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Lanthanide Complexes for Luminescence Imaging Applications

Stephen Faulkner, Simon J. A. Pope and Benjamin P. Burton-Pye
Department of Chemistry, University of Manchester, Manchester, UK

Abstract: In this article, imaging applications of luminescent complexes and recent advances in the design and photophysical behaviour of near-IR responsive complexes are reviewed. Various properties of the luminescent lanthanide complexes are also discussed in detail.

Keywords: Lanthanide complexes, luminescence imaging, europium(III) and terbium(III), near-IR luminescence, photosensitisers

INTRODUCTION

For many years, luminescence from lanthanide ions was almost exclusively the preserve of ions which emit in the visible region such as europium(III) and terbium(III). These ions have luminescence lifetimes in the millisecond region and, time resolved separation of the lanthanide centred emission from scattered light and background fluorescence have allowed the development of imaging techniques. One such example is dissociation enhanced lanthanide fluoroimmunoassay (DELFIA), (Fig. 1) (1, 2), which has been applied to protein (3) and hormone assays (4). Within this review, we will discuss not only imaging applications of complexes active in the visible region utilising techniques such as time-gated imaging microscopy, but also more recent advances in the design and photophysical behaviour of near-IR responsive complexes.

Address correspondence to Stephen Faulkner, Department of Chemistry, University of Manchester, Oxford Road, Manchester M13 9PL, UK. Fax: +44-161-275-4616; E-mail: Stephen.Faulkner@man.ac.uk

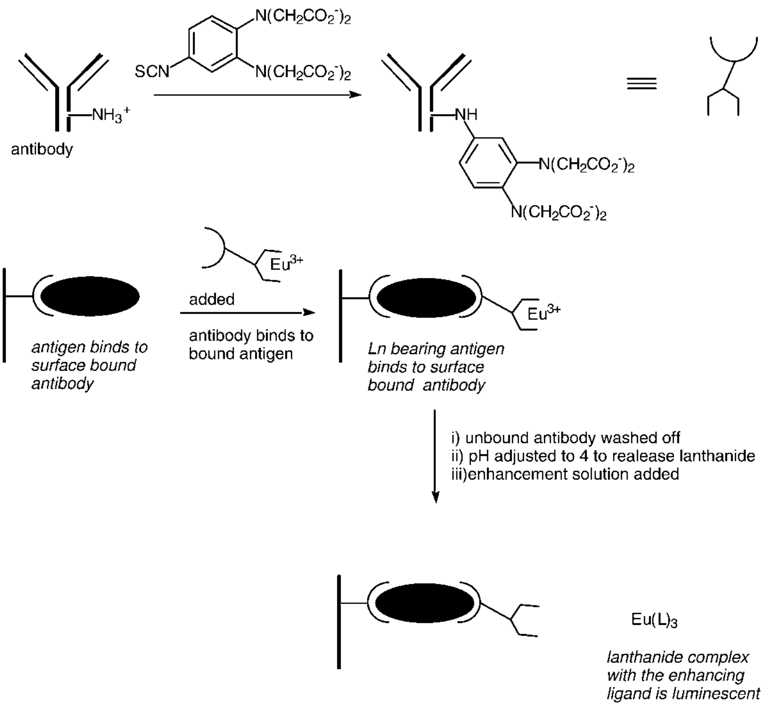


Figure 1. Schematic showing DELFIA.

Europium was the first lanthanide to be studied in solution. Freed et al. (5) discovered that the relative intensities of the spectral emission lines exhibited by europium(III) were affected by the solvent in which it was studied, and subsequently Weissman (6) discovered that these complexes exhibited intense luminescence upon irradiation by UV light. However, it was not until the 1960s that terbium(III) and europium(III) were finally studied in detail (7, 8). They possess long luminescence lifetimes of millisecond order, in contrast to that of biological (i.e., organic) species that exhibit lifetimes in the realm of nanoseconds, because of the relatively few processes in which the luminescence can be quenched. In some systems, europium excited states can be quenched non-radiatively by ligand to metal charge transfer (LMCT) or photoinduced electron transfer (PET), reducing europium(III) to europium(II) (f^6 to f^7) and giving rise to competitive non-radiative deactivation of the excited state. The effectiveness of this type of electron transfer is governed by the magnitude of the free energy of the electron transfer process (ΔG_{ET}) and the proximity of the donor and acceptor (9). Generally, the closer the donor and acceptor are, the more likely it is that the rate of electron transfer is similar to that of photon absorption, and the more likely it is that photon absorption is hindered. Europium emits light with principal emission maxima occurring at 595 and 615 nm, while terbium emits at wavelengths of 480 and 545 nm, which are in the light region that biological media absorb and fluoresce.

IMAGING WITH LANTHANIDE COMPLEXES

The earliest use of lanthanide ions in imaging arose from the need to provide high sensitivity probes for use in bioassay. As lanthanide ions have much longer luminescence lifetimes than conventional fluorescent probes, signals arising from them can be separated from background biological fluorescence (autofluorescence) using time-gating techniques (illustrated in Fig. 2). The need to image such systems, rather than simply obtain their spectra, became more pronounced with the development of high throughput screening techniques and the use of multi-well plates. Lanthanide-based assays have become increasingly commonplace (10–12) and their use as probes for biochemical systems is gathering more interest (13–15) especially, the application of more than one lanthanide in the assay to provide more detailed information on the analytes present in solution (16–20). The spatial resolution of such techniques is such that they are inappropriate for applications which require information about localisation of the material at a sub-cellular level. The use of microscopy can provide the resolution necessary to accurately assign the different analytes as well as their location and their concentration.

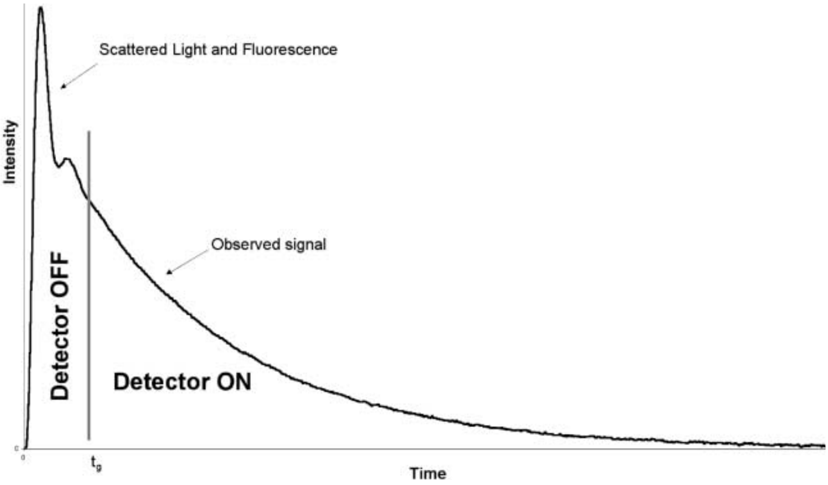


Figure 2. Schematic showing time-gated rejection of stray light and fluorescence.

Luminescence microscopy with lanthanide ions is a relatively simple technique that offers inherent advantages over conventional fluorescence and transmission microscopy (21–23). Once again, time-gating techniques can be employed to separate the lanthanide centred signal from a fluorescent background. In addition, conventional fluorescence microscopy requires the excitation beam to be diverted so that it does not go into the microscope, as it is very difficult to separate the short-lived fluorescence from the short-lived excitation pulse arising from scattered light in the sample. Luminescence microscopy also allows for a simpler experimental set-up, as the short-lived signals can be removed. Time-gated luminescence imaging requires an experimental set-up like that shown in Fig. 3 (24), where a time-gated image intensifier is synchronised with a laser pulse, and is used to control the time province over which measurements are made. A charge coupled device (CCD) camera is used to monitor the emission, as in this experiment the emissions occur in the visible region of the spectrum. A fluorescence image is recorded by the CCD when the image intensifier is set to give a zero time delay, or is switched off. When a time-gate is applied to the image intensifier, the intensifier is only transparent for the duration of the gate, allowing the light incident on the detector to be divided into time-domains. By varying the degree of synchronisation between the excitation pulse and the time-gate, it is possible to apply a time delay to the intensifier, and hence to exclude short-lived processes, which will be complete before the

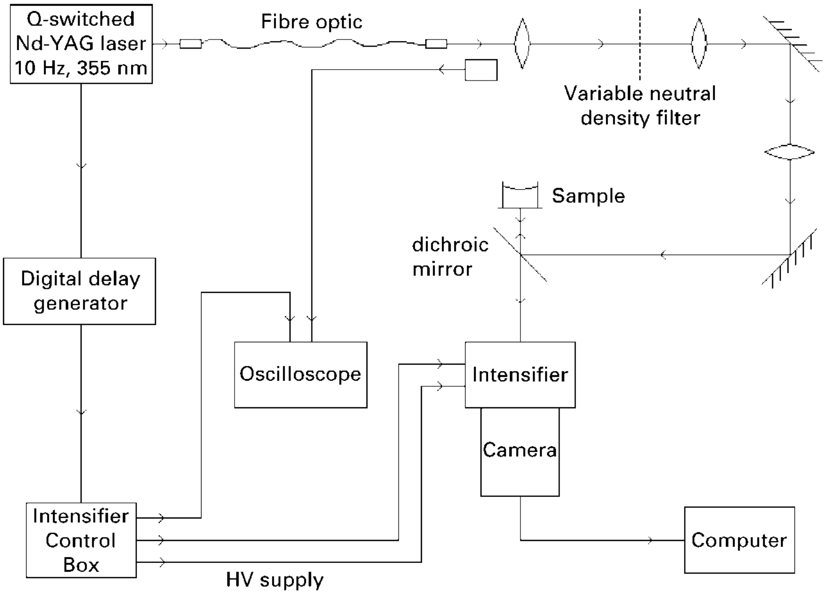


Figure 3. Experimental setup for time-gated microscopy.

intensifier is switched on. Examples of this kind of luminescence microscopy are increasingly common for europium and terbium complexes, which have millisecond order lifetimes (vide supra) (25–33).

For example, in Fig. 4, three particles are shown. The two on the left are labelled with a europium complex (**19**), while that on the right is labelled with rhodamine 6G, which absorbs and emits light at the same wavelengths as the complex (355, 617 nm, respectively). When studied by conventional transmission microscopy the particles are indistinguishable, and fluorescence microscopy can only give information about concentration of the species absorbed on the particles. This effectively demonstrates the problems that can occur with background fluorescence, as the labelled particles cannot be distinguished from one another. However, on application of a short time delay, the only observed signals correspond to luminescence from the lanthanide complex, as fluorescence from the rhodamine 6G is already complete.

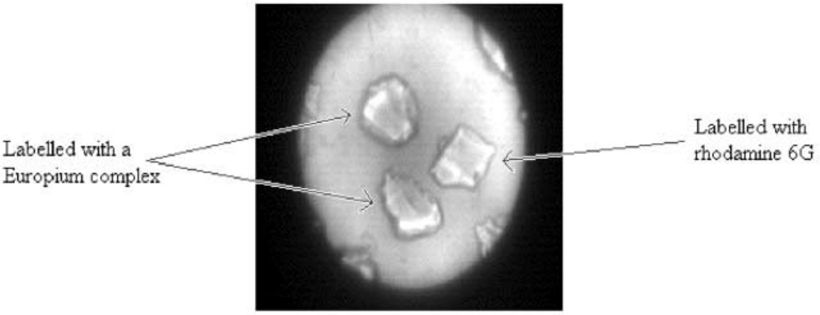


Image obtained using conventional optical microscopy.

Image obtained using conventional fluorescence microscopy.

Time-gated image, gate = 80 μs, delay = 100 μs.

Figure 4. Visible, fluorescence and gated images of Eu and rhodamine labelled particles.

This is illustrated by the absence of any signal corresponding to the particle labelled with rhodamine.

This is one example of the utility of the time-gating technique. In both microscopy and solution-based assays, interference from background fluorescence can be eliminated.

Lanthanide complexes can be tailor-made to exhibit different luminescent lifetimes by exclusion of solvent molecules from the inner hydration sphere of the lanthanide ion. Such complexes can be used in microscopy applications to gain more detailed information. Lanthanide complexes can be distinguished by their characteristic wavelengths of emission (which is wholly due to the metal centre), and their luminescent lifetimes, which are controlled by their coordination environment. A technique that can be used to distinguish between a collection of lanthanide complexes is lifetime mapping (11). This requires a library of data to be collected, and the rate of decay of the luminescence can then be monitored at each pixel in the image producing a falsely coloured image of high contrast. This provides information on the lifetimes of the species under study, allowing different complexes to be identified, though it does not provide information about local concentrations. Lifetime maps such as these allow systems containing more than one label to be studied and these are potentially of great significance for security and encoding applications. Figure 5 demonstrates the application of this technique to distinguish between two particles individually labelled with different terbium complexes, one labelled with a complex of terbium and DOTA, which is an eight-coordinate ligand, and the other with DO3A, which is seven coordinate (34). The lifetime map shows the two different lifetimes and the shapes of the particles.

A similar approach, using interference filters between the microscope and the CCD to select emission wavelengths, can be used to distinguish between complexes of different lanthanides. Figure 6 (34) shows two particles loaded with complexes of europium and terbium, which absorb light in the same region of the spectrum, which allows for simultaneous metal-centred

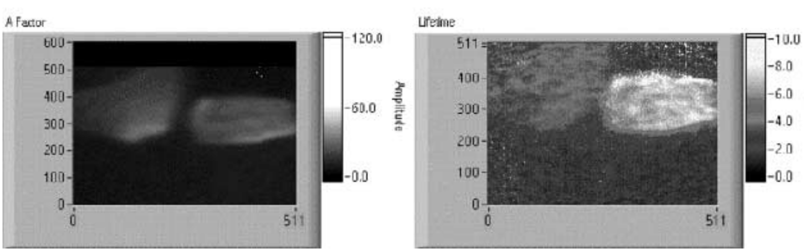


Figure 5. Differentiation of labelled particles carrying different complexes.

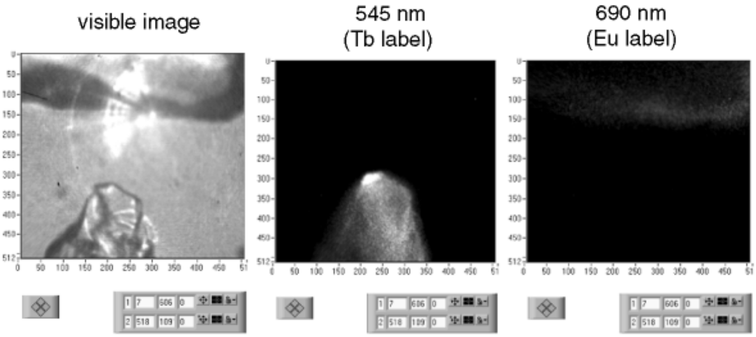


Figure 6. Eu/Tb differentiation by wavelength selection.

emission from the lanthanide centres. The two metal centres have very different emission spectra that overlap at some wavelengths but not at others. It can be seen that, upon application of an interference, filter specific emissions bands can be selected. For instance, for a mixture of europium and terbium complexes, the emission at 545 nm is solely ascribable to terbium, while that at 690 nm is solely due to europium centred luminescence, producing two images corresponding to the different domains of the two ions. Such images can be corrected for the relative intensities of the emission bands, providing a guide to the relative concentrations of the ions.

These techniques can potentially be applied to high throughput screening and security encoding, as well as multiplex imaging of different analytes of varied concentration and have extraordinary prospective applications in cellular imaging experiments using lanthanide complexes that can localise in different parts of a cell.

When considering the use of any molecule for the sole use of imaging applications, one must try to envisage the environment in which it is to be used. Biological media absorb and emit light in the visible region of the spectrum on a short timescale (typically <10 nsec); hence the luminescence lifetime of the reporter group must be significantly greater than that of the backgrounds, ideally being several orders of magnitude longer, if time-gating techniques are to be used. To image cells and tissue, aromatic reporter molecules will be of limited use; long-lived phosphorescence from such compounds is quenched by the interaction with the triplet ground state of oxygen which is obviously abundant in aerated media. By contrast, the emissive lanthanides possess partly filled f-orbitals that interact weakly with their environment, and give rise to excited states which are not commonly quenched by oxygen (vide supra). As such, they offer an ideal tool for time-gated microscopy and assay. However, they have one significant drawback compared with aromatic chromophores in which lanthanide ions have very low extinction coefficients as f–f transitions are Laporte forbidden, which makes direct excitation of the metal centre cumbersome and inefficient. This is commonly overcome by using an aromatic chromophore, to sensitise the emission from the metal centre, whose triplet state energy is comparable with that of the lanthanide acceptor states and thus allows efficient energy transfer (Fig. 7) (13, 15, 35). In theory, although f–f transitions are Laporte forbidden, they become partially allowed as electronic dipole transitions cause admixture of configurations of opposite sign, such as the $4f^{n-1}$ $5d^1$ configuration (36).

For use as tools in imaging applications, lanthanide ions require to be bound in an environment that can act as both a suitable "prison" and a sensitising unit. Lanthanide ions are inherently toxic, and the need for them to be incorporated in kinetically stable complexes is paramount for their use to be viable in imaging applications. The lanthanides have attracted a considerable amount of attention over the years. The small, regular decrease in the ionic radii across the f-block results in only small differences in their coordination behaviour and chemical properties. This decrease in radii is known as the lanthanide contraction, but other metal groups exhibit the same behaviour, such as the transition metal elements. The lanthanides possess relatively high charge densities and have strong electrostatic nature in their bonding, as the ions are polarising and hard Lewis acids. The lanthanides can coordinate to an assortment of ligands, possessing a variety of coordination numbers, but most commonly eight or nine. The hard characteristics of the ions mean that most of the ligands that bind successfully to lanthanides incorporate several oxygen atoms. Indeed, the Lewis acidity of the lanthanides means that they favour binding to atoms that can act as hard Lewis bases, and hence combinations of amine and carboxylic groups are commonly used in lanthanide complexation because the amine groups are easily polarised and are more favourable than groups possessing large ground state dipole moments. The most commonly documented ligand for the complexation of lanthanides is 1,4,7,10-tetrakiscarboxymethyl-1,4,7,10-

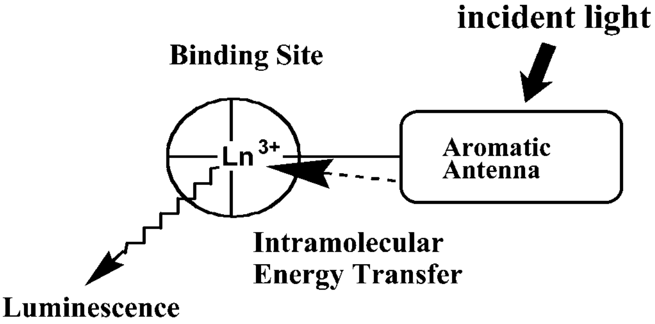


Figure 7. Cartoon representation of energy transfer processes in sensitised emission.

tetraazacyclododecane (commonly known as DOTA). The popularity of this ligand is considerable because of its use as a magnetic resonance contrast agent with gadolinium (37, 38). It forms very stable complexes with lanthanides ([Gd(III)DOTA] has a stability constant of 1028), as the cavity of the four nitrogen atoms is a perfect size to accommodate a lanthanide ion.

An interesting variation of DOTA is the replacement of the carboxylate donor groups with phosphinate groups. The ligand known as GADOBENZ, when complexed with gadolinium, acts as an outer sphere MRI contrast agent because the pendent arms bearing benzyl phosphinate functionalities wrap around the coordinated lanthanide ion to exclude any solvent molecules from the metal centre, which means that only solvent molecules in the second or outer coordination sphere are influenced by the paramagnetic metal centre (39) As well serving as blocking groups to prevent close approach of solvent, the aromatic groups on this ligand serve as good chromophores to transfer energy from its excited triplet state to the metal ion to promote the luminescence process from the electronic transitions from its excited state to the ground states. The conformation of the phosphinate arms means that lanthanide complexes incorporating this ligand have inner sphere hydration numbers of zero, minimising non-radiative quenching by proximate O–H oscillators. A combination of this low q value and the four highly absorbing benzyl groups helps to explain the rather high quantum yield of these complexes.

NEAR-IR LUMINESCENCE FROM LANTHANIDE COMPLEXES

The results discussed earlier relate to systems and imaging techniques that utilise the luminescence properties of europium and terbium (i.e., emission in the visible region). More recently, considerable interest in near-IR luminescence from ytterbium(III), neodymium(III), erbium(III), and praeseodymium(III) has emerged, resulting from a series of technical developments which facilitate the detection of relatively weak near-IR emission spectra. This can be seen from an energy level diagram—Fig. 8 shows the energy levels of the luminescent lanthanides, with the emissive states marked in red. It can be seen that the emissive states which result in near-IR emission are much lower in energy than the emissive states of europium and terbium, greatly increasing the range and variety of potential sensitising chromophores.

Applications of NIR emissive complexes are potentially in two distinct areas. In telecommunications, silica used for fibre optic data transmission requires a window of transparency closely matched by the emission wavelengths of praeseodymium(III) (ca. 1330 nm) and erbium(III) (ca. 1550 nm). Indeed, erbium(III)-doped silica fibre amplifiers are currently used to

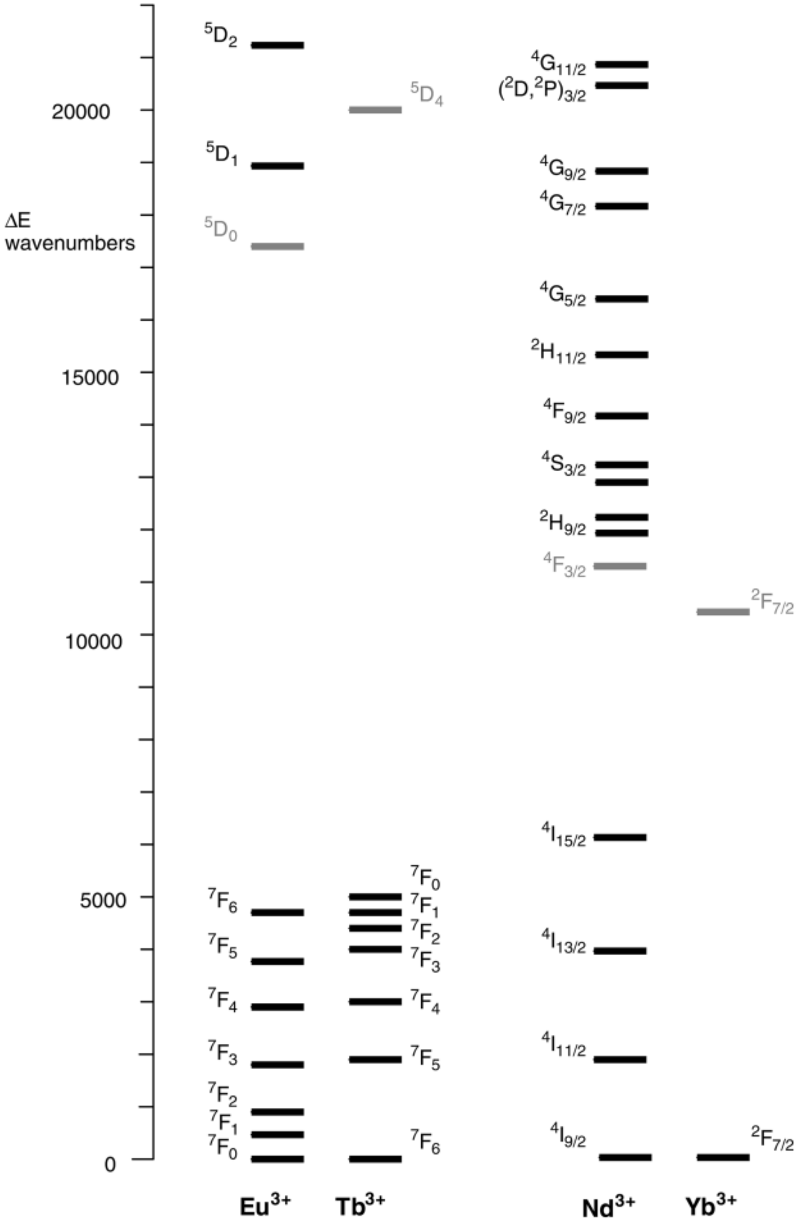


Figure 8. Energy level diagram for the common luminescent lanthanides.

amplify light to 1550 nm (40) and more recently neodymium(III) has been exploited within polymeric wave-guides to amplify signals at 1060 nm (41).

Secondly, although luminescent imaging using visible emitters [e.g., europium(III) and terbium(III)] is well established (vide supra), there are distinct disadvantages to this approach. Such complexes require sensitisation by long-wavelength UV radiation ($<$360 nm) which is the range absorbed strongly by biological chromophores and hence, direct in vivo applications are compromised. Furthermore, the choice of aromatic antennae is limited because of the large energy gap between ground and excited states and therefore, only chromophores possessing high triplet energies will sensitise europium(III) and terbium(III) efficiently (42). In contrast, lanthanides with relatively small energy gaps (i.e., near-IR emitters) encourage the use of a far wider variety of chromophore, and this, therefore, eliminates any competitive excitation by biological molecules. Although the luminescence lifetimes from neodymium(III), ytterbium(III), erbium(III), and praeseodymium(III) are shorter (nanoseconds to microseconds) than their europium(III) and terbium(III) analogues, this should still permit time-gating techniques to be applied, while also providing greater tissue penetration and image resolution in the fluorescence microscopy of biological systems (43). In this section, we will restrict ourselves to discussion of the complexes which have been developed for applications such as those just described; the development of time resolved imaging equipment with high near-IR sensitivity is still ongoing, and widespread application of these systems must, of necessity, await its completion.

The emission spectrum from neodymium(III) in aqueous solution shows three bands at 880, 1055, and 1330 nm attributed to the $^4F_{3/2} \rightarrow {}^4I_{9/2}$, $^4I_{11/2}$, and $^4I_{13/2}$, respectively. In principle, a fourth emission band at ca. 1900 nm ($^4F_{3/2} \rightarrow {}^4I_{9/2}$) exists, but is difficult to detect (44). Lifetime measurements of the luminescence decay can be obtained by monitoring any of the three emission bands but are most commonly obtained from the 1055 nm band since this is frequently the most intense and detectable (43). Lifetime measurements recorded for neodymium are usually in the nanosecond domain. Ytterbium(III) has only one excited state ($^2F_{5/2}$) and it is luminescent at 980 nm corresponding to $^2F_{5/2} \rightarrow {}^2F_{7/2}$. Luminescence lifetime measurements for ytterbium complexes tend to be slightly longer than neodymium and the values are frequently obtained in the microsecond domain.

The luminescence from lanthanide excited states can be quenched through non-radiative energy transfer to stretching vibrations of nearby or coordinated solvent molecules, which is most marked in aqueous media where an abundance of O–H oscillators are present, and Frank Condon overlap of energy levels and stretching vibrations occurs (45–48). Through previous investigations it was found that O–H oscillators are the most efficient quenchers of metal-centred luminescence both in solution and in the solid-state. The extent of this quenching was found to be inversely proportional to the energy gap between the emissive state and the ground state of the metal (46, 47) and that each oscillator quenched the luminescence individually (49, 50). Terbium is less readily quenched by O–H oscillators due to a less prominent Franck Condon overlap. Other vibrational overtones can quench the luminescence of a lanthanide ion. Harmonics of N–H, C–H, and C=O stretching vibrations also have an effect on the luminescence, but not to the extent as O–H oscillators (47). The corresponding frequencies of the deuterated oscillators (O–D, C–D, N–D) prove to be much less effective at quenching luminescence as they are of lower frequency and would require more energy to interact with the emissive state of the lanthanides (51). The rate constant for depopulation of the excited state in water can be split into the sums of the different quenching contributions;

$$k_{\mathrm{H_2O}} = k_{\mathrm{NAT}} + k_{\mathrm{NR}} + \sum k_{\mathrm{XH}} + \sum k_{\mathrm{C=O}}$$

where k_{NAT} is the rate constant for natural radiative processes, k_{NR} is the rate constant for non-radiative de-excitation, and $\sum k_{\mathrm{XH}}$ and $\sum k_{\mathrm{C=O}}$ are the sums of the decay constants of nearby X–H and C=O stretching vibrations. In D_2O, it is assumed that all X–H oscillation do not contribute, as explained earlier and the rate equation in D_2O can be written as:

$$k_{\mathrm{D_2O}} = k_{\mathrm{NAT}} - k_{\mathrm{NR}} + \sum k_{\mathrm{C=O}}$$

Given that in aqueous media the quenching by O–H vibrations dominates any other factors, the equations can be rearranged to give what is known as the Horrocks equation. This can be used to determine the number of water molecules coordinated to a central lanthanide ion, as the rates of quenching from inner-sphere O–H and O–D oscillators vary;

$$q = A(k_{\mathrm{H_2O}} - k_{\mathrm{D_2O}})$$

where A is a proportionality constant specific to each individual lanthanide and its susceptibility to quenching by O–H oscillators. The determination of the proportionality constant was based on a plethora of analyses conducted in the solid-state, where the number of coordinated water molecules was determined by x-ray crystallography (52–56). It has been observed that there are contributions to the luminescence quenching from closely diffusing or "outer-sphere" water molecules and a correction factor has been derived to take into account these interactions (45).

$$q_{\mathrm{corr}} = A'\Delta k_{\mathrm{corr}}$$

Considerable effort has gone into the study of the effect of N–H oscillators in such systems and has concluded that the effects are much more marked for lanthanides such as europium and neodymium, as opposed to the smaller negligible effects to terbium luminescence (45). Table 1 shows the A factors for

Table 1. *A* factors and lifetime ranges for the more common luminescent lanthanides

Ln^{3+} (aq.)	$\lambda_{em}{}^{a}$ (nm)	Lifetime range	$q = A\ (k_H - k_D)$	$q = A\ (k_H - k_D - B)$	
Eu^{3+b}	617	100 μsec–3 msec	$A = 1.05$ msec	$A = 1.2$ msec	$B = (0.25 + 0.075x)\ \text{msec}^{-1}$
Tb^{3+}	545	100 μsec–5 msec	$A = 4.2$ msec	$A = 5$ msec	$B = 0.06\ \text{msec}^{-1}$
Yb^{3+}	980	100 nsec–25 μsec	$A = 0.9$ μsec	$A = 1.0$ μsec	$B = 0.1\ \mu\text{sec}^{-1}$
Sm^{3+}	535	1 to 100 μsec			
Nd^{3+c}	1055	30 nsec–500 nsec			
Er^{3+c}	1530	20 nsec–1 msec			

Note: Shorter lifetimes can be observed where back energy transfer occurs. In such systems, *A* factor determination is largely meaningless. In alcoholic media, the number of solvent O–H oscillators are effectively reduced, and the lifetimes observed tend to be longer. This means that *A* factors become larger, while *B* factors become smaller. It is conventional to multiply and divide by a factor of 2 in such cases. This works to a good approximation.

[a] The wavelength reported corresponds to what is commonly the most intense emission.

[b] The Parker treatment of europium requires that account is taken of x, the number of exchangeable N–H oscillators.

[c] The lifetimes of Nd^{3+} and erbium complexes are dependent on the number of close diffusing C–H as well as on other X–H oscillators. This means that the lifetime can vary considerably from ligand to ligand, limiting the utility of such calculations unless there is considerable similarity between the ligand series.

luminescent lanthanide ions, together with established correction factors and the range of lifetimes normally expected for such systems.

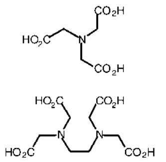
COMPLEXES

By contrast with lanthanide(III) aquo ions (Ln = Nd, Yb, Er), organic ligands can be used to reduce the non-radiative quenching of the metal-centred excited state, with obvious effects on the luminescence lifetimes of lanthanide(III) being profound. For example, with neodymium(III) simple ligands (**1**) such as nitrilotriacetic acid cause a small increase in lifetime (45 nsec), while hexa coordinate acyclic ligands such as EDTA increase it still further (53 nsec). Essentially, ligands that shield the metal from inner sphere solvation prevent the quenching of metal-based luminescence though solvent X–H oscillators, reducing the non-radiative quenching of the metal centred excited state and giving rise to increased lifetimes. The simplest approach to this is to bias ligand : metal stoichiometry in favour of the ligand to enhance lifetime (e.g., by forming ML_2 complexes). A more elegant approach, however, is in the design of ligands which come close to coordinative saturation of the lanthanide centre. To this end, a range of ligands have been synthesised that limit the approach of solvent molecules to the lanthanide ion and these are discussed shortly. Furthermore, ligands that incorporate chromophores or aromatic antennae into their architecture generally benefit from improved extinction coefficients (overcoming inherently weak lanthanide-centred molar absorption) and increased hydrophobicity (and consequently limit the approach of quenching water molecules).

ACYCLIC LIGAND SYSTEMS

Terphenyl-based ligands (**2**) incorporating a triphenylene antenna chromophore are suitable ligands for lanthanide ions (57). The ligand is designed to provide eight oxygen donor atoms allowing encapsulation of the metal ion, and the chromophoric portion of the ligand demonstrates a broad absorption in the UV region up to 350 nm. Thus, excitation at 337 nm resulted in lanthanide-based emission in the near-IR, via a ligand centred triplet state, with microsecond lifetimes in each case. The authors suggest that low quantum yields are obtained due to transferred energy being lost via vibronic coupling of the lanthanide excited state with C–H oscillators in the solvent and within the ligand framework.

Related terphenyl ligands incorporating sulphonamido (**3a**) and amide (**3b**) groups have also been reported providing at least eight donor atoms for metal ion chelation (58). Laser excitation at 350 nm again resulted in metal-based luminescence demonstrating that like europium(III) and terbium(III), neodymium(III), ytterbium(III), and erbium(III) can also accept energy from high lying ligand centred triplet states (in this case the terphenyl based triplet state is ca. 25,000 cm^{-1}). Lifetime measurements for the complexes were obtained in [H$_6$]DMSO and [D$_6$]DMSO with the ytterbium(III) complexes having the longest lifetimes (μsec domain) and neodymium(III) the shortest.



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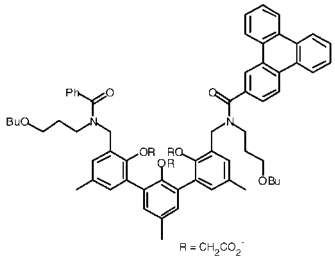

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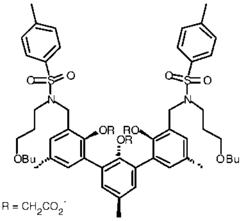
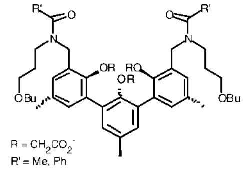


3a



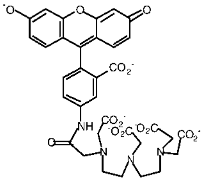
3b

For example, the sulfonamido complexes display long lifetimes in [D$_6$]DMSO of 23.6, 3.5, and 2.5 μsec for ytterbium, erbium, and neodymium, respectively. The lifetimes for each of the metal ion complexes increase significantly when in deuterated media indicating the efficiency of C–H quenching for these species.

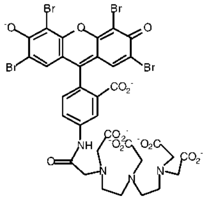
Diethylenetriamine pentaacetic acid (DTPA) is frequently used as a chelating agent for metal ions and the dianhydride derivative can be used as a precursor to many ligand types. Organic chromophores derived from fluoroscein (**4a**) and eosin (**4b**) can be tethered to DTPA (59). This allows the synthesis of ligands combining a potentially eight coordinate donor set with coloured dyes possessing triplet energy levels well suited to NIR emitting lanthanide ions (60). The ytterbium(III), neodymium(III), and erbium(III) complexes were prepared as 1 : 1 chelates with the ligands. All complexes demonstrated lanthanide-based emission upon excitation of the antenna chromophore ($\lambda_{\mathrm{exc}} = 488$ nm), where energy transfer was assumed to proceed via a ligand centred triplet state albeit rather slowly (as determined by molecular oxygen quenching effects). The erbium(III) chelate was the first example to demonstrate erbium(III) based luminescence.

Efficient visible light sensitisation of near-IR emitting lanthanides is also possible by other ligand systems. Fluorexon (4′,5′-*bis*[*N*,*N*-*bis*(carboxymethyl)aminomethyl]fluorescein, **5**) allows the metal ion to be bound in close proximity to the chromophore forming water stable (comparable to

EDTA chelates) 1 : 1 complexes. Sensitisation of the chromophore with visible light (the ligand possesses an absorption maximum at ca. 500 nm) results in efficient population of the metal ion excited states (enhanced by improved intersystem crossing and rapid intracomplex energy transfer) (61).



4a



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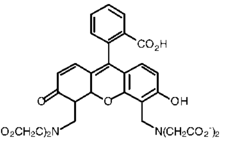

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In related work, flourexon (Fx) carrying an isothiocyanate (ITC) group has been synthesised for fluoroimmunoassay applications (62). Thus, the ligand incorporates a chelating group (iminodiacetic acid), chromophore (fluoroscein), and a functionalised backbone for further reactivity such as protein coupling (ITC group). The 1 : 1 ytterbium(III) complex displays emission at 980 nm with a lifetime of 2 μsec after excitation at 510 nm. Conjugation of the complex with the protein substrate avidin has little effect on the absorption or emission properties of the ytterbium(III) ion, but the absolute quantum yield was low suggesting non-radiative deactivation of the metal ion excited state by molecular vibrations (O–H and C–H) in the surrounding matrix.

Ligands that combine improved chelating strength with chromophoric character towards NIR emitting lanthanide ions are diaza- and tetraazatriphenylenes (**6a–d**) (63). Neodymium(III), ytterbium(III), and erbium(III) complexes were obtained from **6a–c** with a ligand : metal ratio of 2 : 1 while **6d** yielded 1 : 1 chelates. The increase in conjugation and introduction of additional aza-functionality in these ligands broaden the range of excitation wavelength to lower energy, and the absorption spectra do not change significantly upon complexation. The complexes register near-IR emission following absorption of light by the diimine ligands at 280–450 nm. Lifetimes were typically in the microsecond domain. The most highly conjugated tetra-aza ligand (**6d**) was found to encourage the most efficient energy transfer by virtue of minimising the energy gap between the ligand-centred triplet state and the lanthanide ion excited state. The synthetic route to these teraazatriphenylenes allows simple modification and thus tunability of the absorption characteristics, rendering them attractive candidates for efficient energy transfer processes.

The simple, potentially dichelating diimine ligand 3,6-*bis*(2-pyridyl)tetrazine (BPTZ) has also been utilised to chelate lanthanide ions and acts as a chromophore, providing a moderately low-energy absorption maximum at about 550 nm (64). The lanthanide ions are further coordinated by terminal diketonate ligands $[\mathrm{tta}]^-$ [where Htta = thenoyl(trifluoro)acetone] which possess only one C–H oscillator. The larger lanthanide ion neodymium(III) forms a mononuclear complex with BPTZ, whereas the smaller ions ytterbium(III) and erbium(III) form dinuclear adducts (**7**). Luminescence measurements were undertaken in the solid state and in dichloromethane solutions (the complexes dissociate in coordinating solvents). Studies revealed NIR emission on the microsecond timescale (for example, $[\{\mathrm{Yb(tta)_3}\}_2(\mu\text{-BPTZ})]$, $\tau = 13.3\ \mu\mathrm{sec}$) induced by irradiation into the tta-based transition (337 nm) or at 520 nm into the BPTZ-centred transition.

For certain metals, particularly neodymium and erbium, another way in which the luminescence from the lanthanides can be increased or enhanced is to use ligands that have few C–H oscillators in close proximity to the metal centre. The Lehn cryptand (**11**) is one such molecule. It is made up of three bipyridine units linked together by two terminal nitrogen groups, all of which take part in the binding to a metal ion. The aromatic functionalities serve as good chromophores for the transfer of energy to a bound lanthanide upon excitation. Similarly, the authors and co-workers (65) have reported the near-IR luminescence properties of neodymium and ytterbium complexes with poly(pyrazolyl)borate ligands (**8a–b**). The chief advantage of these systems is their relative lack of C–H oscillators close to the metal centre. This has resulted in long luminescent lifetimes particularly for the neodymium species, which are in the microsecond domain. Luminescence spectroscopy was used to determine the degree of inner sphere solvation and in some cases provided evidence of partial ligand dissociation (i.e., increase in q value).

Suppression of quenching effects often requires deuteriation of either C–H or O–H bonds or occasionally substituting C–F bonds for C–H within ligating molecules, which further complicates ligand syntheses and design. Alternatively, Yanagida and co-workers (66) have shown that neodymium(III) centred emission can be significantly enhanced, even in non-deuterated solvent, by incorporating bulky fluorinated moieties into their chelating ligands. The complex Nd(pos) (**9**) possessed a long lifetime of $12\ \mu\mathrm{sec}$ in acetone ($13\ \mu\mathrm{sec}$ in $[\mathrm{D_6}]$acetone) following excitation in the visible at 585 nm. Essentially, the bulky framework of this ligand, preventing approach of solvent molecules to the metal ion, and the lack of ligand-based C–H oscillators, result in exceptionally long neodymium(III) luminescence lifetimes.

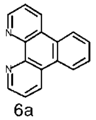
A prerequisite of complexes for use in bioanalytical applications is the stability and solubility of emissive species in water. Bunzli, Piguet, and co-workers (67) have reported the syntheses of lanthanide dimetallic helicates obtained from self-assembly in water. The ligand (*bis*{1-ethyl-2-[(6′-carboxy)pyridin-2′-yl]benzimidazol-5-yl}-methane), (**10**) forms highly stable complexes through triple helicates, which protect the metal ion from solvent approach. The ytterbium(III) complex was emissive following excitation at 355 nm and exhibited a very long lifetime of 40 μsec in deuterium oxide (68). The poor matching of the ligand triplet state and excited state of ytterbium(III) ($^2F_{5/2}$) led to investigations of the energy transfer process between ligand and metal. On formation of the helicate, ligand-centred fluorescence decreased significantly and this together with oxygen having no quenching effect suggested that fast energy transfer might occur via an electron transfer mechanism.

Complexes of ytterbium(III) with proteins (carp parvalbumin) have proved useful tools to aid the probing and understanding of energy transfer mechanisms between (ligand centred) donor and (metal centred) acceptor excited states (69). Irradiation of the ytterbium(III)-bound parvalbumin at 289 nm induced metal-based emission at 977 nm in addition to tryptophan fluorescence. In contrast to all other Ln^{3+} except europium(III), the ytterbium(III)-bound parvalbumin also demonstrated significant quenching of the protein-based fluorescence. Modelling of the tryptophan containing protein revealed the distance between the chromophore (indole ring) and metal ion as 8–11 Å and the spectral overlap integral of Förster theory to be 0. The poor overlap in energy states had previously led to the proposition of metal sensitisation occurring via an LMCT state. However, the LMCT band was not observed for the ytterbium(III) aggregate and, therefore, the mechanism of sensitisation was proposed to proceed via a long-range electron transfer process.

The energy transfer process has also been probed in the ytterbium(III) complex of the Lehn cryptand (70). Irradiation of the bipyridyl chromophore at 355 nm allowed the observation of ytterbium(III) luminescence at 980 nm. The lifetimes were moderate being 0.52 and 5.21 μsec in water and deuterium oxide, respectively, resulting in a derivation of inner sphere solvation (q value of 1.53) (71). As the overlap of the ligand-centred triplet state and ytterbium(III) ion is negligible, an electron transfer process is proposed to explain the intense metal-centred luminescence. Additionally, time-resolved luminescence studies did not reveal a transient absorption correlating with the presence of a ligand-centred triplet, but merely an instantaneous luminescent signal. In contrast, the analogous neodymium(III) complex displayed relatively weak emission at 880, 1055, and 1340 nm following ligand excitation. The lifetimes for the neodymium(III) species in water and deuterium oxide were 100 and 299 nsec respectively, and probably benefit from the absence of C–H oscillators in close proximity with the neodymium(III) ion. The absence of suitable parameters for the derivation of q with respect to neodymium(III) complexes results in an anomalously low value of 0.46 being obtained.

It should be noted that energy transfer in ytterbium complexes can also be explained by invoking a phonon assisted mechanism in which excess energy is dissipated through vibrational harmonics of the ligand and the solvent (72, 73). This mechanism is more general, in that it could occur where electron redistribution is not thermodynamically feasible. However, in cases where redistribution is definitely ruled out (vide infra), there still appears to be a change in mechanism. It is our belief that this issue needs to be resolved spectroscopically, though it is easier to prove the electron redistribution pathway (e.g., by detection of unpaired spin in the intermediate) than to disprove the pathway.

Rare earth ion complexes, in particular those incorporating conju-gated ligands, are increasingly viewed as potential organic light-emitting diodes (OLED) as they combine favourable absorption characteristics with long-lived emission lifetimes. Oligothiophene chelates with erbium(III) (**12**) combine good solubility, good transport properties (electrically excited devices), and polymerisation potential (74). The thiophene carboxylate ligands absorb intensely from 335 to 380 nm and sensitise the erbium(III) ions resulting in near-IR emission. Close matching of the ligand triplet state with the $^4I_{9/2}$ excited state of erbium(III) allows efficient energy transfer. No lifetime data were reported for these species.



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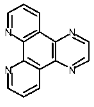

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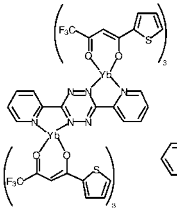

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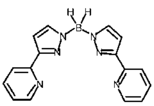

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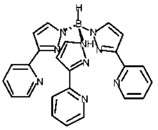

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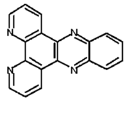

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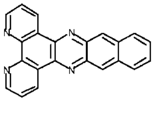

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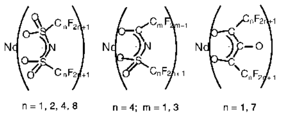

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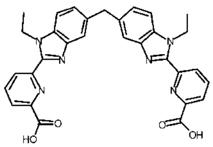

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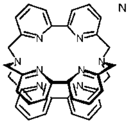

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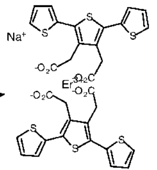

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Lanthanide ions can also be hosted within dendrimeric environments. Balzani and co-workers (75) have demonstrated the encapsulation of such ions including near-IR emitters within polylsine dendrimers containing coordinating amide groups on the interior, and flourescent dansyl groups on the periphery. Excitation at 252 or 338 nm (the absorption maxima of the dansyl chromophores) led to dendrimer flourescence which was quenched in the presence of neodymium(III), erbium(III), or ytterbium(III), and consequently metal-based emission in the near-IR was observed. In the ytterbium(III) case, sensitised near-IR emission is likely to occur via the intermediate formation of an electron-transfer excited state, since the spectral overlap between the excited state of the dansyl units and ytterbium(III) $^2F_{5/2}$ is poor.

Differing philosophies with regard to ligand design have provided numerous complexes. Clearly, the enhancement of lanthanide-based luminescence is primarily a consequence of solvent quenching. Thus, in contrast to the ligands described earlier, non-acyclic ligand systems are desirable because they offer not only kinetic stability, but also more complete metal ion encapsulation.

MACROCYCLIC COMPLEXES

Macrocyclic ligands have long been of interest due to the inherent kinetic stability of their complexes. Aza-macrocycles such as [12]aneN_4 (cyclen) have been developed significantly with regard to lanthanide coordination chemistry and derivatives such as DO3A have been extensively studied. Such ligands are attractive because they offer donor sets of seven or eight atoms that can encapsulate lanthanide ions, which traditionally prefer eight or nine coordination geometry. The ease with which cyclen can be functionalised has also led to the development of ligands that not only prevent the approach of solvent molecules, but also incorporate aromatic chromophores thus overcoming the inherently low molar absorbtivities of lanthanide ions.

Parker and co-workers have devoted a considerable amount of effort into the pursuit of macrocyclic derivatised ligands that incorporate a variety of donor atoms that can bind and encapsulate a lanthanide ion. The examples described here are all based on the tetraaza macrocycle, [12]aneN_4 more commonly known as cyclen. The ring nitrogen atoms can be functionalised with a variety of groups such as benzyl phosphinates and amide derivatives, which incorporate hard donor atoms. Typically, ligands of this type limit the approach of solvent molecules by presenting the lanthanide ion with eight donor atoms. For example, the ytterbium(III) complex of the tetrabenzyl phosphinate ligand (**13**) displays near-IR luminescence upon excitation at 266 nm with relatively long lifetimes of 4.5 and 9.0 μsec in H_2O and D_2O, respectively. Complementary crystallographic studies of this complex revealed the complete encapsulation of the metal ion and the absence of coordinated solvent molecules. Similarly, the degree of solvation, as deduced from the emission lifetimes, gave a q value of 0 (in accordance with the structural data). The analogous neodymium(III) complex demonstrated significantly shorter lifetimes of 159 and 329 nsec in the same solvent system.

It is possible to extend the wavelength of absorption by incorporation of chromophores which absorb light at longer wavelength: for instance, cyclen-derived ligands such as **14**, which incorporate an appended phenanthridine moiety has been synthesised (76). The corresponding ytterbium(III) complex of this ligand has provided a system allowing the effect of pH dependence on the energy transfer between the chromophore and the lanthanide ion to be studied. Both the phenanthridine triplet state (neutral, 22,000 cm^{-1}) and phenanthridinium triplet state (acidic, 21,300 cm^{-1}) have negligible overlap with the excited state of ytterbium and the precise nature of energy transfer in this species was deduced from a thorough photophysical spectroscopic investigation in neutral and acidic, H_2O and D_2O. The effects of triplet energy quenching by oxygen were also investigated. The protonated ytterbium(III) complex demonstrates the typical pathway for lanthanide ion emission, with sensitisation proceeding via energy transfer from a long-lived antenna triplet state. This process is relatively slow owing to the need to dissipate excess energy through the solvent vibrational manifold. At neutral pH (i.e., deprotonated), however, the time-resolved luminescence characteristics are very different demonstrating that extremely rapid energy transfer occurs. It is likely in this case that an electron transfer step occurs from the triplet state of the phenanthridine to the metal ion (77).

TRANSITION METAL COMPLEXES AS PHOTOSENSITISERS

The pursuit of low energy and visible light sensitisation of near-IR lanthanide emitters has been primarily driven for two reasons. First, efficient energy transfer from the sensitiser requires the lowest energy triplet state to lie above the energy state of the emitting lanthanide by $1000-2000\,\mathrm{cm}^{-1}$ (78). Secondly, in medical imaging, biological tissue is relatively transparent to long wavelength light regardless of whether it arises from the excitation source or the probe centred emission. The use of transition metal complex fragments offers an interesting alternative to the incorporation of complex organic chromophores. Such systems have recently been investigated by a number of groups keen to investigate and exploit their use as senitisers. The advantages of such an approach are clear, with transition metal complexes possessing intense, low energy, and readily tunable charge-transfer absorption bands.

van Veggel and co-workers (79) reported the first examples of neodymium(III) and ytterbium(III) luminescence sensitised by the transition metal complexes ruthenium(II) *tris*bipyridine (**15**) and ferrocene (**16**). The absorption properties of $[Ru(bpy)_3]^{2+}$ (the ^{1}MLCT → ^{3}MLCT intersystem crossing is known to proceed with near-unity quantum yield) enable excitation of neodymium(III) and ytterbium(III) with visible light up to 500 nm (into the $M_{Ru}LCT$ λ_{max} 451 nm) while the ferrocene adduct, despite possessing weaker visible light absorption (320 and 440 nm), matches its triplet state well with the luminescent states of those ions. The overall ligand systems are based on the terphenyl-based chelates discussed earlier giving $[Ln_2-Ru]^{2+}$ and [Ln–Fc] complexes. In all cases, the ytterbium(III) luminescence lifetimes were longer than the neodymium(III) species with both in the microsecond domain. For $[Yb_2-Ru]^{2+}$ spectral overlap of the excited states is not as significant as with the neodymium(III) analogue resulting in a weaker emission intensity, although in each example energy transfer is proposed to proceed via the classical antenna triplet state. The ferrocene functionalised complexes demonstrated lanthanide-based emission following excitation at

320 nm in deoxygenated [D_6]DMSO with luminescence lifetimes of 2 and 18.8 μsec for [Nd–Fc] and [Yb–Fc], respectively.

Parker, Beeby, and co-workers have elaborated this methodology by utilising the triplet energy properties of porphyrins and their palladium complexes (**17**) (80). Porphyrins are suitable chromophores (triplet energies 12,000–17,000 cm^{-1}) for sensitisation of lanthanide ions, but palladium porphyrin derivatives display particularly long-lived triplet states. These photophysical characteristics have been utilised in a series of palladium–porphyrin conjugates which are covalently linked to a near-IR emitting lanthanide group. Following excitation in the visible region at 529 nm, emission from either neodymium(III) or ytterbium(III) was observed. In degassed solutions, phosphorescence was observed as broad maxima at ca. 735 and 800 nm and ascribed to Pd–porphyrin in origin and lanthanide-based emissions were enhanced [by a factor of 7 for ytterbium(III) and 2 for neodymium(III)]. The results demonstrate that the rate of energy transfer from the Pd–porphyrin unit to the lanthanide ion and the rate of triplet state quenching by dissolved oxygen are competitive. The more favourable spectral overlap for neodymium(III) species results in more efficient energy transfer than compared with the ytterbium(III) analogue. As a consequence, the ytterbium(III) species are clearly more sensitive to pO_2. These species have also been shown to interact with DNA, the intercalation process suppressing quenching by oxygen and enhancing phosphorescence.

In a further extension of this area, we have recently investigated mixed metal species incorporating platinum(II) [$(PPh_3)_2Pt(\mu$-pdo)Ln(tta)$_3$] (**18**) (where H_2pdo = 5,6-dihydroxy-1,10-phenenthroline; Htta = thenoyltrifluoroacetone) (73, 81). (pdo)$^{2-}$ chelates with Pt(II) via its diolate sites leaving the diimine portion free for lanthanide ion coordination. The absorption properties of [$(PPh_3)_2$Pt(pdo)] show its lowest energy band at 460 nm. In the dinuclear complexes Pt–Ln (Ln = Yb, Nd, and Er), this band is red-shifted to 485 nm with a broad tail extending to about 650 nm. Therefore, an excitation wavelength can be selected to sensitise the lanthanide ions either via the diketonates (337 nm) or via the Pt-based chromphore (>400 nm). Ultimately, excitation at 520 nm of both solid and CH_2Cl_2 solutions revealed near-IR lanthanide-based luminescence on the microsecond timescale. These simple heterodinuclear complexes demonstrate metal–metal, d–f energy transfer by using the "complexes as ligands" approach.

SELF-ASSEMBLED SYSTEMS

In solution, the charge neutral macrocyclic complex [Yb(DO3A)] complex is known to coordinate two solvent molecules (82). By appropriate choice of ligand, we have shown that self-assembly processes can occur in solution with lanthanide complexes (**19**) (83). Tetrathiafulvene (ttf) carboxylate associates (through the hard oxygen donors) with [Yb(DO3A)] in methanolic solution to yield a ternary complex that can be probed using luminescence studies. For this associate, any ytterbium(III)-based emission must be due to energy transfer from the aromatic chromophore ($\lambda_{exc} = 337\,nm$) and lifetimes were 2.02 and 3.49 μsec in CH_3OH and CD_3OD, respectively, giving an inner sphere solvation, $q = 0.21$ (suggesting the ttf adduct prevents approach of solvent). The carboxylate triplet energy gives very little overlap with the ytterbium(III) excited state, but energy transfer is likely to occur via an electron transfer process given that the luminescence signal grow-in is almost instantaneous ($<5\,nsec$) suggesting an efficient process is occurring. This example demonstrates that strong electron donors can act as sensitisers for ytterbium(III) and that self-assembled luminescent systems can be applied to bioassay where the response signal is dependent on the spacial proximity of donor and acceptor units.

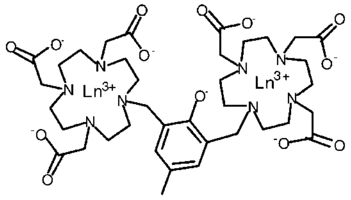
MULTINUCLEAR LANTHANIDE COMPLEXES

In contrast to mononuclear complexes with macrocylic ligands, multinuclear lanthanide examples are relatively rare. The majority of these examples involve kinetically unstable complexes with only a few demonstrating near-IR luminescence. We have been developing new ligand systems that allow the incorporation of more than one lanthanide ion within a kinetically stable ligand framework which is based on macrocyclic ligands.

Cyclen derived bis-macrocycles can be accessed with a variety of bridging groups. We have reported the synthesis of a phenol bridged dinuclear ytterbium complex (**20**), which demonstrates the possibility of differentiated binding sites for each metal ion (84). The coordination environments of the ytterbium(III) ions were probed using luminescence spectroscopy. Following absorption of the excitation wavelength at 337 nm by the phenolic chromophore, metal-centred luminescence was observed at 980 nm. Unusually, for these complexes the emission decays at 980 nm could only be fitted to dual exponential decays. Therefore, two lifetime values were obtained for both non-deuterated (0.51 and 1.67 μsec) and deuteriated (1.17 and 4.95 μsec) aqueous solvent systems resulting in the calculation of two distinct q values of 0.3 and 1, respectively. This evidence suggests either a binuclear complex with two distinct binding sites, or the co-existence of two forms of the complex in solution. One binding site is probably eight-coordinate and involves the phenolate oxygen, while the other is seven-coordinate as the bulk of the macrocyclic groups prevent each metal ion sharing the phenolate oxygen donor atom. In contrast, the corresponding dinuclear ytterbium complex of a bis-macrocyclic ligand system with a xylyl bridging group (**21**), and hence no coordinating properties, gave emission at 980 nm with mono-exponential luminescence decay.

The lifetimes of 1.45 (water) and 6.07 μsec (deuterium oxide) correspond to a single *q* value of 0.4 for both metal ions, while the lipophilicity of the xylyl linkage presumably accounts for the lower than expected *q* value (85).

Finally, we have recently developed a synthetic strategy that allows mixed lanthanide complexes to be accessed. In our system (**22**), two terbium(III) ions are coordinated in macrocylic derived environments, while a DTPA(dianhydride) site binds a ytterbium(III) ion, and benzyl chromophores provide improved absorption characteristics (86). The metal ions are held in close proximity to each other and a comprehensive study of the luminescence has allowed the coordination environments of each metal to be probed. The near-IR emission from ytterbium(III) gave lifetimes of 1.8 (water) and 4.2 μsec (deuterium oxide) and hence a *q* value of 0.2 that corresponds to an eight coordinate donor set for the ytterbium(III) ion from the DTPA derived portion of the ligand. The mixed metal complex is luminescent in both the visible (terbium emission) and near-IR (ytterbium emission), but crucially the design of the complex has allowed terbium sensitised ytterbium luminescence to be demonstrated for the first time. The excited state of terbium(III) can be pumped at 488 nm and this resulted in near-IR emission at 980 nm with lifetime of 4.2 μsec in deuterium oxide. This lifetime is identical to that obtained when $\lambda_{exc} = 337$ nm (i.e., absorption through the chromophore). As neither ytterbium nor the ligand possesses absorption bands at 488 nm, the sensitisation must occur via the terbium ions.



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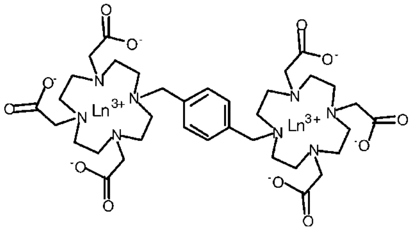

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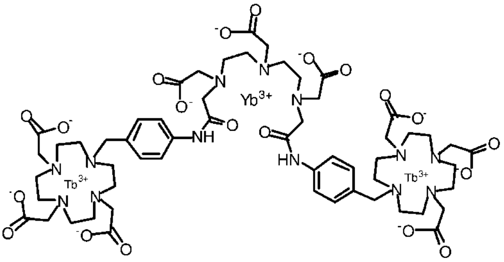

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CONCLUSIONS

The properties of luminescent lanthanide complexes in solution make them eminently suitable for use as imaging agents. Those, which are beginning to be exploited widely in the visible region of the spectrum, have already begun to be applied widely in a range of temporally and spatially resolved techniques. In the last few years, related complexes, which emit light in the near-IR, have also been studied in detail, and their luminescence properties established. Technical developments, particularly in instrumentation for imaging of near-IR emission signals, are still ongoing, but the advantages of near-IR luminescence and visible excitation mean that it is almost inevitable that these systems will become accepted as probes in the same way as their visible cousins have already been.

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