_3 \cdot 2\text{H}_2\text{O}]$  complex was confirmed by elemental and thermal analyses, IR spectroscopy, and NMR. The 1:3 molar ratio (Eu:tta), as well as two water molecules to complete the coordination sphere, was confirmed by percentages provided by the elemental and thermal analyses. IR spectroscopy revealed a displacement in the carbonyl stretching frequency (C=O), from  $1600 \text{ cm}^{-1}$  (tta - free ligand) to  $1604 \text{ cm}^{-1}$ , which is good evidence that the metal ion is coordinated through the oxygen atoms [26, 27].  $^1\text{H}$  and  $^{19}\text{F}$  NMR presented shifted signals in relation to the pure ligand, giving further evidence of complex formation.

**Scheme 1** : Preparation of the  $\text{Eu}(\text{tta})_3(\text{H}_2\text{O})_2$  and the  $\alpha\text{-CD}$  inclusion compound

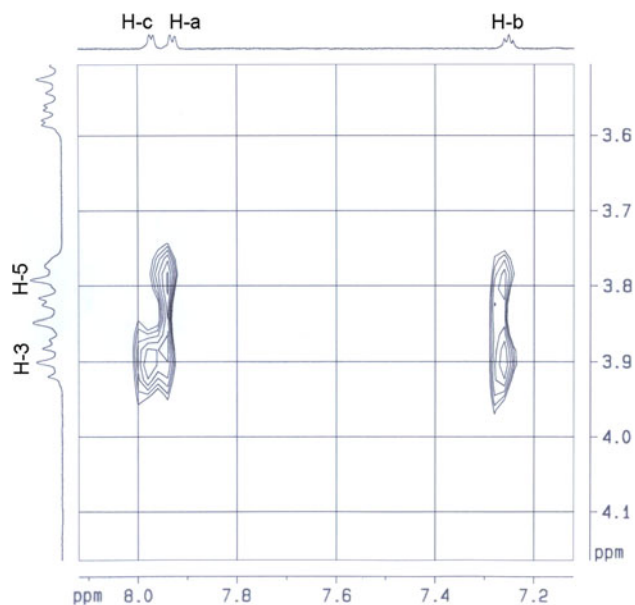


The maximum excitation peak presented by the solid complex, when monitored for the  $^5D_0 \rightarrow ^7F_2$  hypersensitive transition at 612.5, was 378 nm. The emission spectrum displayed the  $^5D_0 \rightarrow ^7F_{0-4}$  characteristic bands of  $\text{Eu}^{3+}$  in the visible region, with sharp lines in the 577, 590, 614, 650, and 697 nm regions. The excitation and the emission spectra of the pure complex in aqueous solution (0.8 mg/50 mL water,  $2.0 \times 10^{-5} \text{ mol L}^{-1}$ ) presented broader bands compared with those of the solid complex, all of which fell in the same wavelength regions.

Scheme 1 shows the synthetic route for the preparation of the  $\text{Eu}(\text{tta})_3:\alpha\text{-CD}$  inclusion compound. The solid complex  $[\text{Eu}(\text{tta})_3 \cdot 2\text{H}_2\text{O}]$  was stirred in aqueous  $\alpha$ -cyclodextrin until the solution became homogeneous. To obtain the  $^1\text{H}$  NMR, the same procedure was performed, changing water for  $\text{D}_2\text{O}$ .

The presence of the thiophenyl ring into the hydrophobic cavity of  $\alpha$ -cyclodextrin was confirmed by  $^1\text{H}$  NMR and 2D ROESY analyses.  $^1\text{H}$  NMR presented shifted signal for the H-3 and H-5 internal protons of  $\alpha$ -CD due to interaction with the guest's aromatic protons [28]. The thiophenyl protons of tta also had their position shifted after complex formation. But the most important evidence of the interaction between the thiophenyl group and the cavity was furnished by 2D ROESY analysis (Fig. 1).

The ROESY experiment is useful to determine which signals arise from protons that are close to each other in the space even if they are not bonded. So this experiment can confirm the interaction taking place in host:guest systems,

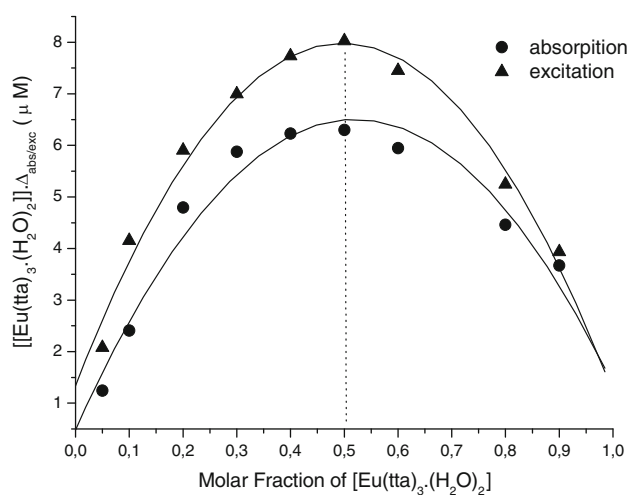


**Fig. 1** 2D ROESY spectrum of the  $\text{Eu}(\text{tta})_3:\alpha\text{-CD}$  inclusion compound in  $\text{D}_2\text{O}$ . The signals confirm the interaction of thiophenyl protons (H-a, H-b and H-c) with the internal H-3 and H-5 protons of  $\alpha$ -cyclodextrin

as in the case of cyclodextrin inclusion compounds [28]. The 2D ROESY spectrum presented a straight interaction between the thiophenyl protons of the tta ligand and the internal H-3 and H-5  $\alpha$ -cyclodextrin protons, confirming the inclusion of this part of the ligand.

To understand the stoichiometry of the formed inclusion compound formed, if 3:1 or 1:1 (complex:  $\alpha$ -cyclodextrin), the Job's method was used [21]. In this method, the total concentration of the species ( $[\text{S}]_0 + [\text{L}]_0 = \text{M}$ ) is kept constant, and the ratio ( $r = [\text{S}]_0 / \{[\text{S}]_0 + [\text{L}]_0\}$ ) between the europium complex (S) and L ( $\alpha$ -CD) species varies from 0 to 1. The maximum complex concentration is reached for  $r = (n + 1)^{-1}$  and does not depend on the concentration M or the binding constant ( $K_a$ ). In absorbance (or fluorescence) studies, the continuous variation method makes use of the difference between the maximum absorbance (or excitation) intensity of the guest in a pure solution and in each mixture with the host. Then, the difference obtained with regard to the pure chelate solution is plotted against the existing molar ratio (Fig. 2).

The stoichiometry of the inclusion compound was achieved by using absorbance and excitation spectra, to confirm the results. In both cases, the inclusion stoichiometry was 1:1. This proportion means that, besides the presence of three tta ligands in the complex and the theoretical possibility of three cyclodextrins in the final inclusion compound, the presence of one oligosaccharide promotes a spatial arrangement that avoids the presence of other cyclodextrins, leading to the inclusion of only one tta molecule in the hydrophobic cavity in the final structure.

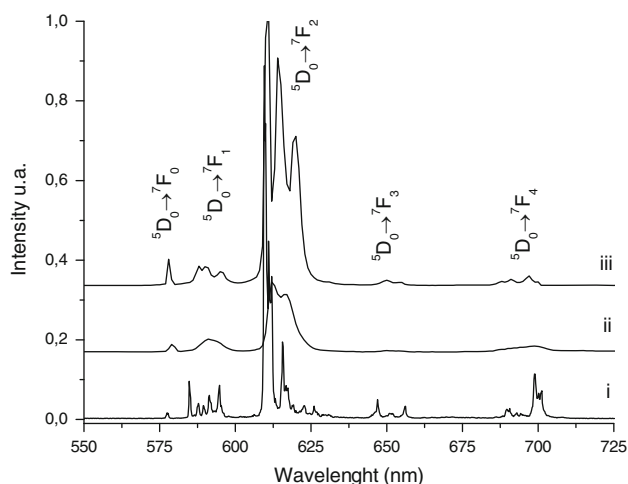


**Fig. 2** Job plot for determination of the stoichiometry of the  $\text{Eu}(\text{tta})_3:\alpha\text{-CD}$  inclusion compound. The solutions were prepared over a range of host/guest ratios and under such a concentration that  $[\text{Eu}(\text{tta})_3(\text{H}_2\text{O})_2]_0 + [\alpha\text{-CD}]_0 = 50 \text{ mmol L}^{-1}$ , and the concentration of  $[\text{Eu}(\text{tta})_3(\text{H}_2\text{O})_2]$  varied from  $5.0 \text{ mmol L}^{-1}$  to  $45 \text{ mmol L}^{-1}$ , in  $5.0 \text{ mmol L}^{-1}$  steps. The position of the maximum indicates the stoichiometry of the complex [18, 19]

The inclusion compound solution presented the  $\text{Eu}^{3+}$  luminescence bands with the same relative intensities, but with some differences with respect to the original complex. The excitation maximum at 378 nm observed for the solid  $\text{Eu}(\text{tta})_3 \cdot 2\text{H}_2\text{O}$  shifted to 350 nm after inclusion, in aqueous medium. The pure complex in water, without cyclodextrin, presented the same excitation maximum as the solid complex (at 378 nm). This change in the excitation maximum can be another evidence of the new spatial arrangement of the complex after the formation of the inclusion compound.

The emission spectrum displayed broad bands in the same regions as the solid complex, namely in the 577, 590, 615, 650, and 700 nm regions. The fluorescence quantum yield of the  $\text{Eu}(\text{tta})_3:\alpha\text{-CD}$  inclusion complex in aqueous medium was 8.8%, while in solid complex is 21.0% [20]. This reduction is due to the non-radiative losses from  $\text{Eu}^{3+}$  to water molecules, indicating that the inclusion in  $\alpha\text{-CD}$  cannot completely isolate the lanthanide coordination sphere from water. The lower luminescent-lifetimes ( $\tau$ ) values of the europium-excited states ( $\tau = 0.162$  ms for the inclusion compound in aqueous solutions and  $\tau = 0.68$  ms for the solid complex) and the higher number of water molecules ( $q$ ) coordinated to metal ion ( $q = 4.14$  in inclusion compound and  $q = 2.21$  in solid complex [29, 30]), are in agreement with the partial inclusion of the complexes into the hydrophobic cavity.

To study the inclusion compound in the solid state, part of the homogeneous solution was dried at  $\sim 40$  °C. The infrared absorption spectrum of the solid compound displayed the typical bands of  $\alpha\text{-CD}$  and the characteristic absorption bands of the guest, showing the structural integrity of the rare earth chelate complex after inclusion. The excitation and emission spectra of the solid are shown



**Fig. 3** Emission spectra of the  $[\text{Eu}(\text{tta})_3(\text{H}_2\text{O})_2]$  solid complex (i,  $\lambda_{\text{exc}}$ : 378 nm) and  $\text{Eu}(\text{tta})_3:\alpha\text{-CD}$  inclusion compound in aqueous solution (ii,  $\lambda_{\text{exc}}$ : 350 nm) and in the solid state (iii,  $\lambda_{\text{exc}}$ : 350 nm)

in Fig. 3 and presents the characteristic  $\text{Eu}^{3+}$  bands. The fluorescence quantum yields are higher than those obtained in aqueous medium, but lower than those achieved with the solid complex, due to water aggregation with the  $\alpha\text{-CD}$  molecule in the inclusion compound.

## Conclusions

The chemical and photophysical properties of the inclusion compound obtained from  $\alpha\text{-cyclodextrin}$  and the europium (III) 2-thienyltrifluoroacetate complex were presented. The luminescence of the  $\text{Eu}^{3+}$   $\beta\text{-diketonate}$  was maintained in aqueous solution, showing that cyclodextrins can aid the use of  $\text{Eu}^{3+}$  complexes in biological assays.

**Acknowledgements** We acknowledge the financial supports from FAPESP, CAPES, and CNPq.

## References

- Vyas, A., Saraf, S., Saraf, S.: Cyclodextrin based novel drug delivery systems. *J. Incl. Phenom. Macrocycl. Chem.* **62**, 23–42 (2008)
- Brewster, M.E., Loftsson, T.: Cyclodextrins as pharmaceutical solubilizers. *Adv. Drug Deliv. Rev.* **59**, 645–666 (2007)
- Braga, S.S., Coelho, A.C., Gonçalves, I.S., Santos, G., Fonseca, F.J., Andrade, A.M., Peres, M., Simões, W., Monteiro, T., Pereira, L.: Luminescence properties of the trimeb inclusion compound of a europium tris- $\beta$ -diketonate. *J. Non-Cryst. Solids* **354**, 2736–2739 (2008)
- Jiang, C.Q., Hou, F.J.: Fluorescence study of the interaction between a modified beta-cyclodextrin and metacycline- $\text{Eu}^{3+}$  and its analytical application. *Anal. Lett.* **37**, 1885–1895 (2004)
- Brito, H.F., Carvalho, A.A.C., Malta, O.L., Passos, J.J., Menezes, J.F.S., Sinisterra, R.D.: Spectroscopic study of the inclusion compound of  $\beta\text{-cyclodextrin}$  and Tris(dibenzoylmethane)europium(III) dehydrate. *Spectrochim. Acta Part A* **55**, 2403–2410 (1999)
- Fernandes, J.A., Braga, S.S., Ferreira, R.A.S., Pillinger, M., Carlos, L.D., Ribeiro-Claro, P., Gonçalves, I.S.: Modification of the luminescence properties of an Europium(III) tris(beta-diketonate) complex by inclusion in gamma-cyclodextrin and 2, 3, 6-trimethyl-gamma-cyclodextrin. *J. Incl. Phenom. Macrocycl. Chem.* **55**, 329–333 (2006)
- Ribeiro, A.O., Serra, O.A.: Spectroscopic study of  $\text{Tb}^{3+}$ (beta-diketonate)<sub>3</sub>: alpha-cyclodextrin inclusion compounds in aqueous solution. *J. Braz. Chem. Soc.* **18**, 273–278 (2007)
- Leonard, J.P., Gunnlaugsson, T.: Luminescent Eu(III) and Tb(III) complexes: developing lanthanide luminescent-based devices. *J. Fluoresc.* **15**, 585–595 (2005)
- Bunzli, J.C.G., Piguet, C.: Taking advantage of luminescent lanthanide ions. *Chem. Soc. Rev.* **34**, 1048–1077 (2005)
- dos Santos, C.M.G., Harte, A.J., Quinn, S.J., Gunnlaugsson, T.: Recent developments in the field of supramolecular lanthanide luminescent sensors and self-assemblies. *Coord. Chem. Rev.* **252**, 2512–2527 (2008)
- Pandya, S., Yu, J.H., Parker, D.: Engineering emissive europium and terbium complexes for molecular imaging and sensing. *Dalton Trans.* **23**, 2757–2766 (2006)

12. Tremblay, M.S., Halim, M., Sames, D.: Cocktails of Tb<sup>3+</sup> and Eu<sup>3+</sup> complexes: a general platform for the design of ratiometric optical probes. *J. Am. Chem. Soc.* **129**, 7570–7577 (2007)
13. de Sa, G.F., Malta, O.L., Donega, C.D., Simas, A.M., Longo, R.L., Santa-Cruz, P.A., da Silva, E.F.: Spectroscopic properties and design of highly luminescent lanthanide coordination complexes. *Coord. Chem. Rev.* **196**, 165–195 (2000)
14. Aime, S., Botta, M., Fasano, M., Terreno, E.: Lanthanide(III) chelates for NMR biomedical applications. *Chem. Soc. Rev.* **12**, 191–217 (1998)
15. Klonkowski, A.M., Lis, S., Pietraszkiewicz, M., Hnatejko, Z., Czarnobaj, K., Elbanowski, M.: Luminescence properties of materials with Eu(III) complexes: role of ligand, coligand, anion, and matrix. *Chem. Mater.* **15**, 656–663 (2003)
16. Petoud, S., Cohen, S.M., Bunzli, J.C.G., Raymond, K.N.: Stable lanthanide luminescence agents highly emissive in aqueous solution: multidentate 2-hydroxyisophthalamide complexes of SM<sup>3+</sup>, Eu<sup>3+</sup>, Tb<sup>3+</sup>, Dy<sup>3+</sup>. *J. Am. Chem. Soc.* **125**, 13324–13325 (2003)
17. Braga, S.S., Ferreira, R.A.S., Gonçalves, I.S., Ribeiro-Claro, P., Pillinger, M., Rocha, J., Teixeira-Dias, J.J.C., Carlos, L.D.: Study of the inclusion compound formed between a luminescent europium(III) beta-diketonate complex and gamma-cyclodextrin. *J. Incl. Phenom. Macrocycl. Chem.* **44**, 261 (2002)
18. Barge, A., Cravotto, G., Robaldo, B., Gianolio, E., Aime, S.: New CD derivatives as self-assembling contrast agents for magnetic resonance imaging (MRI). *J. Incl. Phenom. Macrocycl. Chem.* **57**, 489–495 (2007)
19. Haider, J.M., Pikramenou, Z.: Photoactive metallocyclodextrins: sophisticated supramolecular arrays for the construction of light activated miniature devices. *Chem. Soc. Rev.* **34**, 120–132 (2005)
20. Teotonio, E.E.S., Brito, H.F., Felinto, M.C.F.C., Kodaira, C.A., Malta, O.L.: Luminescence investigations on Eu(III) thanoyltrifluoroacetate complexes with amide ligands. *J. Coord. Chem.* **56**, 913–921 (2003)
21. Fielding, L.: Determination of association constants (K<sub>a</sub>) from solution NMR data. *Tetrahedron* **56**, 6151 (2000)
22. Fery-Forgues, S., Lavabre, D.: Are fluorescence quantum yields so tricky to measure? A demonstration using familiar stationary products. *J. Chem. Educ.* **76**, 1260–1264 (1999)
23. Williams, A.T.R., Winfield, S.A., Miller, J.N.: Relative fluorescence quantum yields using a computer controlled luminescence spectrometer. *Analyst* **108**, 1067–1071 (1983)
24. Brunet, E., Juanes, O., Sedano, R., Rodriguez-Ubis, J.C.: Lanthanide complexes of new polyaminocarboxylate complexes with two chromophores derived from bispyrazolopyridine and aceto or benzophenone: synthesis, characterization and photophysical properties. *Tetrahedron* **61**, 6757–6763 (2005)
25. Malta, O.L., Brito, H.F., Menezes, J.F.S., Silva, F.R.G.E., Alves, S., Farias, F.S., de Andrade, A.V.M.: Spectroscopic properties of a new light-converting device Eu(thenoyltrifluoroacetate)<sub>3</sub>·2(dibenzyl-sulfoxide). A theoretical analysis based on structural data obtained from a sparkle model. *J. Lumin.* **75**, 255–268 (1997)
26. Zhao, X.H., Huang, K.L., Jiao, F.P., Liu, S.Q., Liu, Z.G., Hu, S.Q.: Synthesis and luminescent properties of complexes of Eu(III) with 2-thienyltrifluoroacetate, terephthalic acid and phenanthroline. *J. Phys. Chem. Sol.* **68**, 1674–1680 (2007)
27. Nakamoto, K.: Infrared and Raman spectra of inorganic and coordination compounds. Wiley, New York (1986)
28. Pham, D.T., Clements, P., Easton, C.J., Lincoln, S.F.: <sup>1</sup>H NMR studies of enantioselective host–guest complexation by modified β-cyclodextrins and their europium(III) complexes. *Tetrahedron Asymmetr.* **19**, 167–175 (2008)
29. Supkowski, R.M., Horrocks, W.D.: On the determination of the number of water molecules, q, coordinated to europium(III) ions in solution from luminescence decay lifetimes. *Inorg. Chim. Acta* **340**, 44–48 (2002)
30. Beeby, A., Clarkson, I.M., Dickens, R.S., Faulkner, S., Parker, D., Royle, L., de Sousa, A.S., Williams, J.A.G., Woods, M.J.: Non-radiative deactivation of the excited states of europium, terbium and ytterbium complexes by proximate energy-matched OH, NH and CH oscillators: an improved luminescence method for establishing solution hydration states. *Chem. Soc. Perkin Trans.* **2**, 493–503 (1999)