Synthesis and spectral properties of a new luminescent europium(III) terpyridyl chelate

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A new europium(III) chelate, which uses the 2,2′ : 6′,2″-terpyridyl chelating group as sensitizer, has been synthesized and its spectral properties investigated. The chelate, which can incorporate an isothiocyanate group for biological labelling, has proved useful in time resolved fluorescence studies and for delayed fluorescence resonance energy transfer (DEFRET).

Introduction

Amongst important considerations in modern biological assay design is the need for higher throughput of samples and faster turnaround times. There is a need to generate maximum information in the minimal time and assay combinations, using the concept of 'multiplexing',[1,2] is becoming commonplace. Often both the combination of technologies and the use of intelligent reagents are required to increase assay value.

Currently, fluorescence and luminescence based assays have attracted interest since they can offer more sensitivity and specificity than traditional methods such as radioactive labelling.[3] Versatile reagents are required that have high environmental and photostability, and high quantum yields for emission at longer wavelengths so as to be distinct from those of the biological reagents used in the assay.

Lanthanide probes, such as those based on Eu(III) and Tb(III) chelates, are of interest since the majority of these probes have long lifetimes that allow temporal discrimination between the background signal and the emission signal of the probe. These probes can also be incorporated as energy donors in time resolved or 'delayed' Förster Resonance Energy Transfer (DEFRET) assays,[4,5] where the elimination of 'crosstalk' can greatly increase sensitivity. By appropriate selection of the sensitizer high luminescent quantum yields can be achieved, thus allowing assays employing these probes to be performed at sub-nanomolar concentrations with good resolution. Many europium(III) probes have proven to be robust and resistant to photobleaching.[6]

In order to achieve lanthanide luminescence several criteria have to be met. The metal ions exhibit only weak absorption coefficients since the process is parity forbidden.[7] Therefore a sensitizer, often referred to as a 'light harvester' or 'antennae'[8] molecule, is required within the local environment of the ion. Often triplet sensitizers such as coumarins[9] or quinolinones[10] are used.

A further condition is that the ion has to be shielded from deactivating solvents such as water or methanol since these allow quenching of the excited state of the ion by vibronic deactivation. An insulating sheath[11] around the metal ion is required and is often in the form of a multidentate ligand such as an EDTA derivative; other methods employed for the reduction of vibronic quenching are either the use of deuterated solvents, that are less efficient in the quenching process,[12] or the use of a Lewis base, such as trioctylphosphine oxide,[9,11,13] that shields the central ion from solvent effects *via* the formation of hydrophobic complexes.

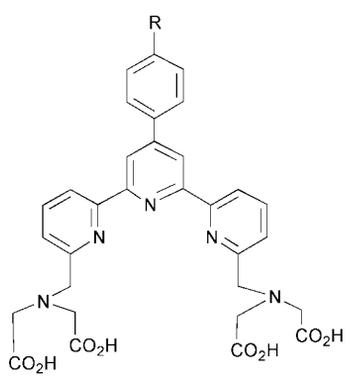
Two main approaches to satisfying these conditions have been used. In the first, Bailey's group has reported[14] the use of a non-sensitizing chelate, such as DTPA (diethylenetriaminepentaacetic acid) as the insulating sheath to which is covalently attached a heterocyclic sensitizer such as 4-aminosalicylic acid, a method developed by Selvin's group[15] using either carbostyril or 7-amino-4-methylcoumarin-3-acetic acid. These act as antennae for the energy shuttle process.

The second approach is to incorporate the sensitizer directly into the shield. The groups of Toner,[16,17] Mathis[18] and Hemmilä[19] employ chelating sensitizers possessing good absorption coefficients that can funnel energy *via* triplet energy transfer into the metal ion. They include bipyridine, terpyridine and phenanthroline derivatives. More recently attention has also been turned to the development of sensitizers that operate at excitation wavelengths >350 nm.[20]

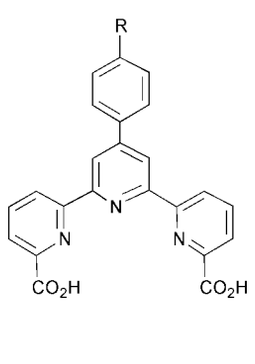
In DEFRET assays the acceptor can be any dye whose absorption energy profile matches that of the donor emission. For europium emission derivatives of the dye allophycocyanin are often employed as the acceptor and this is the basis of at least one commercially marketed assay system.[18]

Preparation of the probes 11–13

In earlier work[21,22] we attempted to synthesize the terpyridyl system **1**, previously reported by Toner.[16] However, the efficiency of the synthesis was reduced by the observed instability of the methoxy group, dealkylation readily occurring when the terpyridyl system or its lanthanide complexes were treated with



1 R = OCH_3
2 R = CH_3



3 R = OCH_3
4 R = CH_3

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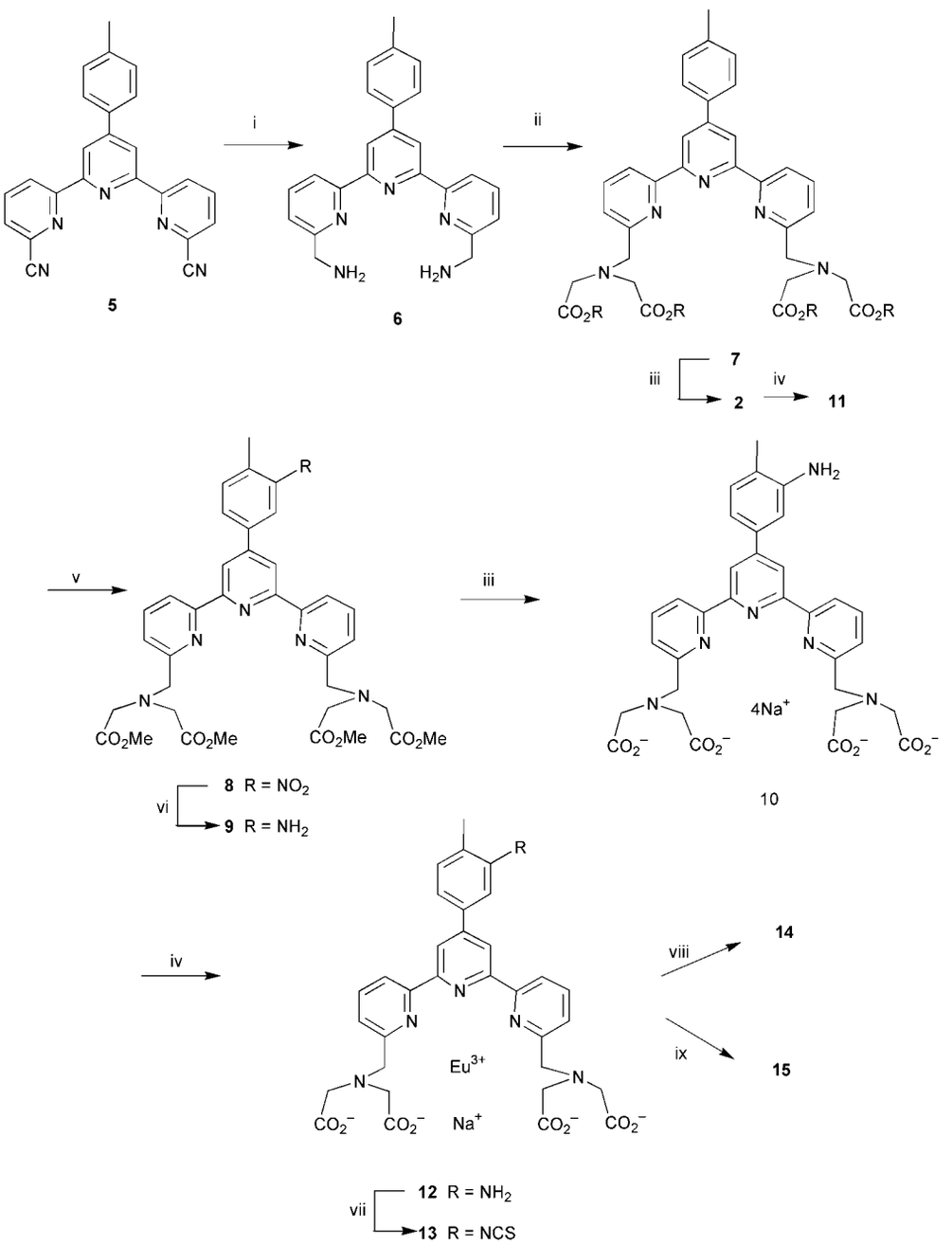

Scheme 1 *Reagents*: i, diborane–THF; ii, $BrCH_2CO_2Me$, diisopropylethylamine, acetonitrile; iii, NaOH; iv, $EuCl_3 \cdot 6H_2O$; v, HNO_3–H_2SO_4; vi, $SnCl_2 \cdot 2H_2O$, aq. ethanol; vii, $CSCl_2$, CH_2Cl_2; viii, 1-butylamine; ix, *N*-(2-aminoethyl)biotinamide **16**.

acid. The resulting phenol proved not to be a useful sensitizer for luminescence.

It was noticed that, in preparing the simpler derivatives, **3** and **4**, the tolyl derivative **4** had the advantage of being more stable towards reagents than the corresponding anisole **3**. We therefore prepared the tolyl analogue of the Toner compound, *viz.* **2** and its Eu(III) complex **11**.

The synthesis of the latter compound was accomplished from the known intermediate **5**,[21] according to Scheme 1. Reduction of the dinitrile **5** with diborane as its THF complex, afforded the bis(aminomethyl) derivative **6**, isolated as its hydrochloride salt. The amine hydrochloride was alkylated with methyl bromoacetate, using diisopropylethylamine as base, to afford, as the major product, the tetra-alkylated chelate ester **7**. A portion of the ester was hydrolysed to give the tetracarboxylic acid **2**, used as a reference chelator–sensitizer. Compound **2** could be complexed with Eu(III) to afford compound **11**. Standard nitration of compound **7** with concentrated nitric–sulfuric acid afforded the nitro-ester **8**. The advantage of using the tolyl derivative, rather than a phenyl-substituted terpyridyl, was that the nitration proceeded smoothly to give just one isomer.

Reduction of **8** with tin(II) chloride gave the amino-ester **9**, which could be hydrolysed to the amino acid **10**. Loading of the ligand **10** with Eu(III) chloride afforded the amino-complex **12**. Compound **12** was reacted with thiophosgene to give the corresponding isothiocyanate **13**. The thiocyanate could be stored at low temperatures (−40 °C) for periods of weeks but could also readily be freshly prepared as required before conjugation to biomolecules. As a model system the thiocyanate was reacted *in situ* with butylamine to afford the corresponding thiourea **14**.

These terpyridyl derivatives are similar but distinct from the series of terpyridyl derivatives described by Hemmilä *et al.*[19]

Spectroscopic characterization of the probes

The luminescent characteristics of **11** were investigated in terms of excitation and emission spectra, lifetime, quantum yield and

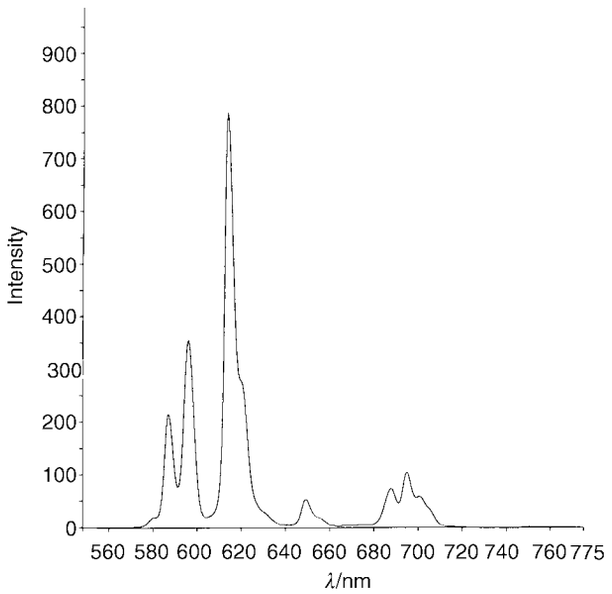

Fig. 1 Emission spectrum of compound **11**, 5×10^{-5} mol dm^{-3}, in HEPES buffer at pH 7.5. Slit widths 2.5 nm, delay 0.1 ms, gate time 1.0 ms. λ_{ex} 295 nm.

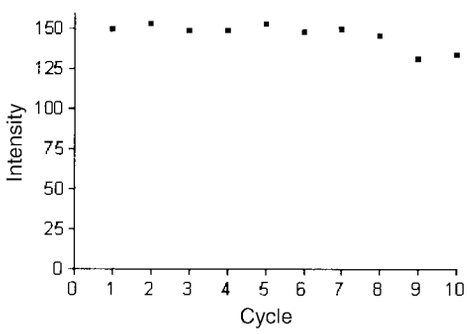

Fig. 2 Heat cycles: heating a solution of the complex **11**, 1×10^{-5} mol dm^{-3}, in HEPES buffer at pH 7.5 to 90 °C for 5 min, then cooling to 25 °C, leaving 5 min and then re-measuring the emission intensity. λ_{em} 615 nm, λ_{ex} 295 nm.

chelation number. Solutions of the probe **11** were prepared at a concentration of 1×10^{-4} mol dm^{-3} in a buffer solution of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (2.283 g in 1 dm^3 water) adjusted to pH 7.5. Aliquots were taken and any dilutions made with further HEPES buffer for spectroscopic measurement (see Experimental).

The excitation spectrum showed two peaks at 295 and 333 nm, in the ratio 1 : 0.56 and the emission spectrum could be obtained by exciting at either of these positions; for bioassays the longer excitation wavelength is preferable. The emission spectrum showed several sharp bands (Fig. 1), a predominant peak occurring at 615 nm. These peaks relate to the $^5D_0$ to ^7F_n emission bands, with the strongest at 615 nm being due to the $^5D_0$ to $^7F_2$ electronic transition. Of note is the relatively emission free window between 660–680 nm. The lifetime of the complex shows a single exponential, with a measured lifetime of 0.9 ms. Temperature studies were carried out to observe the stability of the metal ion complex by subjecting a solution to a repeated heat cycle: heating to 90 °C for 5 min and cooling to room temperature (25 °C) before re-measuring the emission curve. The complex was stable to at least eight such heat treatments (Fig. 2).

The solvation of the species **11**, in particular its hydration state, was then studied. Work by Horrocks and co-workers[12] suggested the use of deuterated water to afford a comparison of the luminescence against that obtained in ordinary water. Vibronic coupling between the O–H oscillators in water and the excited state europium ion causes quenching; each oscillator

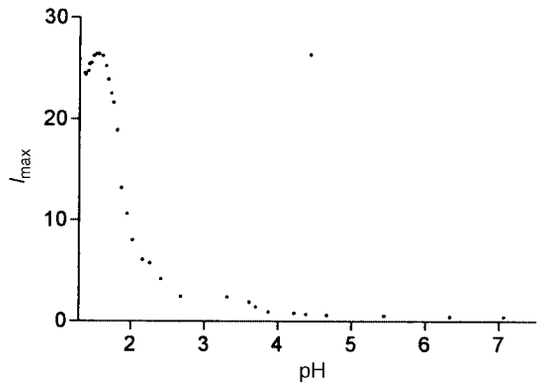

Fig. 3 The pH dependent emission curve of **12** monitored at 615 nm. [**12**] at 1.0×10^{-6} mol dm^{-3}. λ_{ex} at 295 nm.

is assumed to act independently and therefore the rate of quenching is proportional to the concentration of oscillators (principally water molecules) around the coordination sphere. Since O–D oscillators are of lower energy, this energy quenching pathway is less efficient when water is replaced by deuterium oxide. Beeby *et al.*[23] derived the relationship in eqn. (1), where q = the average no. of water molecules in the co-

$$q = A'[(1/\tau_{H_2O} - 1/\tau_{D_2O}) - 0.25] \quad (1)$$

ordination sphere; $\tau_{solvent}$ = the experimental radiative lifetime (milliseconds); and A' is a normalizing factor, found to be approximately 1.2 for the europium(III) species.

The emission lifetimes for D_2O and H_2O were, respectively, 1.6 and 0.9 ms. From equation (1) q computes to be 0.28, indicating, as anticipated, little water on the europium ion in the, nominally, nonadentate chelate **11**.

By comparison, at pH 7.5 the amino-substituted derivative, **12**, exhibited only a very weak luminescence. This is expected, since aromatic amino groups (anilines) are known to quench the excited state of the europium atom by an electron transfer mechanism.[24,25] This quenching mechanism is pH dependent and at sufficiently low pH, when the aniline group is fully protonated, the characteristic lanthanide emission is restored. Thus compound **12** is another example of a pH-sensitive probe. A typical pH emission response curve is shown in Fig. 3. Examination of lifetime curves for the emission at various pH values shows more than one species to be present in solution in the range above 2, a single exponential curve being obtained for the fully protonated species at pH 1.4. The signal from the protonated species is re-quenched by removal of the proton with base, illustrating the reversible nature of the process.

Protection of the amino group, either by acylation or by thiophosgenation, to afford the isothiocyanate derivative **13**, also restores the luminescent behaviour of the chelate, the observed intensity and pattern of emission being very similar to that of the unsubstituted terpyridyl chelate **11**. *In situ* reaction of the isothiocyanate with butylamine, to give the thiourea, **14**, gave a solution that also showed the characteristic Eu(III) luminescence.

Delayed fluorescence resonance energy transfer (DEFRET)

A model energy transfer experiment was carried out, using the lanthanide probe **13** as the donor and the natural allophycocyanin dye (APC dye) as the energy acceptor. APC has a broad excitation band, with a maximum at λ_{max} 650 nm, with a broad, strong shoulder around 615 nm, thus overlapping with the strong europium ion emission peak at *ca.* 615 nm. Its characteristic emission is at λ_{max} 660 nm; since the europium emission signal is weak at this point, monitoring light emission in this region is characteristic of the acceptor.

Table 1 Difference in the emission characteristics upon energy transfer. Conditions as described in Fig. 5. Emission intensities at λ_{615} and λ_{650} with lifetimes in ms

Probe	I_{615}	I_{650}	τ_{615}
15 + APC–streptavidin	5.69	3.57	0.62
15 alone	10.4	0	1.12

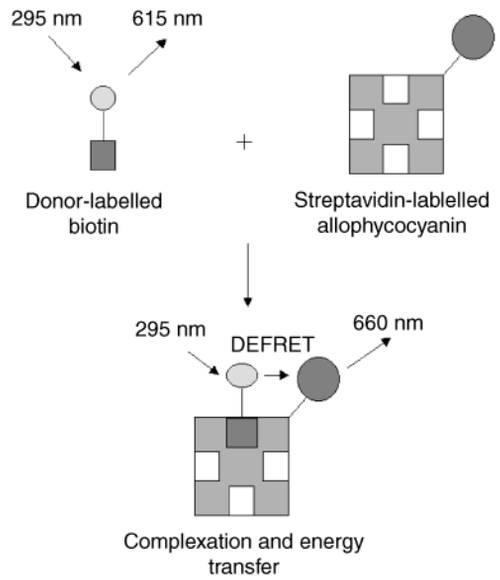

Fig. 4 Basis of delayed energy transfer (DEFRET) assay; up to four biotin units can react with each avidin molecule.

Resonance energy transfer is distance dependent and preferentially occurs when the donor and acceptor are held in the same vicinity, *i.e.* intramolecularly. We used the well-known biotin–streptavidin system to accomplish this (Fig. 4). Thus the isothiocyanate was coupled to *N*-(2-aminoethyl)biotinamide **16** to form the conjugate **15**, used as the donor dye. The acceptor used was streptavidin coupled to APC. Typical results are tabulated (Table 1). Exciting a solution of the donor (λ_{ex} 295 nm) in the absence of the acceptor, using a time delay of 0.1 ms, gave no observed emission signal at 660 nm. Under similar conditions, irradiation of the streptavidin–APC acceptor alone also gave no emission signal after the time delay, since any direct fluorescence of the acceptor stimulated by absorption at 295 nm has decayed to zero. On adding the acceptor, allowing the solution to equilibrate for 30 min to form the biotin–streptavidin complex and re-measuring, the delayed fluorescence spectrum gave a new signal observed at 660 nm and due to energy transfer from the donor to the acceptor.

A consequence of this energy transfer is a quenching of the europium signal at 615 nm. Likewise the lifetime of the emission at 615 nm decreases from 1.12 ms for the donor alone, to 0.62 ms (Fig. 5) in the presence of the acceptor, thus confirming the DEFRET process.

Experimental

Distilled, deionized water was used throughout this work, without deaeration. All volumetric flasks and quartz cells were carefully precleaned with Caro's acid (1 : 1 v/v 30% H_2O_2 and 98% sulfuric acid) at room temperature. These were then rinsed with distilled water, 3 mol dm^{-3} HCl, distilled water and then AnalaR methanol, before air drying and use. Europium(III) chloride hexahydrate was purchased from Sigma-Aldrich. Crosslinked streptavidin–allophycocyanin conjugate and *N*-2-(aminoethyl)biotinamide were purchased from Molecular Probes, Eugene, OH. Other compounds were prepared by the methods described below.

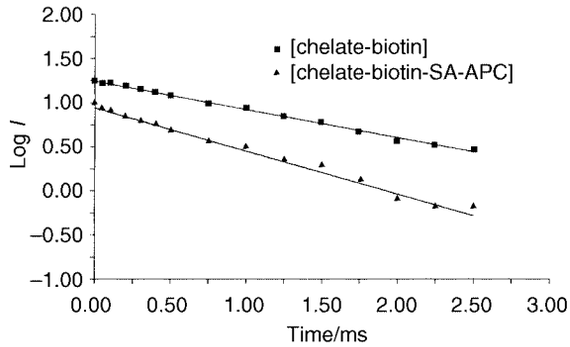

Fig. 5 Difference in the emission lifetimes of the Eu(III) probe–biotin complex **15** in the absence and presence of streptavidin–allophycocyanin; see Experimental for details. λ_{ex} 294 nm, λ_{em} 615 nm, slit width 10 nm, delay 0.05 ms, gate time 5 ms; ■ complex **15** in the absence of streptavidin–allophycocyanin, at 8.3×10^{-7} mol dm^{-3}; ▲ with added streptavidin–allophycocyanin, at 2.5×10^{-7} mol dm^{-3}.

Luminescent measurements were performed on a Perkin-Elmer LS50B spectrofluorimeter fitted with a red-sensitive photomultiplier, using a 1 cm optically flat cuvette at room temperature (20–23 °C) without external temperature control. Europium(III) luminescent measurements were unaffected by small ambient temperature changes. For the europium(III) chelate measurements standard phosphorescent instrument settings were used with a 0.05 ms delay time, excitation slit width of 10 nm and an emission slit width of 10 nm, using a 350 nm emission filter. Output was to an IBM PC interfaced *via* the Perkin-Elmer Fluorescence Data Manager (FLDM) package. For lifetime studies 99.99% deuterium oxide was purchased from Sigma-Aldrich.

Solutions of the probes were buffered with 0.001 mol dm^{-3} 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) at pH 7.5, pH adjustments being made with 0.1 mol dm^{-3} hydrochloric acid, before final dilution of samples to volume. Readings were generally recorded in duplicate. Stock solutions of the probes were prepared at 1×10^{-4} mol dm^{-3} and diluted as required. The stock solutions were stored in the dark and generally freshly prepared each week although stability studies suggested that they retained activity for a period of several weeks.

^{1}H NMR spectra were recorded on a JEOL FX200 instrument using, unless otherwise stated, deuteriochloroform solutions with tetramethylsilane as an internal reference; chemical shifts are in ppm and coupling constants are measured in hertz. Mass spectra were carried out under a variety of regimes (FAB, LSIMS and electrospray) by the EPSRC Mass Spectrometry Laboratory, Swansea.

6,6″-Bis(aminomethyl)-4′-(4‴-methylphenyl)-2,2′ : 6′,2″-terpyridine·5HCl 6

Dinitrile **5**[21] was mixed with dry THF and deaerated BH_3·THF was then added (20 cm^3). The solution was stirred at room temperature for 16 h and then the excess of borane was destroyed with methanol. The solvent was removed *in vacuo* and the white solid obtained was dissolved in HCl-saturated ethanol and heated to reflux for 2 h. Upon cooling a yellow precipitate of the *title salt* formed which was collected and dried (3.2 g, 53%); δ_H (360 MHz, $CDCl_3$) 8.94 (2 H, s), 8.68 (2 H, d, *J* 7.5), 8.16 (2 H, t, *J* 7.5), 8.02 (2 H, d, *J* 7.5), 7.62 (2 H, d, *J* 7.5), 7.49 (2 H, d, *J* 7.5), 4.39 (broad, NH_3^+ and NH^+), 4.04 (4 H, s), 2.44 (3 H, s); *m*/*z* (FAB-MS) 382 (MH^+ − 5HCl), 404 (MNa^+ − 5 HCl). Found: 382.2036 (MH^+ − 5HCl); $C_{24}H_{24}N_5$ requires 382.2048.

6,6″-Bis[*N*,*N*-bis(methoxycarbonylmethyl)aminomethyl]-4′-(4‴-methylphenyl)-2,2′ : 6′,2″-terpyridine 7

The bis-amine **6** (3.0 g, 7.8 mmol) was slurried in dry

acetonitrile (100 cm^3). Diisopropylethylamine (5.4 cm^3, 31.2 mmol) and methyl bromoacetate (2.8 cm^3, 30 mmol) were added and the solution was refluxed for 24 h under a blanket of nitrogen. The solution was cooled, poured into a mixture of saturated aqueous sodium carbonate solution and dichloromethane, the organic layer separated, washed with water and the organic extract dried and evaporated to yield a red gum. This was dissolved in dichloromethane and passed through a short silica column (dichloromethane as eluant) under slight pressure to yield the *title product* (1.2 g, 33%); δ_H (360 MHz) 8.68 (2 H, s), 8.52 (2 H, d, *J* 8), 7.84 (2 H, d, *J* 7.5), 7.78 (2 H, t, *J* 7.5), 7.54 (2 H, d, *J* 7.5), 7.34 (2 H, d, *J* 7.5), 4.31 (4 H, s), 3.73 (12 H, s), 2.47 (3 H, s), 2.16 (8 H, s); *m*/*z* (FAB-MS) 669 (M^+), 670 (MH^+), 692 (MNa^+). Found: MH^+ 670.2872; $C_{36}H_{40}N_5O_8$ requires 670.2877.

6,6″-Bis[*N*,*N*-bis(methoxycarbonylmethyl)aminomethyl]-4′-(4‴-methyl-3‴-nitrophenyl)-2,2′ : 6′,2″-terpyridine 8

The tetra-ester **7** (500 mg, 0.7 mmol) was dissolved in conc. sulfuric acid at 5 °C. Fuming nitric acid (0.7 mmol) was added and the solution was stirred for 20 min. The solution was then quenched into ice and neutralized with 10% w/v aqueous sodium carbonate solution. The organics were extracted with dichloromethane and the solvent removed under reduced pressure to yield a dark brown oil. This was passed through an alumina column (dichloromethane as eluant) to yield the *title compound* as a yellow oil (403 mg, 75%); δ_H (360 MHz) 8.71 (2 H, s), 8.56 (2 H, d, *J* 7), 8.50 (1 H, s), 8.08 (1 H, d, *J* 7), 7.89 (2 H, d, *J* 7), 7.63 (2 H, d, *J* 7.5), 7.53 (1 H, d, *J* 7), 4.31 (4 H, s), 3.73 (12 H, s), 2.47 (3 H, s), 2.16 (8 H, s); *m*/*z* (LSIMS MS) 715 (MH^+), 737 (MNa^+). Found: MH^+ 715.2739; $C_{36}H_{38}N_6O_{10}{\cdot}H^+$ requires 715.2728.

6,6″-Bis[*N*,*N*-bis(methoxycarbonylmethyl)aminomethyl]-4′-(4‴-methyl-3‴-aminophenyl)-2,2′ : 6′,2″-terpyridine 9

Tetra-ester **8** (400 mg, 0.55 mmol) was dissolved in ethanol and heated to 50 °C with stirring. $SnCl_2{\cdot}2H_2O$ (0.68 g, 3 mmol) was added and the solution became orange. The mixture was stirred for 1 h at which point it was quenched over ice and dilute sulfuric acid. The solution was neutralized with 10% w/v aqueous sodium carbonate solution and an excess of EDTA disodium salt was added. The organics were extracted with dichloromethane, dried and the solvent removed by rotary evaporation to yield a brown gum. This was passed through a short silica column (dichloromethane as eluant) to yield the *title compound* as a yellow oil (223 mg, 58%); δ_H (360 MHz) 8.81 (2 H, s), 8.54 (2 H, d, *J* 7), 7.87–7.72 (3 H, m), 7.61 (2 H, d, *J* 7.5), 7.26 (1 H, s), 7.14 (1 H, d, *J* 7.5), 4.16 (4 H, s), 4.1 (broad s, NH_2), 3.72 (12 H, s), 2.50 (3H, s), 2.16 (8 H, s); *m*/*z* (LSIMS MS) 685 (MH^+), 707 (MNa^+).

6,6″-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4′-(4‴-methyl-3‴-aminophenyl)-2,2′ : 6′,2″-terpyridine sodium salt 10

The amine **9** (0.20 g, 0.292 mmol) was dissolved in methanol (2 ml). An aqueous solution of sodium hydroxide (47 mg, 1.16 mmol in water, 2 cm^3 and methanol, 3 cm^3) was added and the solution was stirred for 2 h at room temperature. The solvent was then removed to yield a pale white solid (0.22 g). This was taken through to the following stage without further purification.

{6,6″-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4′-(4‴-methyl-3‴-aminophenyl)-2,2′ : 6′,2″-terpyridine}europium(III) monosodium salt 12

The tetra-carboxylic acid salt **10** (110 mg, 0.16 mmol) was dissolved in water (5 cm^3). Europium(III) chloride hexahydrate (86 mg, 0.24 mmol) was added and the solution stirred at room temperature for 1 h. The solution was then basified with a small volume of 0.1 mol dm^{-3} sodium hydroxide solution. The excess of europium precipitated out as the insoluble hydroxides. The mixture was filtered and the filtrate treated with cold acetone to yield the *monosodium salt* product as a white precipitate (92 mg, 75%), which was dried over P_2O_5. The salt was soluble in water and, when made acid (pH 2), showed the typical red europium luminescence under UV light. A sample was redissolved in the minimum of water and reprecipitated with acetone for the spectroscopic studies. The complex was found to be the mono-sodium, mono-europium salt; *m*/*z* (FAB-MS) 799 (^{151}Eu) and 801 (^{153}Eu) (MH^+), 821 (^{151}Eu) and 823 (^{153}Eu) (MNa^+). Found: 799.1083; $C_{32}H_{28}N_6O_8{\cdot}^{151}Eu{\cdot}Na{\cdot}H^+$ requires 799.1085.

{6,6″-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4′-(4‴-methyl-3‴-isothiocyanatophenyl)-2,2′ : 6′,2″-terpyridine}europium(III) 13

The sodium salt of the europium(III) complex **12** (80 mg, 0.09 mmol) was dissolved in water (5 cm^3). To this was added dichloromethane (1 cm^3) containing an excess of thiophosgene. This was stirred for 2 h at room temperature and then the aqueous layer was extracted and washed with dichloromethane. The aqueous layer was added to cold acetone whereupon the *isothiocyanate product* precipitated out as an off-white solid (55 mg, 66%); *m*/*z* (FAB-MS) 841 and 843 (MH^+, both Eu^{3+} isotopes), 863 and 865 (MNa^+, both Eu^{3+} isotopes). The product was dried over P_2O_5 under reduced pressure and stored in a freezer at −20 °C before use.

6,6″-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4′-(4‴-methylphenyl)-2,2′ : 6′,2″-terpyridine sodium salt 2

The ester **7** (200 mg, 0.30 mmol) was dissolved in methanol (2 cm^3) and the solution was added to an equivalent quantity of a solution of sodium hydroxide (48 mg, 1.2 mmol) in water (5 cm^3). The solution was stirred for 2 h at room temperature before removing the solvent under reduced pressure to leave the *sodium salt* as a pale white solid (230 mg). This was passed through to the next stage without further purification.

{6,6″-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4′-(4‴-methylphenyl)-2,2′ : 6′,2″-terpyridine}europium(III) sodium salt 11

The sodium salt **2** (100 mg, 0.14 mmol) was dissolved (100 mg) in water (5 cm^3). This was stirred with europium(III) chloride hexahydrate (183 mg, 0.5 mmol) at room temperature with sodium carbonate buffer (pH 7.5) for 1 h. The solution was then basified with a small volume of sodium hydroxide. The excess of europium precipitated out as europium hydroxide. The mixture was then filtered and the filtrate treated with cold acetone. The *title product* precipitated out as the monosodium salt (65 mg, 58%); *m*/*z* (FAB-MS) 784 and 786 (MH^+, both Eu^{3+} isotopes), 806, 808 (MNa^+, both Eu^{3+} isotopes). Found: 808.0866; $C_{32}H_{27}N_5O_8{\cdot}^{151}Eu{\cdot}Na{\cdot}Na^+$ requires 808.0867.

Conjugation of the *N*-(2-aminoethyl)biotinamide 16 to the probe 13

Freshly prepared solutions of the conjugate **15** were prepared by stirring the isothiocyanate **13** (0.90 mg portions) at room temperature with the biotin derivative **16** (0.30 mg) in HEPES buffer (1 cm^3) at pH 7.5 for 2–6 h. The resulting solution was then made up to 10 cm^3 with more buffer to give a solution $1 \times 10^{-6} \pm 10\%$ mol dm^{-3}. A solution of the streptavidin–allophycocyanin conjugate was also prepared in HEPES buffer (2×10^{-6} mol dm^{-3}). Portions of the solutions were mixed in the ratio 8 : 1 to give a 4 : 1 molar ratio of the biotinamide–streptavidin system. The solution was allowed to stir at room temperature for at least 30 min before measurements were made.

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