Engineering emissive europium and terbium complexes for molecular imaging and sensing†

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Emissive f-block coordination complexes constitute an important class of optical probes, with applications ranging from sensing of bioactive species, high throughput assays and screening protocols *in vitro*, to time-resolved imaging studies *in cellulo* or *in vivo*. The key chemistry issues to be addressed in complex design and characterisation are defined, with an emphasis on the use of emissive europium and terbium complexes and their conjugates in molecular imaging. Both luminescent 'tags' useful in energy transfer studies and 'responsive' systems for sensing are discussed.

Introduction

Luminescent molecular probes that are used as sensor materials or in imaging applications are mostly based on well established condensed heterocyclic dyes, such as fluorescein or rhodamine.[1,2] They are fluorescent and typically possess a short lived singlet ex-

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cited state with a lifetime of up to a few nanoseconds. Absorption and emission bands are broad, with bandwidths of the order of tens of nm, and they usually possess rather small shifts between these bands, *i.e.* a small Stokes shift. The wide range of useful and commercially important fluorescent probes has been expanded and enhanced over the past two decades by the introduction of Alexa Fluor® and BOPIPY® dyes,[2,3] the latter based on the charge neutral 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene core, **1**. These dyes are more resistant to photobleaching (a light induced change in a chromophore, resulting in loss of absorption of light at a given

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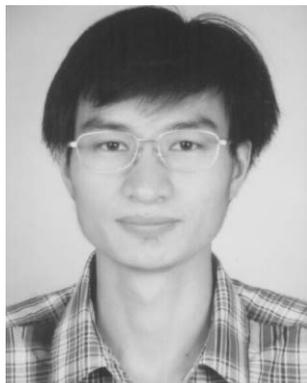
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wavelength), exhibit a pH-independent fluorescence and possess high extinction coefficients (200 000 M^{-1} cm^{-1}) with emission quantum yields that are often >0.7. Absorption and emission wavelengths can be determined by appropriate substitution of the core fluorophore.[3] These dyes, or their bio-conjugates, have some limitations. They are prone to interference from light scattering in biological media (Rayleigh, Tindall or Raman) and from the natural fluorescence of certain biomolecules, as well as being limited by occasional technical problems (*e.g.* autofluorescence) associated with their small Stokes shift. Such limitations have driven the search for improvements in luminescent probes over the past few years. Important advances have been made in the controlled synthesis and enhancing photo-stability of the strongly absorbing ($\varepsilon > 10^6$ M^{-1} cm^{-1}) semi-conductor quantum dots' based on *e.g.* InN, CdS and CdSe. These materials also possess emission bands whose bandwidth is a function of particle size, allowing an element of control that is absent in the traditional organic dyes.[4–6]

Metal coordination complexes comprise another important class of luminescent probes and considerable efforts have been made to exploit their unique excited state properties.[7] In this article, attention is focussed on lanthanide complexes, and consideration of the key issues to be addressed before coordination complexes can be engineered to act as practicable sensors or as imaging probes for microscopy.[8–14]

The excited state lifetimes of lanthanide ions range from the order of microseconds for the rear-IR emitting lanthanides (Yb(III), Er(III) and Nd(III))[8] to milliseconds for Eu(III) and Tb(III) complexes. These long lifetimes immediately offer an advantage as they allow time-resolved spectroscopy and microscopy to be used. The introduction of a time delay (say 1 or 100 μs), prior to detection of the emitted light eliminates interference from light scattering and auto-fluorescence and greatly enhances the signal/noise ratio (detection limits are conservatively in the 10^{-12}–10^{-15} M range) and hence the reliability of detection and monitoring.[15,16] Such features encouraged the development in the 1980's of various time-resolved assays, using lanthanide components, first commercialised by Wallac-Oy in Finland following the innovative work of Hemmila and Soini in heterogeneous systems.[16] Homogeneous assays followed, developed mainly by CIS-bio International in France.[15]

Lanthanide emission bands are very sharp compared to organic fluorophores, typically with a full width at half maximum of less than 10 nm. To a first approximation, the metal-based emission profile is similar for each lanthanide complex. However, for Eu(III) and Yb(III) complexes in particular, the form and relative intensity of the main emission bands are a sensitive function of the local coordination environment, allowing the signalling of changes in metal complex speciation[12c,17–20] by ratiometric analysis.

Every lanthanide complex requires the incorporation into the ligand structure of a sensitising chromophore, capable of transferring its excited state energy to the encapsulated lanthanide(III) ion (Scheme 1). This is essential as direct excitation of the Ln^{3+} ion is very inefficient, leading to low values of ε (≤ 1 M^{-1} cm^{-1}) as the
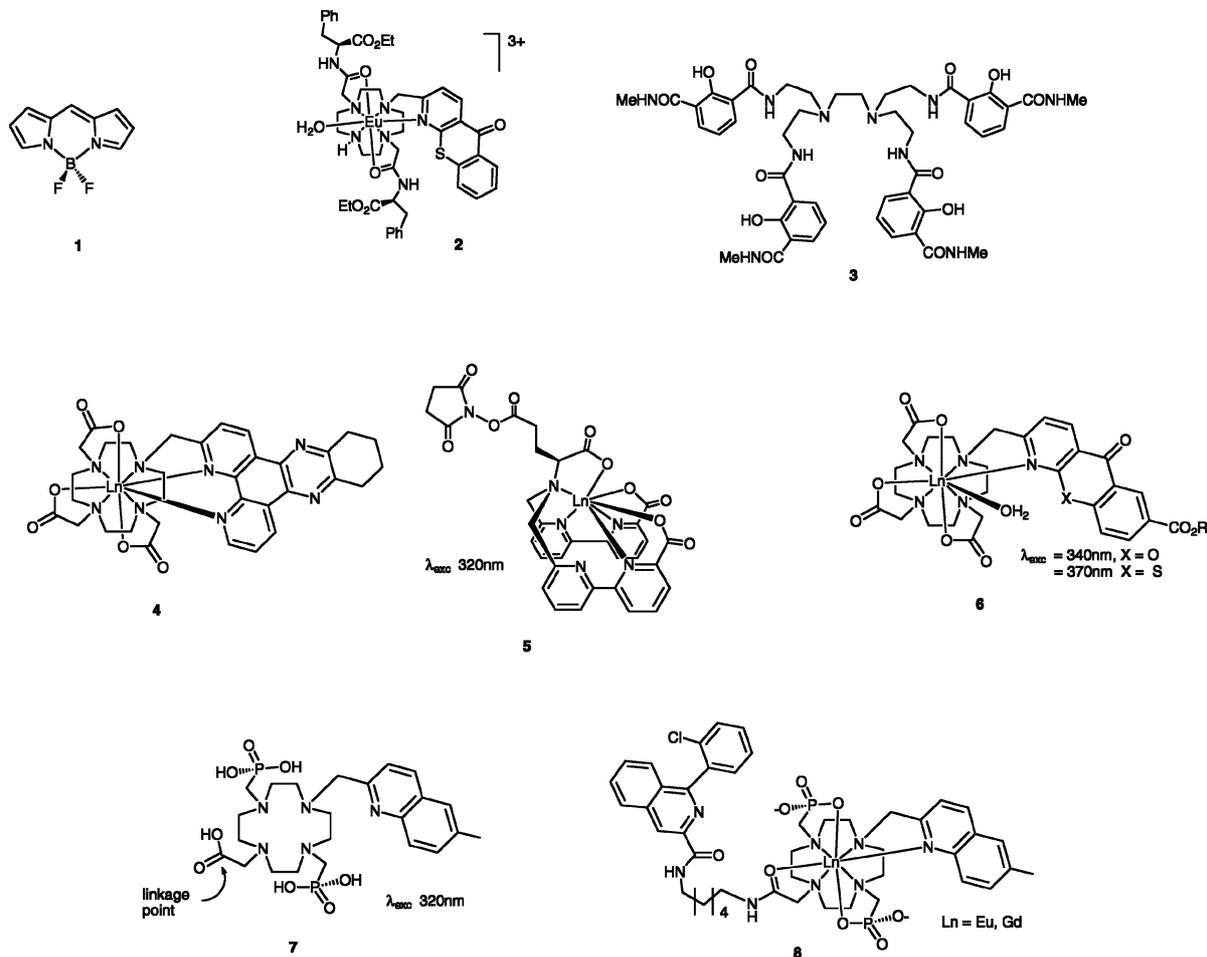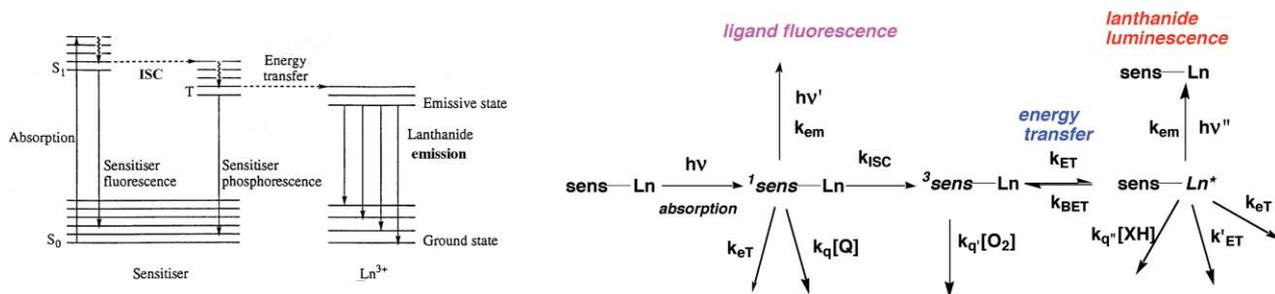
Scheme 1

relevant f–f transitions are parity (Laporte) forbidden. Since the early work on single component emissive systems of Lehn, Mathis and Alpha,[21] an extensive range of aromatic and heterocyclic chromophores has been examined. These moieties need to be engineered into the ligand and serve as an antenna to harvest incident light. Detailed analyses of their structural and mechanistic features have been reviewed in depth[8,12,13,15–17,20] elsewhere.

The essential requirements for this sensitising moiety can be summarised as follows: a high extinction coefficient at an excitation wavelength appropriate for the application—*e.g.* in the range 337–420 nm for single photon excitation of biological samples with Eu^{3+}/Tb^{3+} systems; a fast inter-system crossing step to ensure the aromatic triplet is populated efficiently—minimising ligand fluorescence; a fast energy transfer step leading to population of the lanthanide excited state; a seven- to nine-coordinating ligand to encapsulate the Ln(III) ion, minimising the quenching of the lanthanide excited state by vibrational energy transfer to matched oscillators, *e.g.* O–H, N–H or C–H stretching vibrations.

The lanthanide excited state may persist for several milliseconds, during which it is prone to quenching by electron or energy transfer processes. The $^5D_0$ and $^5D_4$ excited states of Eu^{3+} and Tb^{3+}, for example, lie at 206 (17 200 cm^{-1}) and 244 kJ mol^{-1} (20 400 cm^{-1}) above the ground state. This free energy can be used to drive various electron transfer processes, for example in a collisional encounter with electron rich species such as I$^-$, urate or ascorbate ($E_{ox} < 0.5$ V).[22] The latter two anions are ubiquitous low molecular weight anti-oxidants in many biosystems, typically existing in 0.1 to 2 mM levels in different cell types.[23] Inhibition of this quenching process requires that electron transfer to the complex be disfavoured, either by anionic repulsion, *e.g.* introducing peripheral negatively charged groups to suppress

collisional encounters, by adding a very high local concentration of a non-oxidisable anion to the medium (*e.g.* > 0.1 M F$^-$),[15] or by reducing the electron affinity of the overall complex.

Recent examples of highly emissive lanthanide complexes absorbing above 340 nm, **2**,[24] [Ln·**3**][14] and **4**,[22] serve to illustrate these design issues of desirable sensiser characteristics and appropriate ligand structure. The absorption and total emission spectrum of **2** (Fig. 1), highlights the relatively long excitation wavelength (λ_{exc} 384 nm), shows that inter-system crossing is competitive with fluorescence from the aza-thiaxanthone singlet excited state (residual ligand fluorescence at 450 nm) and reveals the highly structured red europium emission spectrum, which can be observed selectively upon application of a 100 μs time delay in acquisition.

Critical design features for luminescent probes

Emissive lanthanide complexes that are to be used either as 'responsive' systems or as luminescent 'tags' in bio-conjugates, for example as fluorescence resonant energy transfer (FRET) components, must be designed with the following features in place, in order to gain generic utility. A high overall emission quantum yield ($\geq$10% in aqueous media) is needed following excitation into a strongly absorbing chromophore at wavelengths greater than 337 nm. Of particular interest are excitation wavelengths that correspond to readily available laser lines (337, 355 or 405 nm for Eu/Tb; 532 nm for Yb/Nd), or are close in energy to the growing range of powerful light emitting diodes, *e.g.* 365 or 390 nm. Some of these LEDs now have power-ratings in the 100 milliwatt range. This requires a small singlet triplet energy gap for Eu- and especially Tb-sensitised emission. Suitable chromophores include quinolones, tetraazatriphenylenes,[25] substituted
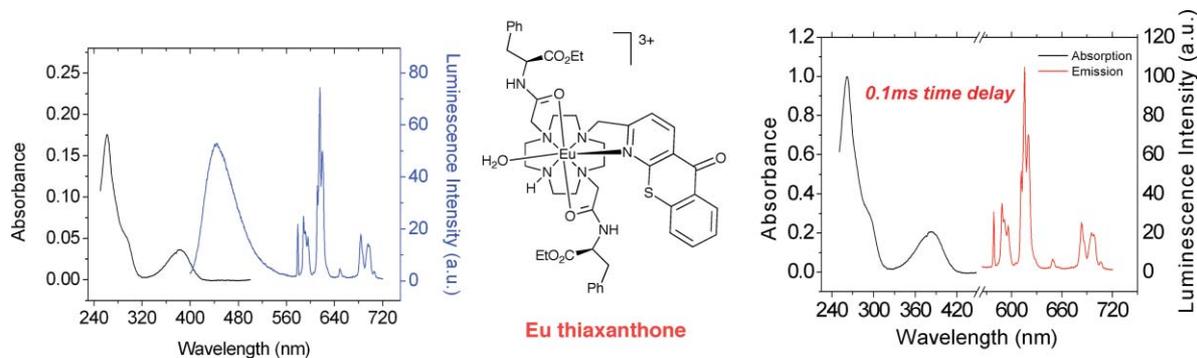

Fig. 1 Absorption and total emission spectra for the aqua–europium complex, **2**, (λ_{exc} 384 nm, ϕ_{em} 9% H$_2$O) highlighting the short-lived ligand-based fluorescence and the long-lived europium luminescence, the latter being observed uniquely when a 100 μs time delay is used (right).

bipyridyls/terpyridyls and their *N*-oxides,[9,15,16,26] xanthones and their derivatives[24] (acridones are often limited by inefficient triplet population in polar media).[27,28] The ligand should be synthesised efficiently, from readily available precursors and at reasonable cost, and the complex should be thermodynamically and kinetically stable with respect to metal ion loss, photofading and oxidative degradation in air or in aqueous media. When the lanthanide complex is to be used as an emissive 'tag', it must possess a suitable functional group to expedite conjugation, *e.g.* a pendant acid or primary amine group. Recent examples include **5**,[9] **6** (Fig. 2) and **7**. The simple permutation of the lanthanide ion allows bi- and multimodal analyses to be undertaken, *e.g.* using emissive Eu/Tb/Sm and Dy analogues differing in emission lifetime and spectral output for multi-parametric analysis.[8,30,31] In addition, Eu/Tb and Gd complexes of a given ligand may be expected to show similar biodistribution and/or cellular uptake profiles so that combined MRI and optical imaging protocols become possible with a 'common' complex or conjugate.[32–34] A recent example is provided by **8**, in which the conjugated *o*-chlorophenylquinoline moiety targets (with apparent nanomolar affinity) a peripheral benzodiazepine receptor that is over-expressed in glioblastoma, breast cancer and Alzheimer's disease.[29]
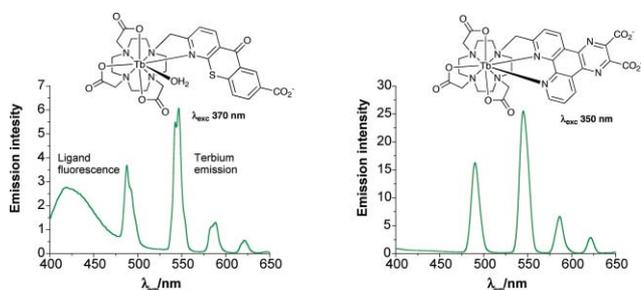
Fig. 2 Emission spectra for selected terbium complexes (298 K, 0.1 M NaCl), showing residual ligand fluorescence from the aza-thiaxanthone chromophore (left).

Using lanthanide complexes for imaging *in cellulo*

Given the favourable impact that time-resolved homogenous assays has had in analytical biochemistry and high throughput screening,[15,16] a topical challenge is to devise complexes that luminesce efficiently within living cells. Extensions to *in vivo* work can then be confidently undertaken, whilst appreciating that light in the 710–820 nm window penetrates skin and tissue most effectively. This may limit developments *in vivo* to the use of near-IR emitting luminophores (*e.g.* cyanine dyes and Nd^{3+}/Yb^{3+} complexes), unless effective two-photon excitation protocols are devised for

Eu^{3+}/Tb^{3+} luminescent probes. Two different classes of probe may be distinguished at the outset: stable luminescent complexes that serve as 'tags' in bioconjugates for use in FRET or 'tracking' assays, and responsive systems wherein the spectral emission profile, lifetime or circular polarisation varies as a function of the local concentration of a target intracellular analyte. In each case, the complex or conjugate must resist excited state quenching by local donors/acceptors. In this respect, suppressing quenching of the lanthanide excited state by photoinduced electron transfer from ascorbate, urate or glutathione is again likely to be important in defining practicable systems.[22]

An obvious pre-requisite for *in cellulo* imaging is that the complex must be non-toxic and cell permeable, preferably with a distinctive compartmentalisation profile, *i.e.* a preference to localise in a given organelle.[2] For conjugates, this localisation is likely to be determined by the nature of the protein/peptide/synthetic vector, with cell-penetrating peptides, *e.g.* penetratin and its analogues offering much scope in this respect.[35] However, many of these systems recognise a cell surface receptor and use receptor mediated endocytosis as the mechanism of cell entry, so that the conjugate then resides in an endosome (or a related vesicle structure). Endosomal escape is then essential, if an organelle is being targeted or if the cytosol needs to be labelled, otherwise the endosome will age to become a lysosomal compartment (low pH, enhanced likelihood of degradation), isolated from the rest of the cell. For example, the use of **8** (Eu complex, λ_{exc} 320 nm) to label glioblastoma cells (Fig. 3) is believed to follow an endocytosis mechanism,[29] consistent with the finding that uptake was minimal at 4 °C. It was suggested that this conjugate might localise in the outer mitochondrial membrane, where the target receptors are expressed, although this was not unequivocally demonstrated. Earlier work from the same group had highlighted the opportunities in optical imaging of surface lesions using a related Tb–pyridyl complex (λ_{exc} 280 nm), following topical administration using a hamster oral cancer model.[36]
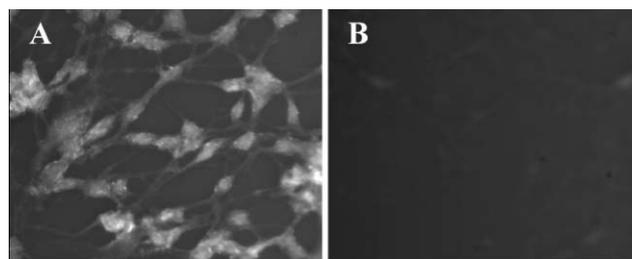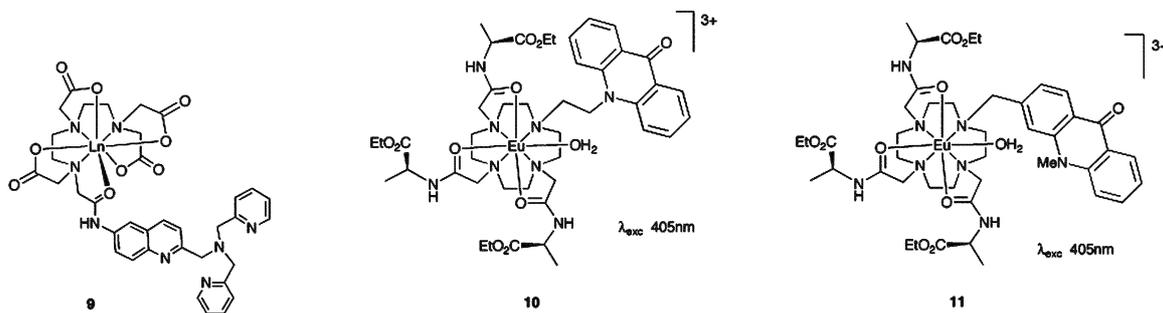
Fig. 3 Epifluorescence microscopy images of C6 glioblastoma cells; (left) following incubation with **8** (Ln = Eu, λ_{exc} 320 nm, 60 minute incubation 25 μm complex); (right) is a blank control.

Tracking changes in Zn^{2+} concentrations in real time in living cells is an important current area of study in bio-inorganic chemistry.[37,38] Bifunctional europium complexing agents have been defined incorporating a zinc binding moiety so that the observed luminescence emission intensity increases as a function of $[Zn^{2+}]$.[39,40] Using non-ratiometric methods, proof-of-principle zinc-dependent imaging studies have been reported with **9** (λ_{exc} 320–340 nm) following direct injection of the complex into HeLa cells. Other workers interested in plant nutrition have examined direct uptake of Eu^{3+} aqua species in oat roots and interrogated the process using laser excitation time-resolved confocal microscopy.[41]

Predicting cellular compartmentalisation profiles for low MW luminescent species is not straightforward. A good example is provided by the differing behaviour of the constitutionally isomeric complexes **10** and **11**.[42,43] In mouse skin fibroblasts (NIH-3T3 cells), confocal microscopy has been used to examine the intracellular distribution of these complexes. They were developed to report upon local concentrations of HCO_3^- by measuring the changes in the intensity ratio of two (or more) Eu emission bands. Bicarbonate is an important intracellular species as it binds reversibly to the enzyme adenylylcyclase, thereby regulating release of the key secondary messenger cyclic-AMP. The two isomeric complexes **10** and **11** (λ_{exc} 405 nm) exhibit very different behaviour *in cellulo*! The 'C-linked' isomer (Fig. 4) distributes quite well in the cytosol and shows a tendency to stain the endoplasmic reticulum revealed in co-staining experiments with a conjugate of Brefeldin A. The 'N-linked' complex **11**, in contrast, is taken up by an endosonal pathway, and in co-localisation studies with Lysotracker-green, exhibits good correspondence with this organic dye that is known to localise in the acidic lysosomal compartment. Useful bicarbonate analyses are therefore precluded in this case (pK_a ~6.2 for $HCO_3^-/H_2O–CO_2$).
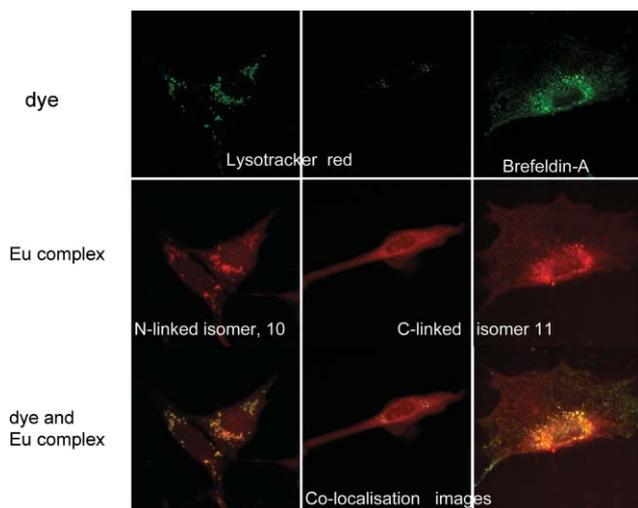

Fig. 4 Comparison of confocal fluorescence microscopic images of constitutionally isomeric Eu complexes **10** and **11** in NIH-3T3 cells highlighting their differing localisation profiles.

The citrate anion is a key component of many biological systems including fatty-acid synthesis, nitrogen metabolism and photorespiration. Simple terbium salts, such as the citrate, have been noted to bind *in vitro* to single-stranded nucleic acids, as revealed by the enhancement of terbium emission intensity following binding to RNA.[44] In addition, terbium citrate has been used to examine the visualisation of RNA by electron microscopy. Using ultra-thin sections staining of nucleoli, interchromatin and perichromatin granules and coiled bodies in the cell nucleus have been revealed, whereas ribosomes are the only contrasted structures in the cytoplasm.[45] A pH-insensitive ratiometric chemosensor for citrate has been reported,[46] in which the europium emission spectral form changes as a function of local citrate concentration in competitive media ($K_d = 0.28$ mM), allowing ratiometric analysis (λ_{exc} 384 nm). This complex is also taken up by live cells *via* a non-endocytotic pathway, and stains the intranuclear cell nucleoli with some selectivity (Fig. 5). Control experiments suggested that the complex is not binding to RNA itself (addition of RNA quenches Eu emission *in vitro*). The local speciation of the europium complex, **2**, has been probed by examining the spectroscopic signature of the complex in cells, and the integrity of the sensitiser in the complex confirmed by excitation spectroscopy. The species observed *in cellulo* by confocal microscopy is certainly not a simple RNA, aqua or citrate adduct, but may be a protein-bound entity.[47]

The amphipathic cationic lanthanide complexes related to **12** and incorporating an efficient tetraazatriphenylene chromophore, (λ_{exc} 350 nm) also exhibit good cell uptake and a strong intracellular emission profile.[22] Originally, these enantiopure complexes were examined *in vitro* for their ability to bind the most common helical form of DNA (*B*-DNA) stereoselectively[48] ($K_a > 10^6$ M^{-1} with different affinities for Δ *vs.* Λ isomers), but cell localisation work in HEK (embryonic kidney) 3T3, COS and CHO cells revealed good cell penetration. At higher concentrations of complex in the incubating medium, the complex localises in the cell nucleus (perhaps DNA-bound), and egress of the complex from the nucleus back to the extranuclear organelles has been observed by confocal microscopy. Cell uptake is almost non-existent for the corresponding charge neutral and anionic analogues of **12** (*i.e.* with carboxymethyl or 'glutarate' side chains). Given that the cationic complex also localises well at 4 °C and binds fairly strongly to albumin in the cell growth medium (effective $K_d \sim$ 0.1 mM), it may be entering the cell *via* a pore or channel possibly as a reversibly bound protein adduct.

Many examples have been reported of the use of conjugates of europium complexes in high throughput screening, cell surface labelling or FRET studies.[8b,15,16] Several examples[15] relate to the use of the europium cryptates (λ_{exc} 337 nm), for example in FRET systems labelled with Alexa-Fluor 647 (as an acceptor) to analyse membrane protein–protein interactions at the surface of living cells.[15d] Examples include the use of europium-labelled annexin V to study the cell-death cascade in adherent cell cultures[49] and a 'europium tagged' antibody in the screening of inhibitors that block phosphorylation of the tumour-suppressor retinoblastoma protein.[50] In contrast to some earlier work using the effective DTPA–carbostyril[8b,51,52] conjugates described by Selvin(λ_{exc} 337 nm), some reports use very inefficient sensitisers such as 4-aminosalicylate[53a] or a simple anilide[53b] (λ_{exc} 325/320 nm), exciting at the long-wavelength edge of each absorption band. Finally, the lipophilic europium complex europium tris(thenoyltrifluoro-acetonate), has been shown to stain liposomal membranes.[53c] The emission intensity was temperature dependent in the range 15–40 °C, suggesting its use in cellular thermal imaging, for example as a consequence of
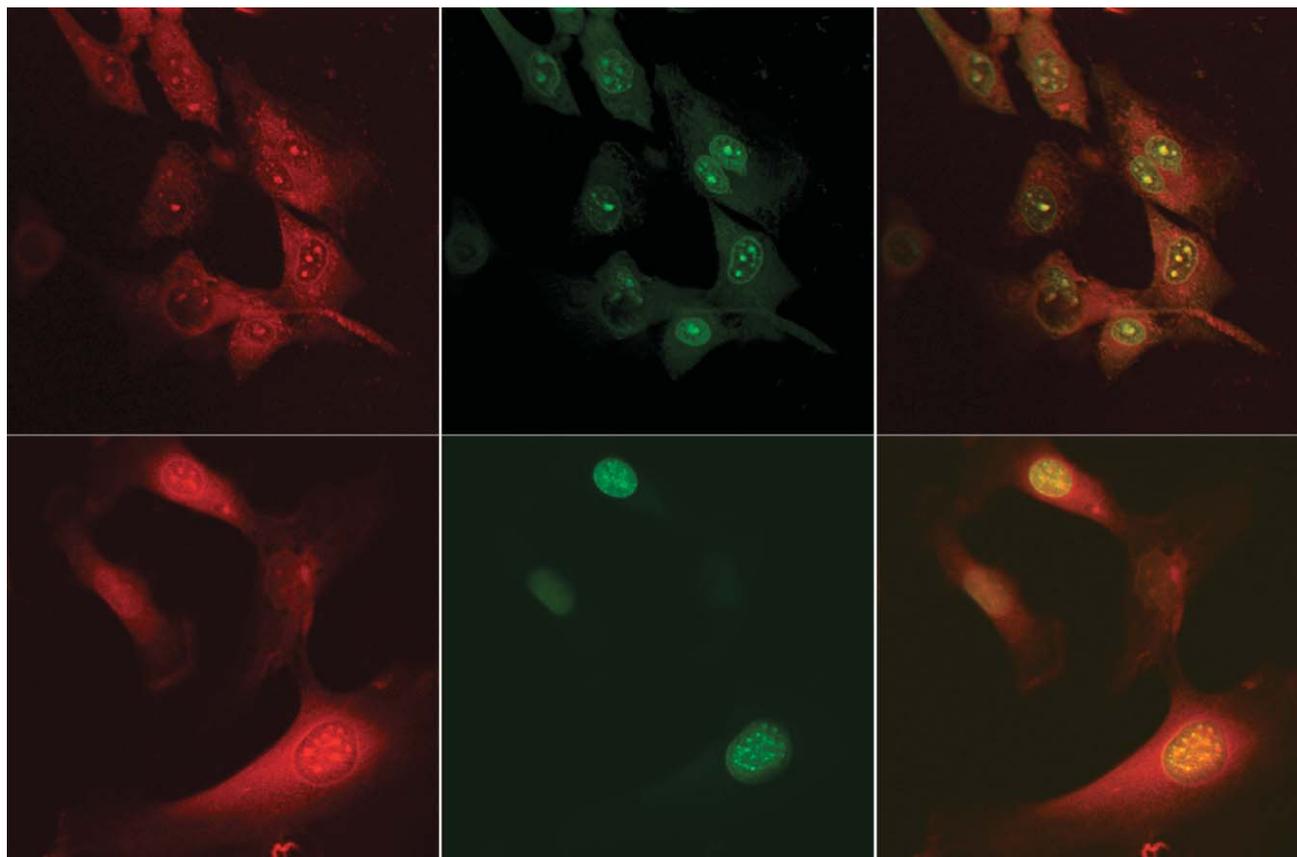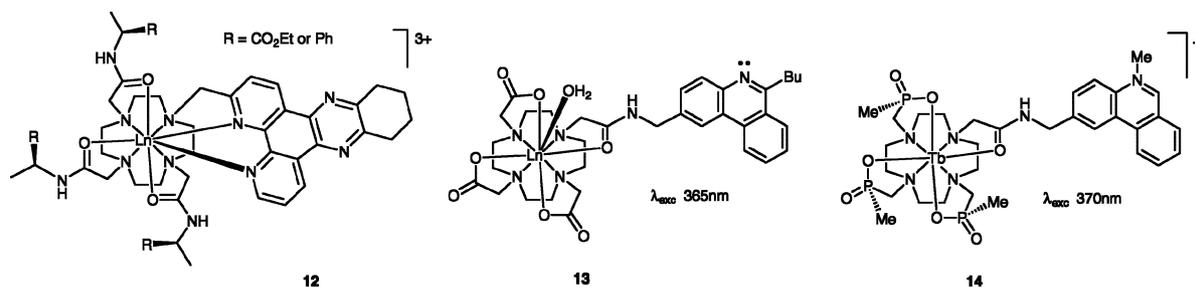
Fig. 5 Confocal fluorescence microscopy study showing the cellular localisation behaviour of the europium complex **2** (λ_{exc} 405 nm) in living (upper row) and fixed (lower) cells, revealing the staining of the nucleolus inside the cell nucleus, consistent with the observed co-localisation of the green fluorescent dye 'SYTO-RNA-select'.

the local enthalpy change following activation of the muscarinic receptor by introduction of acetyl choline.[54] Issues of calibration (observed intensity is a function of local complex concentration) and europium speciation have not been overtly clarified.

Lanthanide complexes as functional components of sensors operating in aqueous media

In devising good sensors based on the long lived emission of lanthanide complexes additional design criteria and practical considerations need to be borne in mind and several recent reviews have highlighted some of these issues.[12,13,55–57] Most published reports are concerned with issues of chemo-selectivity in binding, often under particular solvent conditions which bear little relation to the analysis of a target species in an operational sensor. Two different cases can be considered. First, sensors that are spatially localised are required for flow-analysis and immobilisation in laterally structured thin layers, *e.g.* on silicon with pre-determined integrated electronics for signal read-out, or in a sol–gel glass for extended operational lifetimes. An intrinsic feature here is that a wide range of signal transduction mechanisms can be employed, provided that the sensing moiety (Ln complex) is stable to storage, adheres to the support and does not leach, resists photo-degradation and gives a strong signal with high signal/noise ratio. Intensity, lifetime, phase modulation or emission polarisations are all practicable parameters to monitor if these criteria are met. For systems that involve 'freely mobile' probes in solution—as needed for certain *in cellulo*/*in vivo* and environmental applications—then the analytical signal must be referred intrinsically to another signal. This avoids a dependence on concentration for emission intensity measurements and can be another emission or excitation wavelength, thereby giving rise to a ratiometric sensor.

Immobilised lanthanide sensors

An optical sensor for hydrogen peroxide has been devised by immobilising a rather ill-defined europium tetracycline complex (λ_{exc} 405 nm) in a polyurethane hydrogel. The europium emission intensity increases linearly in the range 2–200 µM and is accompanied by a lifetime increase, but is subject to interference by citrate and phosphate.[58] Coupled assays for glucose and glucose-oxidase have been developed, as well as a fluorescence imaging method for monitoring glucose oxidase activity *in vitro*.[59] Flow-injection analyses for the broad-spectrum antimicrobial agents norfloxacin and ciprofloxacin (each is a quinolone) have been developed.[60,61] Here, terbium ions are simply immobilised on a cationic Sephadex resin and luminesce when reversibly bound to these quinolones in the flowing analyte, with a working range of 10–150 ng L^{-1}.

Sol–gel matrices are excellent supports for sensor immobilisation due to their inherent mechanical and chemical stability, optical transparency and case of formation. Typical fabrication conditions require ageing of the sol–gel precursors (*e.g.* Si(OR)$_4$, R = Me or Et) under acidic conditions (effective pH in range 2–3), so that kinetic stability of the complex with respect to acid-catalysed dissociation is essential. Robust sensors for pH[62,20] based on Eu and Tb complexes with a phenanthridinium chromophore have been devised (*e.g.* 13, Fig. 6) for use in static or flow analyses and a stable pH response (range of pH 5 to 8) was observed for periods of at least 6 months. In this case, protonation of the phenanthridine N leads to suppression of the photoinduced electron transfer (PET) process that quenches the sensitiser singlet excited state,[20] resulting in enhanced Eu emission. Related examples of ratiometric changes in excitation spectra have been defined with similar immobilised terbium complexes,[62] where the 370/311 nm intensity ratio varied by a factor of 8 over the pH range 4 to 8. The sol–gel matrix is highly suitable for pH and oxygen sensors, due to the ease of proton or O$_2$ transport through the medium. Examples of oxygen sensors include the terbium complex 14, for which excellent sensor stability and performance was reported,[63] based on the analysis of changes in the lifetime of terbium emission intensity of 545 nm (λ_{exc} 370–375 nm). In this example, reversible back-energy transfer from the terbium ion to the phenanthridinium triplet leads to competitive dynamic quenching of the long-lived triplet by oxygen, signalled by variations in terbium intensity and lifetime (N.B. the Eu analogue
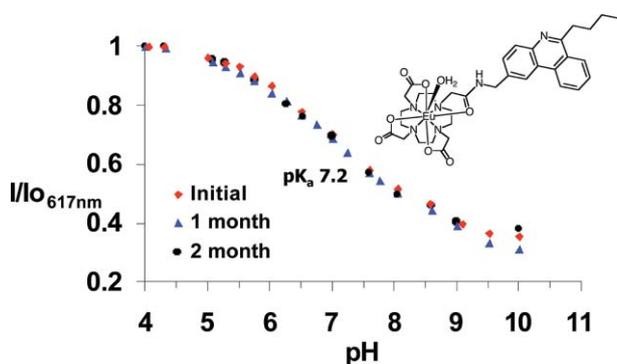

Fig. 6 Variation of the europium emission intensity of complex 13 (λ_{exc} 365 nm, λ_{em} 617 nm) with pH, following immobilisation of the complex in a sol–gel matrix and fabrication of an optically transparent thin film sensor for flow-injection analysis.[20,62]

is insensitive to pO$_2$ variations, suggesting a simple ratiometric analysis of emission intensity in co-complexes).[30]

Thin film applications have also been devised with more lipophilic lanthanide complexes. An ultrathin polymer optode has been reported involving Langmuir-Blodgett (LB) films of poly-dodecylacrylamide and the Eu–phen complex 15,[64] which operates as a temperature sensor through variations of the intensity of the hypersensitive Eu emission band at 613 nm. Related work has explored LB films of octabutyl bis(phthalocyaninate) rare earth complexes (Pr, Gd, Ln) as components of fibre optic sensors.[65] The near-IR absorption spectra of the Ln complexes changed form in the presence of nitrogen oxide gases and certain volatile aldehydes and esters. No emission variations were reported.

Lanthanide sensory systems operating in solution

The photophysical pathway defining sensitised lanthanide luminescence (Fig. 1 above) offers three 'windows of opportunity' for sensing, as either the two short-lived excited states of the sensitiser or the lanthanide excited state may be perturbed. A more detailed analysis has been described[66,12,20] and further clear reviews of the chemistry of such systems reported.[56,13,16] Related work highlighting the scope of chiroptical analyses has also been defined,[67,68] encouraged by the strong circularly polarised luminescence of chiral lanthanide complexes.[12,69] In addition, systems exhibiting well defined sensitivity to two or more analytes, either intrinsically[30,70] or by the introduction[71–73] of 2 receptor sites in the complexing agent have been studied. For example, complexes 16[72] and 17[73] exhibit emission intensity variations in the presence of H$^+$/Cu^{2+} and H$^+$/K$^+$ respectively, allowing their consideration as components of optical logic gates.[71]

Perhaps the most challenging work at present addresses anion sensing in aqueous media, as the large free energies of anion hydration, pH-dependent speciation and geometrical similarity of many common anions renders the development of chemoselective systems difficult. Various strategies are being explored and many involve reversible binding to the lanthanide centre.[74,75] They range from a europium-containing iron–sulfur cluster species showing apparent 'selectivity' for phosphate species,[76] to allegedly selective sensors for 2,3-bisphosphoglycerate based on a ditopic receptor[77] (λ_{exc} 280 nm, MeOH–MeCN). A suitable heptadentate ligand incorporating an appropriate sensitiser will usually form either a CN = 9 diaqua or a CN = 8 mono-aqua complex. In each case, the bound waters may be displaced by certain anions. Anion affinity is determined by the charge,[43] polarisability[18] and chelating ability of the different species.[19,75]

The charge density and steric demand at the lanthanide centre are also key issues and are complex dependent, giving rise to differing anion affinity profiles for each lanthanide complex. Thus, complex 2 binds citrate strongly and selectively[46] and has a low affinity for HCO$_3^-$ whereas 10 and 11 favour HCO$_3^-$ in ratiometric analysis (λ_{exc} 405 nm). Complexes related to 10 and 11, but lacking the ring N-alkyl substituent show *ca.* 40 : 1 chemoselectivity for phosphorylated-tyrosine residues over phospho-serine/threonine sites in phosphorylated-peptides.[78,79]

A key point to emphasise is that the desired chemoselectivity and affinity can be engineered into the lanthanide complex, according to the problem posed by the nature of the interferents presented in

15 λ$_{exc}$ 350nm

16

17 λ$_{exc}$ 313nm

triplet-triplet quenching

singlet-O$_2$

no quenching

18
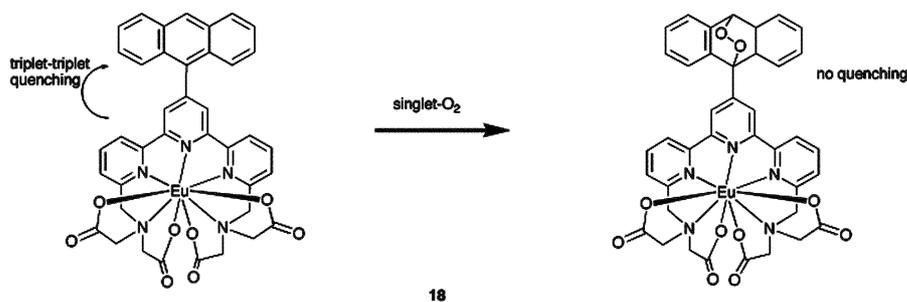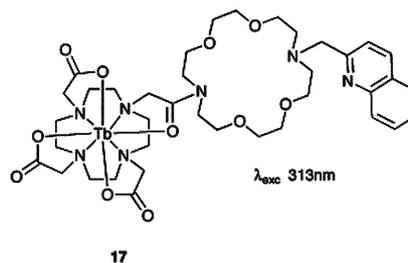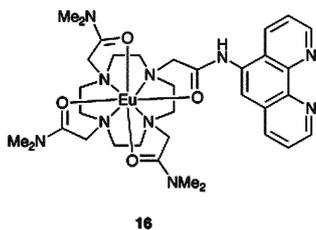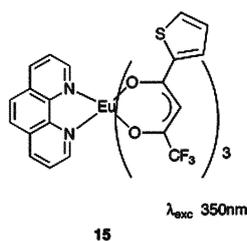
the chosen analyte. Thus, caution must be exercised in assessing the significance of a complex reputedly 'sensing' a given anion, unless the potential anionic interferents in the sample to be addressed are properly examined.[80–82]

Finally, a europium complex, **18**, has been designed to report on the presence of singlet oxygen.[83] The reaction of singlet oxygen, $^1\Delta O_2$, with substituted anthracenes generates the related *endo*-peroxide. In complex **18**, europium emission is quenched by intramolecular triplet–triplet energy transfer between the terpyridyl moiety and the anthracene. Triplet anthracene is unable to sensitise Eu emission as its energy lies well below that of the $^5D_0$ Eu emissive state (206 kJ mol^{-1}). This quenching pathway is not available in the *endo*-peroxide adduct so that each encounter of **18** with a molecule of $^1\Delta O_2$ switches on the Eu luminescence. Detection limits of 2–8 nM were reported (λ_{exc} 337 nm), rendering the system suitable for snapshot imaging purposes, provided that the retro-cyloaddition reaction is not competitive on the experimental timescale. The absence of reversibility of the cyclo-addition, of course, limits the application, as an *in situ* calibration of measured Eu emission intensity is essential.

Conclusions

Developments in molecular imaging optical, MRI and PET probes are set to enhance our ability to monitor key biological processes *in cellulo* and assess injury and disease states *in vivo*. There remains much to be done to devise emissive probes based on metal-coordination complexes, taking advantage of time-resolved sensing and imaging methodology. Key issues will be to understand better excited-state quenching of the lanthanide excited state, to gain a working structure/activity appreciation of the permeability of emissive, *responsive* complexes *in cellulo*, to encourage the development of available instrumentation for time resolved multiphoton microscopy and to work in harmony with bioscientists, addressing their challenges by using systems in which the understanding of the chemistry of the probes is secure.

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