Synthesis and properties of nanospheres copolymerised with luminescent europium(III) chelates

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Europium(III) chelates based on β-diketone and thienylpyridine subunits tethered to an acrylamide function were synthesized. They were polymerised with styrene and acrylic acid in the presence or absence of trioctylphosphine. The photophysical properties of the nanobeads obtained are also discussed.

Introduction

The high specific activity and very low background signal has made time-resolved fluorescence (TR-F) based on lanthanide(III) chelates an extremely successful detection technology.[1–3] Indeed, lanthanide(III) chelates have been used in *in vitro* diagnostics over two decades. The commercially available DELFIA® assay method uses a non-luminescent europium(III) chelate as the label.[4] After immunoreaction, the europium(III) ion is dissociated from the non-luminescent chelate by lowering the pH to 3.2 and the luminescence is enhanced with a mixture of β-diketone (4,4,4-trifluoro-1-(2-naphthyl)-butane-1,3-dione), detergent (Triton X-100) and chelator (trioctylphosphine oxide, TOPO). The new chelate formed has a very high luminescence, giving a detection sensitivity of *ca.* 50×10^{-15} M. However, the DELFIA® technology cannot be used in applications requiring site-specificity. Furthermore, the use of the chelator, TOPO, is essential for high luminescence. To overcome these limitations, stable luminescent lanthanide(III) chelates have been developed.[2,3] These chelates consist of a ligand with a reactive group for covalent conjugation to bioactive molecules, an aromatic structure which absorbs the excitation energy and transfers it to the lanthanide ion, and additional chelating groups such as carboxylic acid moieties and amines. Unlike organic chromophores, these molecules do not suffer from Raman or Rayleigh scattering or concentration quenching. Although numerous stable luminescent lanthanide(III) chelates have been synthesized, none of them has a quantum yield as high as the best β-diketone based chelates.

It has been shown that detection sensitivity can be dramatically enhanced by incorporating the lanthanide(III) chelates into particles.[5–17] The beads containing lanthanide(III) chelates as dye molecules have most commonly been prepared simply by swelling chelates into the polymer. Few of this type of labelled beads are currently commercially available. Since the lanthanide(III) chelates are not covalently bound to the polymer particles, the signal obtained from the particles may decrease as a function of the time because of leaking. This problem can be avoided by linking the chelate covalently to the matrix. Indeed, this type of silica based nanobead has recently been published.[17] However, the use of the silica-based nanoprobes for bioassays has the problem that the silica network may dissolve in basic aqueous conditions.[18] We report here the first synthesis of polystyrene based nanoparticles where the europium(III) chelates are covalently linked to the bead. Also, the chemical and photophysical properties of the nanospheres are discussed.

Results and discussion

Synthesis of the polymerisable europium(III) chelates

β-Diketones are widely studied ligands in the research of europium(III) chelates. Their synthesis is simple starting from a monoketone and a carboxylic acid ester using a strong base.[19] The highest luminescence enhancement is achieved with ligands having a fluorinated alkyl group in the α-position of the keto function.[4] In the present work, the β-diketone-based chelate, which allows polymerisation, was synthesized as shown in Scheme 1. Accordingly, acylation of 3-aminoacetophenone with methacroyl chloride yielded the amide **1** in a moderate yield. Claisen condensation of **1** with ethyl trifluoroacetate gave rise to the β-diketone **2**. Treatment of this with 0.25 equiv. of europium chloride in the presence of piperidine yielded the desired europium(III) chelate **3**.

Lanthanide(III) chelates are often extremely hydrophilic, and their solubilities in organic solvents are low. Naturally, this is a serious problem when developing luminescent chelates which should be polymerised to nanobeads. We have found that certain europium(III) chelates having furylpyridine subunits have moderate solubility in organic solvents such as THF.[20] The lipophilicity can be further increased by substituting furane moieties with thiophene units. The synthesis of such a polymerisable luminescent europium(III) chelate is depicted in Scheme 2. Accordingly, 4-bromo-6-bromomethyl-2-pyridylmethylene-nitrilobis(acetic acid) di(*tert*-butyl ester), **4**,[21] was allowed to react with *N*-*tert*-butyloxycarbonylhexane-1,6-diamine to give **5**, the reaction of which with tributylstannylthiophene in the presence of Pd(0) yielded **6**. Acid catalyzed removal of the protecting groups gave the ligand **7**, which was converted to the corresponding europium(III) chelate, **8**, by treatment with europium(III) chloride. Finally, the reaction of the amine **8** with methacroyl chloride gave the chelate tethered to a methacroyl function, **9**.

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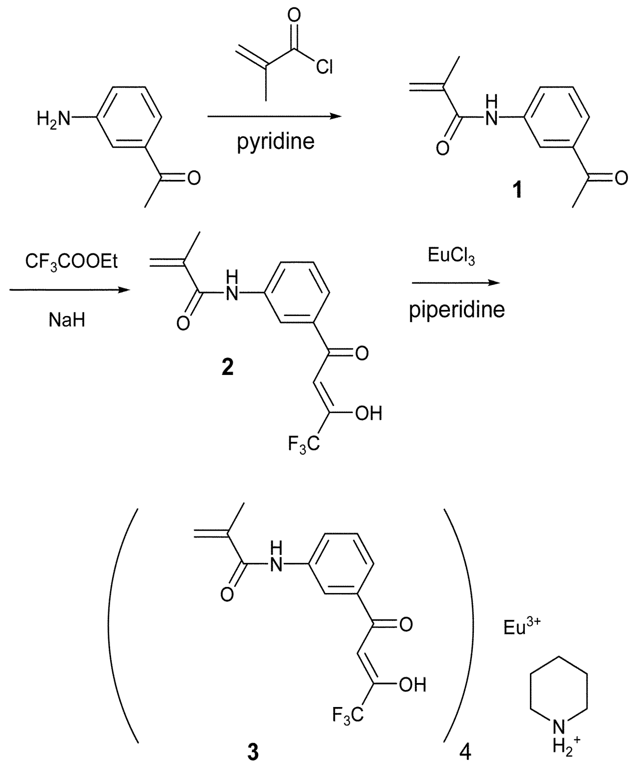


Scheme 1 Synthesis of the β-diketone-based europium(III) chelate.

The polystyrene based nanoparticles were synthesized using the method of Härmä *et al.*,[14] except the polymerisable chelates (**3**, **9**) were included in the copolymerisation mixture. In the case of the β-diketone **3**, the polymerisation was performed in the presence and absence of trioctylphosphine oxide. The amount of the chelate **3** was either 10 or 20% of the weight of the polymer components. It is worth noting that higher chelate concentrations could not be used because of the limited solubility of **3** in the polymerisation matrix. In the case of **9**, only a single concentration was used.

Photophysical properties of the nanoparticles

The nanoparticles prepared (**A–D**) showed typical fluorescence spectra of europium chelates:[4] excitation and emission maxima were at 336 and 614 nm, respectively for beads **A–C**. The corresponding maxima for bead **D** were 318 and 615 nm.

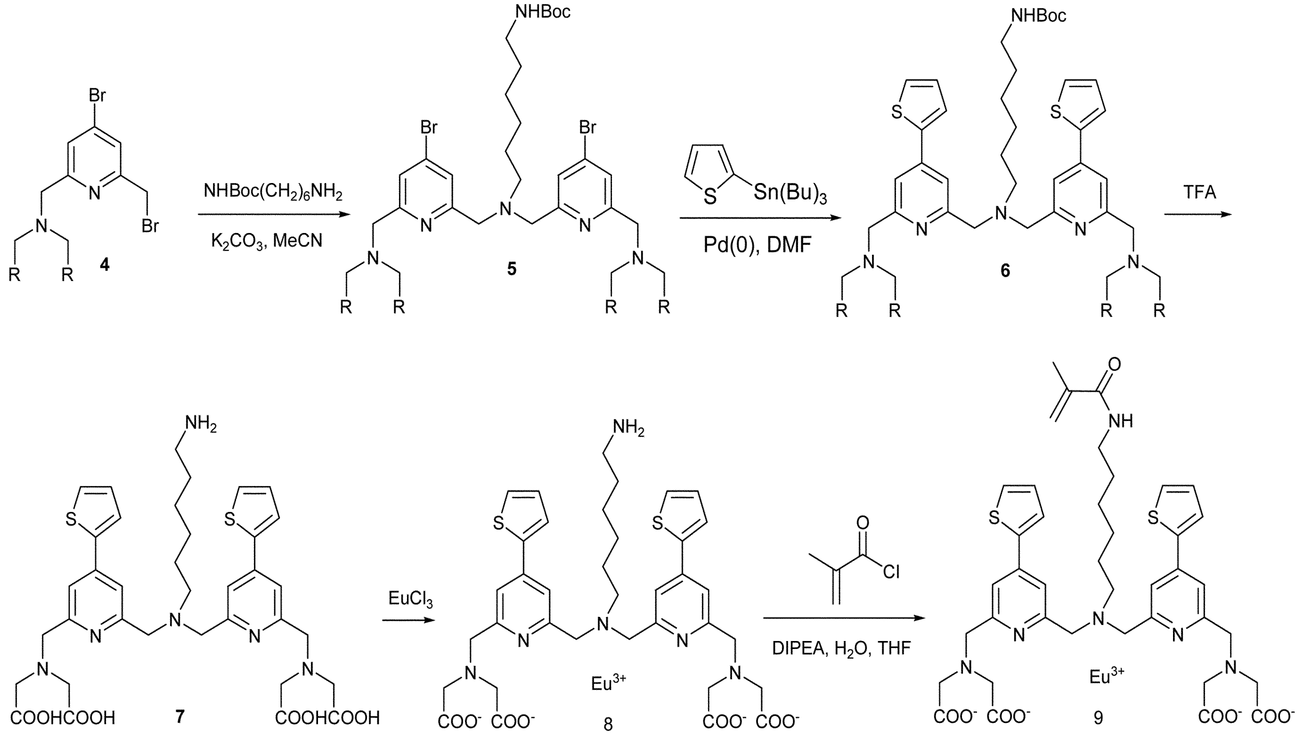
The relative fluorescences of the beads prepared are presented in Table 1. It is clearly seen that the higher the loading of the chelate in the particle, the higher is the relative fluorescence.

As expected,[4] the addition of TOPO has a significant effect on the fluorescence of the beads polymerised with **3**: addition of TOPO to the polymerisation mixture caused an almost sevenfold increase of the fluorescence (compare beads **B** and **C**). The enhancement could be obtained, although to a smaller extent, even when TOPO was not added to the polymerisation mixture, but it

Table 1 Properties of the nanospheres prepared

Bead	Size/nm	Chelate (w/w)[a]	Relative fluorescence[b]
A	101	**3** (10%)	0.097[c] (0.19)[d]
B	115	**3** (20%)	0.210[c] (0.39)[d]
C	111	**3** (20%) + TOPO	1.52[c] (1.40)[d]
D	93	**9** (27%)	0.42[c] (0.42)[d]

[a] Percentage of dry weight of the chelate in the polymerisation mixture. [b] Compared to DELFIA enhancement solution. [c] Measured in Tris–saline buffer, pH 7.75. [d] Tris–saline buffer, pH 7.75 + TOPO + Triton X-100.



Scheme 2 Synthesis of the europium(III) chelate based on thienylpyridine subunits. R = –COO-*t*-Bu.

was present in the Tris–saline buffer where the relative fluorescence was measured. This indicates that TOPO is able to swell into the beads. The relative fluorescence of bead **D** (*i.e.* where TOPO was not in the polymerisation mixture), did not increase in the presence of external TOPO.

As shown above, the addition of TOPO is essential for the high fluorescence of β-diketone-based nanobeads. Although the chelate is covalently bound to the matrix, TOPO is not. Accordingly, there is a danger that TOPO may leak from the beads especially if the beads have to be used or stored in non-aqueous or partially aqueous media. This, in turn, may lead to a decrease of fluorescence as a function of the time. The problem can be avoided by using a stable luminescent europium(III) chelate, such as **8**. As shown in Table 1, the nanobeads containing the chelate **8** have a higher relative fluorescence than beads **A** and **B** without an external chelator. Although the fluorescence of bead **D** is remarkably lower than that of bead **C**, it has the advantage that no TOPO is needed in the polymerisation mixture. According to our knowledge bead **D** is one of the brightest directly luminescent stable europium(III) chelates synthesized.

Experimental

General

Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). All dry solvents were from Merck and they were used as received. NMR spectra were recorded on a Bruker 250 or on a Jeol LA 400 spectrometer operating at 250.13 and 399.7 MHz for ^{1}H, respectively. The signal of TMS was used as an internal reference. Coupling constants are given in Hertz. ESI-TOF mass spectra were recorded on an Applied Biosystems Mariner instrument. Luminescence measurements were measured with a PerkinElmer LS-5 luminescence spectrometer. IR and UV–VIS spectra were recorded on PerkinElmer Spectrum One and Shimadzu 2400 instruments, respectively. Particle size analyses were performed on a Coulter LS-230 instrument, and are based on volume statistics. Photophysical properties of the nanoparticles prepared were measured as described previously,[22] but the chelate concentration measurements were based on the weight of dry beads.

N-(3-Acetylphenyl)methacrylamide (1). 3-Aminoacetophenone (20.9 g, 0.15 mol) was dissolved in dry pyridine (60 mL) on an ice–water bath. Methacryloyl chloride (26.1 cm^3, 0.24 mol) was added dropwise during $\frac{1}{2}$ h, and the mixture was stirred for an additional $\frac{1}{2}$ h. The stirring was continued for 3 h at RT. All volatiles were removed *in vacuo.* The residue was dissolved in dichloromethane (150 cm^3), washed with 0.5 M HCl (2 × 100 cm^3) and water (2 × 100 cm^3) and dried (Na_2SO_4). Purification was performed on silica gel. The column was first eluted with CH_2Cl_2 to elute fast migrating impurities, and then with 5% (v/v) methanolic dichloromethane to elute the product. The yield was 18.1 g (59%). ^{1}H NMR ($CDCl_3$): δ 8.10 (1H, m); 8.00 (1H, br); 7.95 (1H, m); 7.43 (1H, m); 7.23 (1H, m); 5.85 (1H, s); 5.51 (1H, s); 2.61 (3H, s); 2.08 (3H, s). ESI-TOF MS: required for $C_{12}H_{14}NO_2^+$ 204.10 $(M + H)^+$, found 204.08.

4,4,4-Trifluoro-1-[3-(methacrylamido)phenyl]-1,3-butanedione (2). To a stirred solution of compound **1** (11.9 g, 58.55 mmol) in dry THF (100 cm^3) sodium hydride (3.52 g, 88 mmol; 60% dispersion in oil) was added portionwise. After 5 min, ethyl trifluoroacetate (13.9 cm^3, 0.18 mol) was added, and the mixture was stirred for an additional 1 h before being concentrated *in vacuo.* The residue was suspended in ethyl acetate (220 cm^3) and acidified with 10% aqueous H_2SO_4 (80 cm^3), and washed with water. The organic layer was separated and dried over Na_2SO_4. Purification on silica gel (eluent, petroleum ether, bp 40–60 °C–ethyl acetate, 1 : 1, v/v) yielded 8.71 g (50%) of the title compound. ^{1}H NMR ($CDCl_3$): δ 8.14 (1H, s); 7.87 (1H, d, *J* 7.5); 7.74 (1H, br s); 7.74 (1H, d, *J* 7.8); 7.48 (1H, t, *J* 8.0); 6.58 (1H, s); 5.85 (1H, s); 5.54 (1H, s); 2.09 (3H, s). λ_{max}(EtOH)/nm: 208, 250, 326. ESI-TOF MS: required for $C_{14}H_{12}F_3NNaO_3^+$ 322.07 $(M + Na)^+$, found 322.03.

Synthesis of the europium chelate (3). Compound **2** (9.8 g, 32.7 mmol) was dissolved in the mixture of abs. ethanol (100 cm^3) and piperidine (3.2 cm^3), and the mixture was warmed to 45 °C. Europium chloride hexahydrate (2.40 g, 6.54 mmol, predissolved in 20 cm^3 of water) was added dropwise. The mixture was allowed to cool to RT, and water (100 cm^3) was added dropwise. The precipitation formed was collected by filtration, washed with water, and dried *in vacuo.* The yield was 7.9 g. λ_{max}(H_2O + 1% DMF, v/v)/nm: 326 (ε 50552). ν_{max}(KBr)/cm^{-1}: 3440; 1663; 1620; 1586; 1534; 1489; 1301; 1187; 1138; 780; 580. Ex_{max} 353 nm; Em_{max} 614 nm (tris–saline buffer, pH 7.75). Attempted characterization on ESI-TOF mass spectrometry was not successful. Accordingly, the structure shown in Scheme 1 is based on the molecular ratio of the reagents used in the preparation of **3**.

Tetra-(*tert*-butyl)-2,2′,2″,2‴-{[6-(*tert*-butyloxycarbonylamino)-hexylimino]bis(methylene)bis(4-bromopyridine-6,2-diyl)bis(methylenenitrilo)}tetrakis(acetate) (5). 4-Bromo-6-bromomethyl-2-pyridylmethylenenitrilobis(acetic acid) di(*tert*-butyl ester) (**4**, 8.50 g, 16.7 mmol) and 6-*tert*-butoxycarbamoylhexane-1,6-diamine (1.80 g, 8.4 mmol) were dissolved in dry acetonitrile (60 cm^3). K_2CO_3 (9.2 g, 66.8 mmol) was added, and the mixture was heated overnight at 50 °C. The precipitation formed was removed by filtration, and the filtrate was concentrated. Purification on silica gel (eluent: petroleum ether, bp 40–60 °C–ethyl acetate, from 10 : 1 to 5 : 2, v/v) yielded 5.9 g (65%) of compound **5**. ^{1}H NMR ($CDCl_3$): δ 7.73 (2H, d, *J* 1.9); 7.58 (2H, d, *J* 1.9); 4.00 (4H, s); 3.74 (4H, s); 3.47 (8H, s); 3.08 (2H, q, *J* 5.5); 2.51 (2H, t, *J* 7.2); 1.53–1.42 (4H, m); 1.46 (36H, s); 1.44 (9H, s); 1.33–1.22 (4H, m). ν_{max}(film)/cm^{-1}: 3401 (N–H); 1734 (C=O); 1565 (arom. C–C). λ_{max}(EtOH)/nm 268. ESI-TOF MS for $C_{49}H_{78}Br_2N_6O_{10}$ $(M + 2H)^{2+}$: calcd, 536.22; found, 536.18.

Tetra-(*tert*-butyl)-2,2′,2″,2‴-{[6-(*tert*-butyloxycarbonylamino)-hexylimino]bis(methylene)bis(4-{thiophen-2-yl}pyridine-6,2-diyl)-bis(methylenenitrilo)}tetrakis(acetate) (6). Compound **5** (2.85 g, 2.66 mmol) and 2-(tributylstannyl)thiophene (1.86 cm^3, 5.86 mmol) were dissolved in dry DMF (25 cm^3) and deaerated with argon. $(Ph_3P)_4Pd$ (0.215 g, 0.22 mmol) was added, and the mixture was stirred at 90 °C for 6 h in the dark. The mixture was cooled to room temperature and concentrated *in vacuo.* Purification was performed on silica gel (eluent: petroleum ether, bp 40–60 °C–ethyl acetate–triethylamine, from 5 : 1 : 1 to 5 : 3 : 1, v/v/v). The yield was 2.2 g (76%). ^{1}H NMR ($CDCl_3$): δ 7.76 (2H, s); 7.70 (2H, s); 7.55 (2H, d, *J* 3.1); 7.36 (2H, d, *J* 4.9); 7.09 (2H, m); 4.05 (4H, s); 3.82 (4H, s); 3.50 (8H, s); 3.03 (2H, m); 2.60 (2H, m); 1.49–1.43 (4H, m); 1.45 (36 H, s); 1.42 (9H, s);

1.39–1.32 (4H, m). ν_{max} (film)/cm^{-1} 1730 (C=O). λ_{max}(EtOH)/nm 293. ESI-TOF MS for $C_{57}H_{85}N_6O_{10}S_2(M + 2H)^{2+}$: calcd, 539.29; found, 539.23.

2,2′,2″,2‴-{(6-Aminohex-1-yl-imino)bis(methylene)bis[4-(thiophen-2-yl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis(acetic acid) (7). Compound **6** (2.16 g, 2.00 mmol) was dissolved in trifluoroacetic acid (25 cm^3), and the mixture was stirred for 2 h at room temperature before being concentrated. The residue was triturated with diethyl ether. The precipitation formed was filtered, washed with diethyl ether and dried *in vacuo.* The yield was quantitative. ^{1}H NMR (DMSO-d_6): 7.90 (2H, s); 7.78 (2H, d, *J* 4.9); 7.73 (2H, d, *J* 3.4); 7.71 (2H, s); 7.25 (2H, dd, *J* 3.4 and 4.9); 3.95 (4H, s); 3.86 (4H, s); 3.30 (8H, s); 3.22 (2H, m); 2.74 (2H, m); 1.78 (2H, m); 1.50 (2H, m); 1.33–1.23 (4H, m); ν_{max}(KBr)/cm^{-1} 1735; 1675; 1609 (C=O); 1559 (arom. C–C). λ_{max}(EtOH)/nm 300.

2,2′,2″,2‴-{(6-Aminohex-1-yl-imino)bis(methylene)bis[4-(thiophen-2-yl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis(acetic acid) europium(III) (8). Compound **7** (1.9 g) was dissolved in water (30 cm^3), and the pH of the solution was adjusted to 6.5 with solid $NaHCO_3$. Europium chloride hexahydrate (0.81 g, 2.2 mmol; predissolved in 30 cm^{3}of water) was added dropwise keeping the pH at *ca.* 6. The mixture was stirred for 1.5 h at RT. The pH was raised to 8.5 with aq. NaOH. The precipitation was removed by centrifugation. The clear solution was collected and concentrated *in vacuo.* It was used for the next step without further purification. ν_{max}(KBr)/cm^{-1} 1684; 1638; 1615 (C=O); 1552 (arom. C–C). λ_{max}(EtOH)/nm 312. ESI-TOF MS for $C_{36}H_{40}EuN_6O_8S_2^-(M - H)^-$: calcd, 901.16; found, 901.16.

2,2′,2″,2‴-{(6-Methacroylamidohex-1-yl-imino)bis(methylene)-bis[4-(thiophen-2-yl)pyridine-6,2-diyl]bis(methylenenitrilo)}tetrakis-(acetic acid) europium(III) (9). Compound **8** (2.6 g, 2.8 mmol) was dissolved in a mixture of water (20 cm^3), THF (40 cm^3) and DIPEA (1.7 cm^3). Methacroyl chloride (0.42 g, 4.0 mmol) was added, and the mixture was stirred for 5 min at RT before being concentrated *in vacuo.* The residue was suspended in chloroform (30 cm^3). The precipitation formed was removed by filtration. The filtrate was concentrated to give the title compound. ESI-TOF MS for $C_{40}H_{44}EuN_6O_9S_2^-$ $(M - H)^-$: calcd, 969.18; found, 969.18. The partition coefficient of compound **9** between H_2O and $CHCl_3$, was *ca.* 1 : 1.

Polymerisation. A typical procedure

A mixture of styrene (1.83 cm^3), acrylic acid (234 mm^3), hexadecane (94 mg), divinyl benzene (0.12 g) and compound **3** (0.512 g, 0.358 mmol; 20% of the dry weight; predissolved in 2.0 cm^3 of chloroform) and TOPO (0.208 g, 0.534 mmol) were dissolved in water (40.0 cm^3) containing sodium dodecyl sulfate (0.09 g) and sodium borate decahydrate (0.017 g). The resulting suspension was deaerated with argon and homogenized using ultrasound (1 min, 215 W). The resulting emulsion was transferred into a reactor, and it was stirred mechanically (140 rpm) at 60 °C for 20 min under argon (pH 3). The polymerisation was initiated by the addition of potassium persulfate (0.05 g, predissolved in 3.00 cm^3 of degassed water). The reaction was allowed to proceed for 5 h. The mixture was allowed to cool to RT and was purified by dialysis. The following beads were prepared:

10% (w/w) chelate **3** [bead **A**]
20% (w/w) chelate **3** [bead **B**]
20% (w/w) chelate **3** + 1.5 equiv. TOPO [bead **C**]
27% (w/w) chelate **8** [bead **D**]

where % w/w is the percentage of the weight of the chelate from the dry weight of the polymerisation mixture.

Conclusions

Presented here is the synthesis of nanospheres with covalently linked europium(III) chelates. The chelates were either derivatives of a β-diketone or a stable luminescent thienylpyridine. The highest relative fluorescence was obtained with the β-diketone copolymerised with the chelating agent TOPO. Although the relative fluorescence of the thienylpyridine derivative was lower than that of the β-diketone derivative, no additives were required. Accordingly, the present method for particle derivatization can be, in all likelihood, used in the future when developing brighter stable nanoparticles loaded with lanthanide(III) chelates.

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