7 Luminescent Lanthanide Complexes as Sensors and Imaging Probes

D. Parker, Y. Bretonniere

7.1 Introduction and Background

The use of luminescent probes is now commonplace in biological and clinical applications for the detection and monitoring of a wide range of chemical species, from simple ions to complex bioactive molecules. A wide variety of fluorescent organic molecules is available for this purpose, either incorporating or attached to suitable binding sites for the target species (Haugland 2002). Although this area of research is dominated by purely organic molecules, a considerable research effort in the development of new emissive sensory systems is directed towards luminescent metal complexes, including those of transition metal ions (Demas and de Graff 2001) and of the

lanthanide(III) ions (Parker and Williams 2003). Metal complexes often possess an extensive excited state chemistry, owing to the presence of metal-centered (e.g., *dd* and *ff*) and charge-transfer states involving the metal, in addition to the singlet, triplet, and charge transfer excited states associated with the ligand. Thus, they not only offer additional opportunities for perturbing the emission characteristics, but also may give rise to longer-lived luminescence.

The lowest energy, metal-centered excited states of several of the lanthanide(III) ions have long radiative lifetimes of between 0.1 and 10 ms. As long as deactivation by nonradiative processes is minimized, the observed decay of light emission in solution at ambient temperature may also be of this order. Such long-lived emission is an attractive feature in a luminescent probe, as it allows time-resolved detection methods to be used, offering excellent discrimination between probe and background emission (Lakowicz 1999). A time delay can be introduced between the pulsed excitation of the sample and the measurement of the probe's luminescence, during which the shorter-lived background fluorescence characteristic of biological and clinical samples decays to negligible levels. Such procedures obviate experimental problems associated with light scattering and the large Stokes' shifts of emissive lanthanide complexes also negate the problem of autofluorescence. Although not unique to the lanthanides, long-lived emission *under ambient conditions* is unusual. The triplet-derived phosphorescence of many aromatic molecules, for example, is characterized by long *natural* lifetimes, but is usually quenched too efficiently to be observed in fluid solutions at ambient temperature: deoxygenation and low temperatures are normally required to reduce competitive deactivation processes, rendering such molecules unsuitable for time-gated procedures.

The contracted, "core-like" nature of the 4f orbitals in the tripositive lanthanide ions has three major effects on the optical spectra, leading to markedly different characteristics when compared to the d-d spectra of transition metal ions. First, ligand-field splittings are small, typically around 100 cm^{-1} (1.2 kJ mol^{-1}). Therefore, excited states in compounds or complexes of the lanthanides are effectively the same as for the gas-phase ions. The energy of the *ff* transitions in the spectra is, to a first approximation, independent of the coordination environment. From the point of view of the design of respon-

sive complexes for chemical sensing, this restricts the use of emission wavelength changes as a signaling mechanism, in contrast to many fluorescent sensors. On the other hand, it allows optics to be set up and optimized for fixed emission wavelengths and, in fact, changes in the fine structure of the bands, and especially their relative intensities, can be very informative, especially for Eu(III) complexes. Second, the weakness of the interaction of the *f* orbitals with ligand orbitals leads to minimal mixing of the electronic excited states with ligand vibrations. As for the *dd* transitions of d-block metal ions, it is only through coupling with asymmetric ligand vibrations that the formally forbidden *ff* transitions become partially allowed, so the weakness of the coupling ensures that the transitions remain weak. In absorption, this leads to very low extinction coefficients (ε typically 0.1 $mol^{-1}dm^3cm^{-1}$); in emission, it explains the low transition probabilities and hence the long natural lifetimes. Finally, the emission bands are almost "line-like," i.e., very sharp and narrow, even in solution, because the vibrations of the ligands and concomitant changes in M–L distances have very little effect on the energies of the excited states.

A consequence of the low molar absorption coefficients of the lanthanide(III) ions is that the excited states are not efficiently populated by direct absorption of light by the metal ion, unless laser excitation is used. The most widely-used emissive lanthanides, terbium and europium, can be excited in this way by the 488-nm line of the argon ion laser (matching the Tb $^7F_6 \rightarrow {}^5D_4$ energy gap) and by rhodamine 110 in a dye laser (for the Eu $^7F_0 \rightarrow {}^5D_0$ transition at 580 nm or the $^7F_2 \rightarrow {}^5D_1$ at 557 nm). An alternative solution, which allows lower intensities of light to be used and which opens up additional strategies in the design of responsive luminescent complexes (Sect. 7.2), is that of sensitized emission. A chromophore is built into the structure of a ligand forming a stable complex with the lanthanide ion. It is chosen so that it absorbs light in the range 300–420 nm and transfers its excitation energy to the metal ion (Parker and Williams 1996). If the chromophore has a high extinction coefficient and energy transfer occurs efficiently, then the "effective" molar absorption coefficient of the metal is greatly increased and intense luminescence can result following excitation with conventional lamps or LEDs.

The choice of sensitizer is determined primarily by the energy of the emissive excited state of the metal: the sensitizer must possess an excited state, from which energy transfer can occur, whose energy is at least equal to that of the metal emissive state. The efficiency of the process is dependent on the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor; in other words, it should be favored by a matching of the energy levels of the donor and acceptor. A further requirement is that the donor excited state must be sufficiently long-lived for energy transfer to compete effectively with other deactivation pathways. An energy transfer rate constant of 10^5–10^7 s^{-1} is typical of Eu^{3+} and Tb^{3+} complexes containing aromatic sensitizers within about 5 Å of the metal ion. This is too slow to allow energy transfer to occur from short-lived singlet states (τ_{obs}^{-1} typically 10^9–10^8 s^{-1}) and most studies in which the energy transfer process has been investigated in detail have revealed the predominance of the longer-lived *triplet* state as the donor (Beeby et al. 2001).

Whilst a triplet energy comparable to, or a little higher than that of the metal emissive state favors the efficiency of sensitization, too small an energy gap (<1,700 cm^{-1}) is detrimental, since thermally activated back energy transfer from the metal to the sensitizer then competes with emission, leading to reduced lifetimes and quantum yields. This limits the choice of sensitizer to those with triplet energies in excess of about 19,000 cm^{-1} for Eu^{3+} and 22,000 cm^{-1} for Tb^{3+} (the emissive states, $^5D_0$ and $^5D_4$, lie at 17,200 and 20,400 cm^{-1} respectively), although a much larger range of sensitizers is possible for lanthanides with lower energy emissive states like Yb^{3+} ($^2F_{5/2}$ at 10,200 cm^{-1}). Further desirable features in the sensitizer are a high quantum yield of triplet formation and a relatively low singlet excited state energy (i.e., a small S_1–T_1 energy gap), to allow excitation to be achieved at long wavelengths (>350 nm), and so avoid the need for quartz optics and competitive absorption by simple biological molecules.

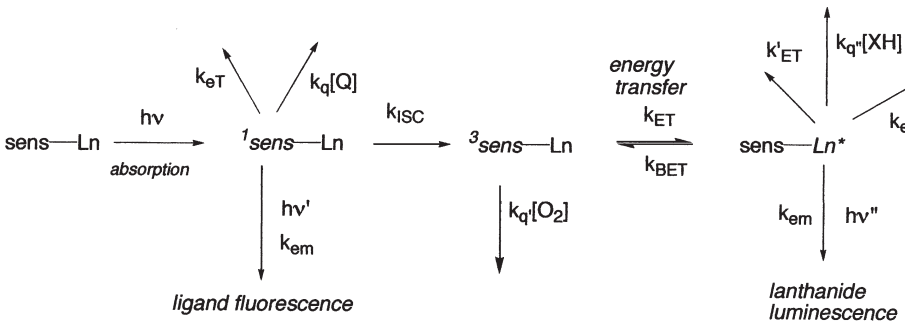
A variety of aromatic chromophores has been considered as sensitizing moieties. Simple naphthyl groups suffer from several problems: they generally possess only very weak absorption beyond 300 nm; their triplet states are only a little higher in energy than the $^5D_4$ of Tb^{3+}, leading to severe back energy transfer; the singlet excited states are quenched by photoinduced electron transfer to Eu^{3+}.

A functionalized 1,10-phenanthroline ligand is used in the commercially available FIAgen bioassay reagent. More recently, phenanthridines (Parker et al. 1998), acridones (Sammes et al. 2000), benzophenones (Beeby et al. 2002), and tetraazatriphenylenes (Werts et al. 2000a, b) have been investigated, which offer efficient sensitization for both europium and terbium, while allowing excitation at wavelengths in the range 340–420 nm.

The next key issue to address is the nature of the ligand to bind the lanthanide. Most applications require the function of the probe complex in aqueous solution and, in the biosciences, in the presence of significant concentrations of competing endogenous ligands (e.g., proteins, phosphorylated anions, lactate, citrate). Complexes are therefore required which have high kinetic and thermodynamic stability with respect to metal ion dissociation, preferably over the pH range 4.5–8. In this respect, the requirements are similar to those demanded by gadolinium-based contrast agents in MRI (Caravan et al. 1999). The high coordination numbers favored by the lanthanide(III) ions necessitate use of multidentate ligands, preferably at least heptadentate, while the high charge density of the tripositive ions is best accommodated by ligands offering anionic or polarizable nitrogen or oxygen donors or a combination thereof. Polyaminocarboxylate ligands based on the acyclic DTPA system or the closely related macrocyclic ligand DOTA are examples of ligands that satisfy each of these requirements, and with analogs incorporating phosphinate or amide groups in place of the carboxylates, constitute a versatile group of ligands, modified to incorporate a variety of sensitizing groups. Other ligand frameworks which have been used as the basis for ligand modification include terpyridines (Toner et al. 1993), bipyridyl-functionalized cryptands (Alpha et al. 1990), and calixarenes (Sabbatini et al. 1995).

7.2 Mechanistic Approach to Modulation of Luminescence

The photochemical pathway that defines the mechanism of sensitized lanthanide luminescence reveals that there are three excited states which may be perturbed, leading to modulation of the emission from the lanthanide ion (Scheme 1; Parker and Williams 2003).



Scheme 1. Lanthanide luminescence

First, the singlet excited state of the sensitizing chromophore may be quenched by an electron or charge-transfer process which can be inter- or intramolecular in nature. This quenching process competes with intersystem crossing, depletes the population of the singlet excited state, may be accompanied by fluorescence quenching, and is echoed by a reduction in the intensity of emission from the lanthanide ion. Another way of perturbing the singlet excited state involves a reversible binding process of the chromophore itself, e.g., following protonation or metal coordination, leading to a change in the energy of the singlet excited state. This will modulate the relative intensity of lanthanide emission but more significantly leads to a change in the corresponding excitation spectra, allowing ratiometric measurements to be performed (Blair et al. 2001). Second, the intermediate triplet of the chromophore may be perturbed. Its energy may also be changed by reversible metal binding or protonation at an integral basic site leading to differences in the rate of forward or reverse energy transfer (Parker et al. 1998; Parker and Williams 2003). This process also modulates the lifetime of the lanthanide excited state. Aromatic triplet states are particularly sensitive to collisional quenching by molecular oxygen. In cases where $k_q[O_2]$ is of the same order as k_{ET}, the lanthanide emission lifetime and intensity is sensitive to variations in dissolved oxygen concentration (Blair et al. 2002). In addition, enhanced oxygen sensitivity may occur when there is a finite rate of reverse energy transfer from the Ln^{3+} excited state, leading to a repopulation of the chromophore triplet state. This

thermally activated process is only significant when the energy gap between the two states is less than 1,500 cm^{-1}. Most examples have involved the $^5D_4$ excited state of Tb^{3+}. It lies at 20,400 cm^{-1} and is close in energy to a large number of aryl triplet states. Pronounced modulation of the lifetime of the lanthanide excited state results from collisional deactivation of the aryl triplet by oxygen. The measured dependence of lanthanide emission/lifetime variation on oxygen concentration may be kinetically complex, being determined by two coupled first-order differential equations.

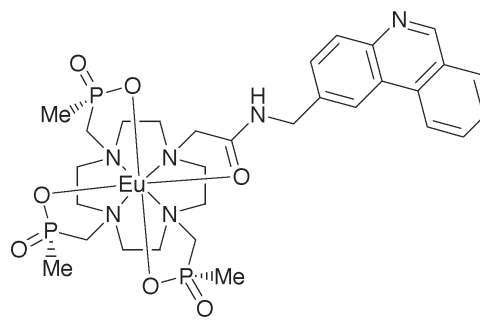
Finally, the lanthanide excited state itself may be quenched. Here, there are three different cases: vibrational energy transfer involving energy-matched XH oscillators [especially hydroxy (OH) and amino (NH) groups]; energy transfer to an acceptor of appropriate energy; and electron transfer involving the metal-centered excited state. In the first case, the displacement of bound water molecules by inter or intramolecular anion binding leads to an increase in Ln emission intensity and lifetime and modifies the form of the emission spectrum. This allows ratiometric analyses to be undertaken (Bruce et al. 2000; Lowe et al. 2001). Analysis of emission spectra changes is easiest for europium complexes, because of the absence of degeneracy of the ^5D_o emissive state. Examples involving energy transfer are less common; suitable acceptors for Eu^{3+} include the dye bromothymol blue or the protein allophycocyanine which fluoresces at 665 nm with the lifetime of the donor europium complex (Mathis 1995). Cases of charge transfer quenching of the Ln^{3+} excited state are even less common but include deactivation of a cationic terbium-tetraazatriphenylene complex, following its binding to GC base pairs in poly (dGdC) or calf thymus DNA (Bobba et al. 2002).

7.2.1 Singlet Excited State Perturbation

The best-defined examples of singlet excited state quenching in lanthanide conjugates involve halide anions. The chloride ion fulfils a series of key functions inside a variety of different cell types, including ion transport, pH homeostasis and the regulation of cell volume and intermediary metabolism. The accumulation of chloride above its equilibrium state has been recognized in smooth muscle

contraction (Chipperfield and Harper 2000) and is hypothesized to be linked with modulation of (Na^++K^++Cl^-) cotransport and the perturbation of HCO_3^-/Cl^- exchange. Considerable interest exists in devising better ways of monitoring intracellular chloride concentrations (30–70 mM range) using optical methods. So far this has been achieved by examining the quenching of fluorescence of zwitterionic N-alkylated quinolinium or acridinium species. This phenomenon involves collisional quenching of the singlet excited state, but gives intensity modulation only, with short wavelength excitation and interference from proteins via PET quenching from protein amino groups.

In the stable Eu complex [Eu**1**]$^+$, addition of halide ions led to a reduction in phenanthridinium fluorescence (405 nm), echoed by a diminution in europium emission intensity (e.g., at 616 nm). With chloride, Stern-Volmer quenching constants (K_{SV}^{-1}) were in the range 40 to 50 mM, and were independent of added lactate, phosphate, citrate and hydrogen carbonate (Parker et al. 1998). Quenching followed the order I>Br>Cl. In the analogous terbium complexes while phenanthridinium fluorescence was modulated to the same extent, the Tb emission intensity was much less sensitive to added Cl^-, because of competitive quenching of the T_1 state by O_2 (Sect. 7.2.2), suggesting a possible use of Tb/Eu complexes in tandem to assay chloride by ratiometric methods.

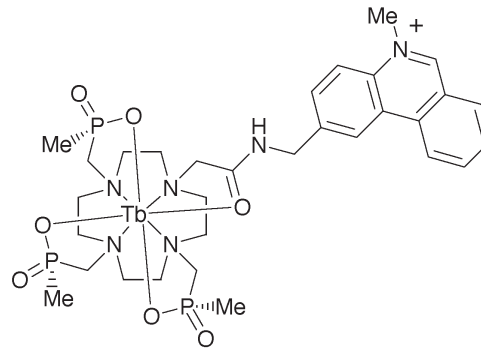


[Eu.**1**]

7.2.2 Triplet Perturbation

Measurement of the level of dissolved oxygen in water is important in many biological and environmental systems: in river waters, for example, hypoxia may lead to the death of fish when the oxygen concentration falls from 0.27 mM (normal) to below 0.04 mM. Knowledge of oxygen gradients in complex biological samples is required for many processes that involve aerobic energy metabolism. Optical methods are used widely (Demas et al. 1999) as they are amenable to miniaturization (e.g., fibre-optic optodes), exhibit fast response times, and rarely are subject to interference. The majority are based on quenching metal to ligand charge transfer (MLCT) states in Ru or Os di-imine complexes of ligand-centered triplet states in Pd and Pt porphyrins. In emissive lanthanide complexes, the long-lived lanthanide excited state is not sensitive to O_2. It is the triplet state of the sensitizing moiety which may be subject to collisional quenching either because of a slow rate of competing energy transfer or because it is long-lived as a result of repopulation by a back energy transfer process.

A series of near-IR luminescent complexes has been described (Nd, Er, Yb) in which an integral fluorescein, eosin, or metalloporphyrin group acts as a sensitizing chromophore (Werts et al. 2000a, b). Intramolecular energy transfer competes with triplet quenching by O_2, giving rise to O_2 sensitivity in emission lifetime and intensity. Examples of oxygen sensing using Eu complexes operating at the gas/solid interface have been reported. Europium β-diketonate complexes bearing a phenanthroline chromophore (λ_{exc} 340 nm) have been immobilized in polystyrene films and exhibit modest oxygen sensitivity (Imao et al. 2000). Perhaps the greatest opportunity for devising practicable pO_2-dependent Ln^{3+} luminescence involves sensitized terbium emission. The long-lived Tb $^5D_4$ excited state ($\tau \sim 1$–3 ms) lies at 20,400 cm^{-1}, within range of many common aryl triplet states. When the energy gap is less than about 1,000 cm^{-1}, thermally activated back energy transfer significantly depletes the emissive $^5D_4$ level, and oxygen sensitivity results. Examples of aryl triplets with such energy include substituted coumarins, N-alkyl or protonated phenanthridiniums and quinoliniums, triphenylenes, and naphthalenes. The dependence of the Tb emission



[Tb.**2**]$^{+}$

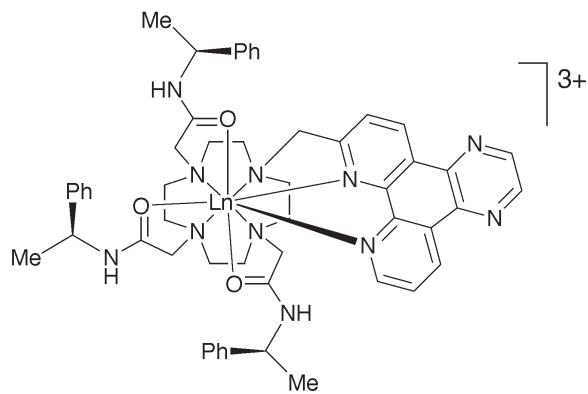
lifetime on pO_2 may follow Stern-Volmer behavior in homogeneous solution, but can be kinetically complex in immobilized systems. Simple calibrations have been established (Blair et al. 2002), e.g., for Tb-N methylphenanthridinium conjugates such as [Tb.**2**]$^{+}$, immobilized in sol-gel matrices, which possess an overall quantum yield efficiency of 20%, with respect to O_2 quenching in aqueous media.

7.2.3 Lanthanide Excited State Perturbation

Each of the excited Ln^{3+} ions is subject to quenching by vibrational energy transfer involving harmonics of energy-matched XH oscillators. Directly coordinated water molecules afford the most common example of this quenching, so if a species can bind directly to the Ln^{3+} center, reversibly displacing these waters, both the emission intensity and lifetime increase and the form of the emission spectrum will also change, allowing ratiometric analyses. With chiral complexes, the local helicity at the metal center may be perturbed as well, allowing modulation of the circular polarization of the emitted light (Lopinski et al. 2002). Examples of intermolecular binding include the reversible binding of anions (HCO_3^-, lactate, citrate, phosphate, DNA) at diaqua-lanthanide centers (see Sect. 7.3.1). Intramolecular examples have been reported in which an integral sulfona-

mide nitrogen binds reversibly, in a pH- (or metal ion-) dependent manner (Lowe et al. 2001).

The Ln^{3+} excited state may also be quenched by a nonradiative charge-transfer interaction. Such a process leads to a reduction in emission lifetime and intensity and has been reported in cases where the Ln^{3+} ion is directly coordinated to an electron-poor heteroaromatic, e.g., the chiral Eu and Tb complexes of the emissive ($\phi_{H_2O}^{Eu} = 21\%$); ($\phi_{H_2O}^{Tb} = 40\%; \lambda_{exc}$ 355 nm) nine-coordinate tetraazatriphenylene conjugate, [Ln.**3**] (Bobba et al. 2002). The complex binds strongly to DNA and engages in a charge transfer interaction which is more well defined with GC base pairs, building up charge on the heteroaromatic moiety. This is signaled by a marked hypochromism and a shift to the red of the aromatic absorption band. At the same time, a change in the polarizability of the Ln-bound N donors occurs, leading to a build-up of positive charge at the metal center. Such a process is more efficient for Tb^{3+} than Eu^{3+} (i.e., an MLCT type of process) and is accompanied by a change in the form of the hypersensitive $\Delta J = 4$ manifold in Eu^{3+} emission spectra, allowing ratiometric analysis of the binding interaction. This behavior is consistent with modulation of the polarizability of the "axially" bound tetraazatriphenylene N in the monocapped-square antiprismatic complex (Bruce et al. 2003).



(SSS)-Δ-[Ln.**3**]$^{3+}$

7.3 Tailoring the Lanthanide Complex for Use "In Cellulo"

The above discussion summarizes the guiding chemical principles that have been used to establish the probe and sensor properties of responsive lanthanide complexes over the past 10 years. To date, most applications of luminescent lanthanide complexes in biochemistry and biology have related to the development of time-resolved immunoassays (Mathis 1995) or the introduction of lanthanide complexes as long-lived donors in fluorescence resonance energy transfer (FRET) analyses (Jones et al. 2001). For use in live cell imaging, the requirements of the luminescent probe are more exacting: luminescence at wavelengths shifted from the excitation wavelength; an excitation wavelength well removed from absorption by endogenous cellular chromophores; good absorption characteristics (high ε), and a large overall emission quantum yield; cellular permeability or better still, preferential uptake or retention by different cell types or cell compartments; no cell toxicity; high chemical stability with respect to complex degradation and resistance to photo-bleaching and photo-fading. In addition, the responsive complex must not only bind the target analyte with appropriate avidity (match K_d) and selectivity, but also signal that selective binding event by sufficient changes in emission form (ratiometric methods), polarization, or lifetime to allow calibration.

7.3.1 Anion Analysis: Selectivity for Hydrogen Carbonate

Most work to date has addressed the reversible displacement of one or both waters in diaqua complexes by anion ligation to the lanthanide center (Bruce et al. 2000; Dickins et al. 2002; Parker et al. 2002). This event is signaled by changes in the intensity and form of Ln emission, allowing ratiometric analyses to be undertaken. Such methods are most appropriate in analyses of Eu emission spectra. The relative intensity of the magnetic-dipole-allowed $\Delta J = 1$ transitions (around 590 nm) is insensitive to the associated change in coordination environment while the intensity of the electric-dipole-allowed $\Delta J = 2$ (616 nm) and $\Delta J = 4$ (around 702 nm) manifold changes considerably, particularly if the "hard" axial water molecule

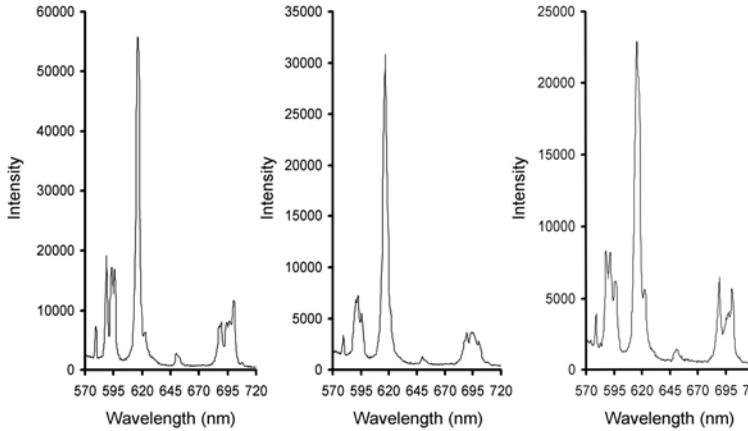
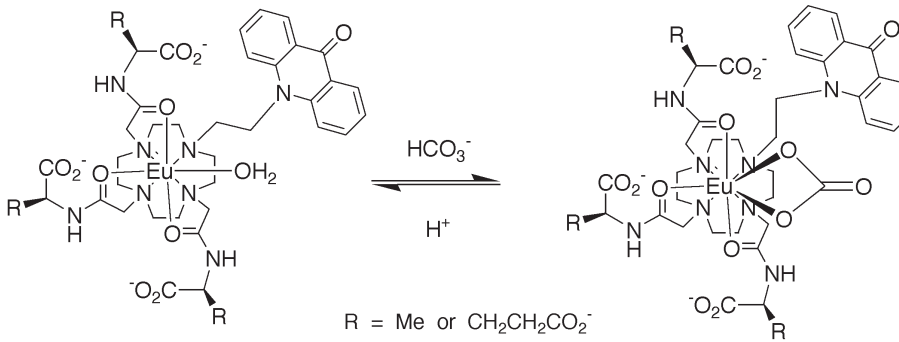


Fig. 1. Europium emission spectra for $[Eu.\mathbf{4}]^{3-}$ (0.1 mM, 1 mM anion, pH 7.4, λ_{exc} 410 nm) in the presence of (*S*)-lactate (*left*), bicarbonate (*center*) and hydrogen phosphate, showing the changes in the form of the emission spectrum signaling reversible anion coordination

is displaced by a more polarizable charged donor. An example of such emission spectra (Fig. 1) highlights the increase in the relative intensity of the $\Delta J=2$ band (618 nm) for the carbonate-bound species – compared to lactate and citrate – and exemplifies how each bound anion gives a "fingerprint" emission spectral profile, allowing simple identification and ratiometric analysis. This information is required in the application of such methods in intracellular media – where a large number of anions may compete in binding to the lanthanide center. The structure of several of these ternary complexes has been defined by X-ray crystallography (e.g., in chelated adducts with lactate, citrate, several amino-acids, acetate; Dickins et al. 2002) and by NMR studies on Eu and Yb analogs. Relative binding affinities have been assessed in simple competitive analyses or in fixed interference studies. They reveal that in the millimolar range, it is the more polarizable (higher energy HOMO) oxygen in HCO_3^- (bound as CO_3^{2-}) and various phosphorylated di-anions (e.g., glucose-6-*O*-phosphate; AMP but *not* cAMP) which bind most avidly, and at ambient pH, it is the bicarbonate anion (range 3–25 mM) which wins the competition between endogenous anions.

The bicarbonate ion is an essential component of biological systems and is vital to many cellular processes in mammals, such as intracellular pH homeostasis, kidney function, and sperm maturation. However, almost nothing is known about how bicarbonate concentrations fluctuate within a cell in response to cell stimuli and such analyses are currently limited to radiochemical measurement of overall $H^{14}CO_3^-$ uptake and inferences based upon changes in intracellular pH. Information about how HCO_3^- is localized and varies within cell compartments is needed in order to understand the diverse physiological processes it may control, e.g., cyclic-AMP regulation, through pH-independent, reversible binding to a soluble adenylyl cyclase enzyme (Cann et al. 2000). Moreover, the pathological consequences of perturbing some of these physiological processes may be very significant: mis-expression of carbonic anhydrase (CA) is linked to the presence of a variety of tumor types and CA-II deficiency syndrome in humans is characterized by renal tubular acidosis, osteoporosis, and mental retardation. There is a pressing need for a direct method to allow changes in HCO_3^- concentration to be measured within a cell, and to monitor the changes in different compartments (e.g., mitochondria) in real time.

A series of europium-acridone conjugates has been reported (Bretonniere et al. 2002) that reveal a fourfold change in the intensity ratio of the Eu 618/588 nm emission bands in the physiologically relevant bicarbonate concentration range, 5–25 mM. The system has been calibrated in the presence of competing anions and protein, e.g., in a cell lysate. The affinity for bicarbonate has been controlled by changing the peripheral electrostatic gradient in the Eu complexes. Thus, anionic, zwitterionic and cationic complexes have been examined, ([Eu.**4**]: the cationic version is the ethyl ester analog of those shown). The zwitterionic and anionic complexes possess the desired affinity and selectivity for bicarbonate (Fig. 2). The complexes are nontoxic to cells (5 mM), and the imaging of cells by confocal microscopy reveals uptake and clear intracellular staining. Preliminary colocalization studies with established compartmentalized organic dyes, such as Mitotracker Green and Lysotracker Green are consistent with localization in lysosomes and possibly mitochondria (Fig. 3). Key experiments remain to be undertaken to address the following: can a ratiometric method be used in real time to im-



[Eu.**4**]

age bicarbonate fluctuations? Can the Eu complex be targeted to other cell compartments? What is the mechanism of cellular uptake? The early work is encouraging and augurs well for the application of these probes to image intracellular HCO_3^- fluctuations, and hence enhance our understanding of the biological significance of the most important C_1 oxyanion.

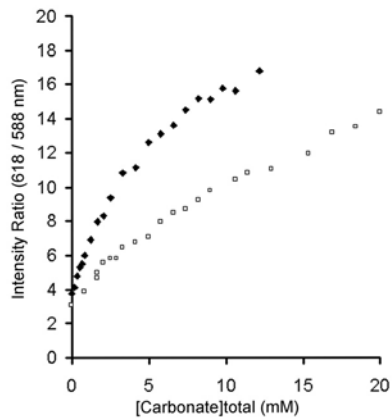


Fig. 2. Calibration curves showing the response of the europium complexes ([Eu.**4**]: 0.1 mM; *open squares*, glutarate derivative; *filled diamonds*, alanine (zwitterionic); 1 mM anion, pH 7.4, λ_{exc} 410 nm) to total added bicarbonate/carbonate in a cell lysate (NIH 3T3 cells)

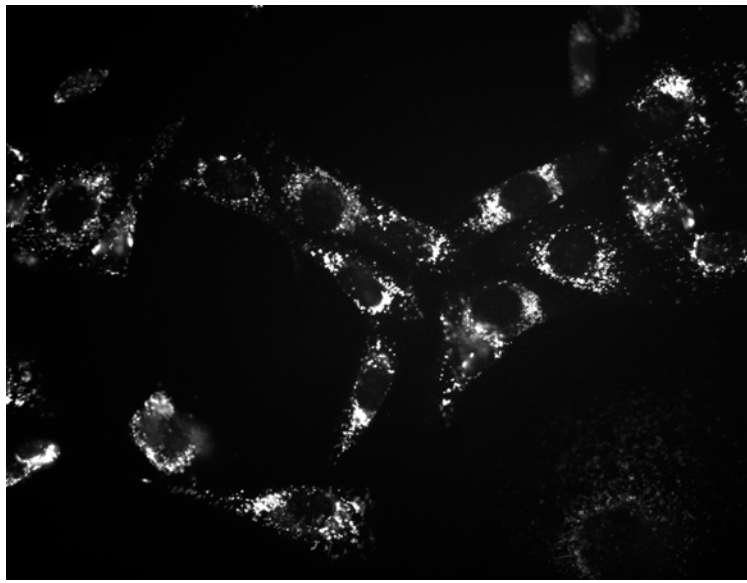


Fig. 3. Microscopy image showing the localization of the anionic complex, [Eu.**4**] (as the glutarate derivative), 3 h postinoculation in NIH 3T3 cells

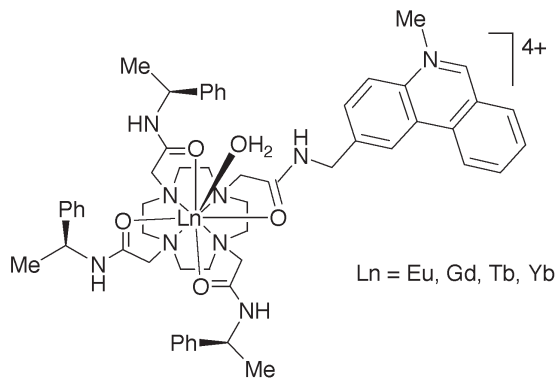
7.3.2 Targeting the Cell Nucleus: DNA Binding Complexes

Although chiral, octahedral complexes of the late d-block metals have been studied for over 20 years in vitro as structural and reactive probes for nucleic acids, relatively little has been reported on their behavior in living cells. Typical examples include the Δ and Λ isomers of $Ru(phen)_3^{2+}$ and a large series of mixed ligand dipyridophenazine (dppz) complexes of ruthenium, rhodium, and osmium, e.g., $[Os(bpy)(dppz)]^{2+}$. These complexes are reputed to bind to DNA by preferential intercalation of the phenazine moiety from the minor groove and the perturbation of the metal-ligand charge transfer transitions that accompanies DNA binding provides a sensitive spectroscopic handle to study the nature of the bound complex.

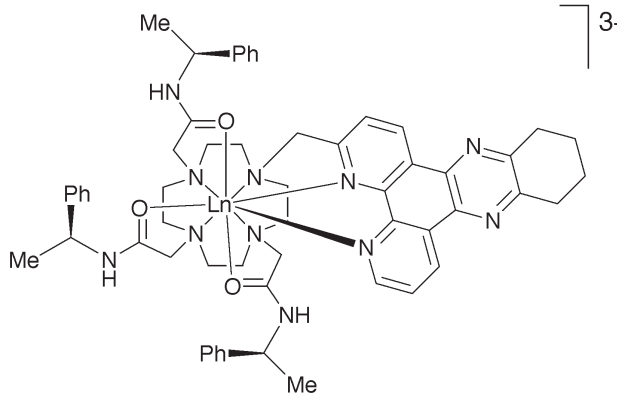
Until very recently, there were no well-defined examples of chiral lanthanide complexes suitable for binding to the polyanionic nucleic acids. In general, the literature reports that discuss lanthanide-nu-

cleic acid interactions have been dominated by the interactions of the aqua ions themselves. Such work has often focused on the selective sensitization of selected ions, e.g., Tb^{3+}, and advantage has been taken of this effect in several assays directed at the detection of selected nucleic acids. Another theme of current activity relates to the ability of certain lanthanide ions to cause nucleic acid cleavage and attempts have been made to target certain sequences using an antisense strategy.

Recently, a series of cationic lanthanide complexes has been defined incorporating a phenanthridinium moiety, in which the configuration of a remote carbon stereogenic center determines the helicity of the overall complex. In the prototypical example, $[Ln.\mathbf{5}]^{4+}$, a primary component of the free energy of binding to oligonucleotides and DNA, has been linked to an intercalative interaction, supported by absorption, CD, and fluorescence quenching behavior. The lanthanide coordination environment and local helicity remained unchanged but the oligonucleotide underwent distinctive changes in local helicity and pitch that were sensitive to the handedness of the Ln complex and in certain cases to the nature of the Ln ion (Yb versus Eu). A limitation of this system is that the singlet excited state of the phenanthridinium complex is quenched on DNA binding via a nonradiative charge transfer interaction with GC base-pairs. The



(SSS)-Δ- $[Ln.\mathbf{5}]^{4+}$



(SSS)-Δ-[Ln.**6**]$^{3+}$

lanthanide emission is therefore also switched off on binding, limiting the scope for assessing the intracellular activity of such probes (Dickins et al. 2002).

More promising behavior is shown by the highly emissive, enantiopure Δ and Λ tetrazatriphenylene complexes, [Ln.**3**]$^{3+}$ and [Ln.**6**]$^{3+}$, which bind to DNA stereoselectively via an electrostatic and intercalative mechanism (Bobba et al. 2002). The lanthanide emission reduces in intensity (approximately 50%) but is not "switched off" on DNA binding. Moreover, it is modulated in form. Of particular interest is the ability of these complexes to enter live cells, target the cell nucleus, and on prolonged irradiation lead to cell death (Frias et al. 2003). Control experiments reveal $<10\%$ cell death in the absence of complex or irradiation. Cells were examined by fluorescence microscopy from 1–48 h after incubation with the complex. Images taken over a period of 48 h showed that cells internalize each complex but that uptake of the Eu complex appears faster than the Tb analog. The microscopy images (Figs. 4, 5) reveal the migration of the probes through the cytoplasm, across the nuclear membrane into the nucleus, revealing substructures within the nucleus. Surprisingly, the Tb complex appeared to remain in the cytoplasm for longer periods and was apparently slower to migrate to

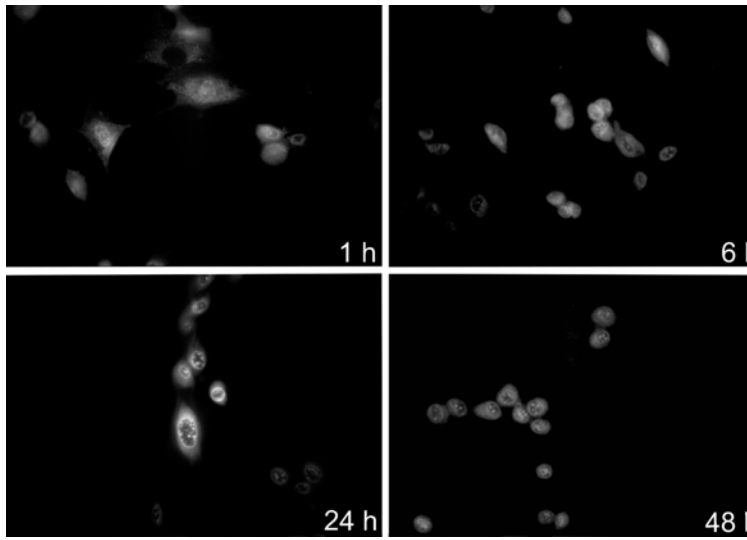


Fig. 4. Microscopy images, at different time points postinoculation, showing the uptake of the DNA-binding complex $[Eu.\mathbf{6}]^{3+}$ into the cell nucleus of NIH 3T3 cells

the nucleus. Parallel in vitro studies had indicated that emission from the Tb complexes was quenched to a greater extent when bound to DNA than for the Eu analogs. When uptake of the Δ and Λ enantiomeric complexes was compared, no significant differences were noted.

In a separate experiment, the cells were inoculated for a period of 3 h, to ensure that the complex had been internalized and was located in the nucleus (Fig. 6). Unbound complex was removed by washing with phosphate-buffered saline solution. The images revealed a transfer of the luminescence from the nucleus to the cytoplasm after a few hours and indicated that the complex remains in the cytoplasm for sustained periods. The egress of the complex from the nucleus back into the cytoplasm was not observed in localization studies carried out with an excess of complex, suggesting that entry into the cell nucleus requires a favorable concentration gradient of the complex.

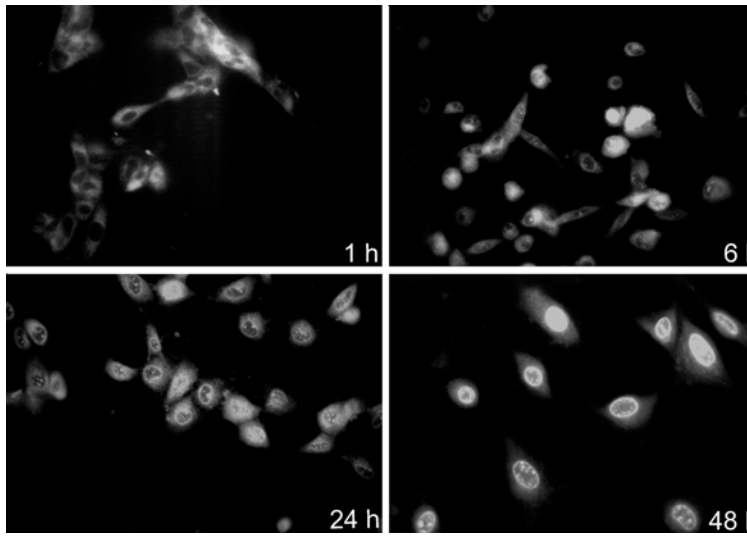


Fig. 5. Microscopy images, at different time points, postinoculation, showing the uptake of the DNA-binding complex $[Tb.\mathbf{6}]^{3+}$ into the cell nucleus of NIH 3T3 cells

The mechanism by which the cells take up the complex remains to be defined. These probes (tripositive charge and MW >1,000) are unlikely to enter by any passive diffusion process across the plasma membrane and therefore active uptake is more likely. No obvious vesicular structures were apparent in the cytoplasm by fluorescence microscopy, so an endocytotic uptake mechanism also seems unlikely.

7.4 Conclusions and Future Perspectives

There remains a great deal of work to do before luminescent lanthanide complexes are used in practice in cell biology. Nevertheless, they show considerable promise as luminescent probes, and once time-gated microscopy equipment is more generally available, and charge coupled device (CCD) cameras can be added to facilitate ratiometric analyses, one might expect further applications to develop.

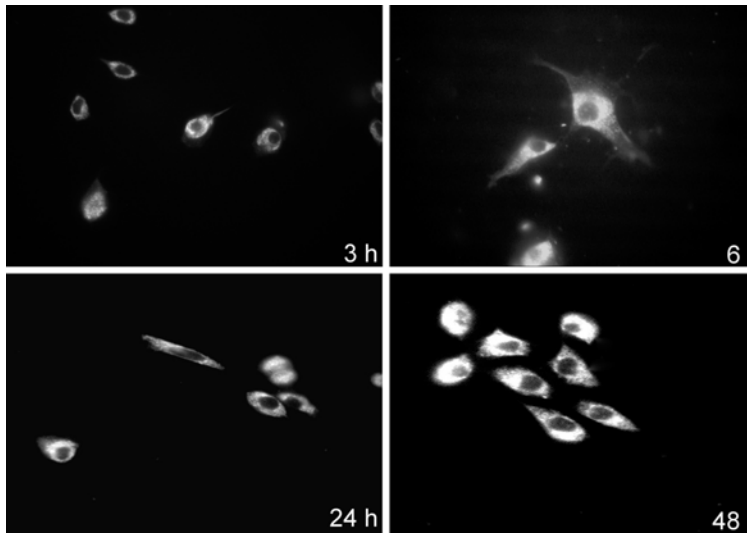


Fig. 6. Microscopy images showing the transit of the complex, $[Eu.\mathbf{6}]^{3+}$ out of the cell nucleus over a period of time, in the absence of added complex

The more important challenge for the molecular scientists is to devise practicable methods for monitoring the spatio-temporal variations in the concentration of essential bioactive ions. Great progress was made in cell biology following the development of Ca-sensitive fluorophores by Tsien, but we still need *new chemistry* and biochemical assays to be discovered, to allow us to understand the biological role of many simple ionic species such as bicarbonate, phosphopeptides and small proteins and molecules such as carbon monoxide and nitric oxide.

References

Alpha B, Ballardini R, Balzani V, Lehn J-M, Perathones S, Sabbatini N (1990) Antenna effect in luminescent lanthanide cryptates: a photophysical study. Photochem Photobiol 52:299–306

Beeby A, Faulkner S, Parker D, Williams JAG (2001) Sensitised luminescence from phenanthridine appended lanthanide complexes: analysis of

triplet mediated energy transfer processes in terbium, europium and neodymium complexes. J Chem Soc Perkin Trans 2:1268–1273
Beeby A, Bushby LM, Maffeo D, Williams JAG (2002) Intramolecular sensitisation of lanthanide(III) luminescence by acetophenone-containing ligands: the effect of para substituents and solvent. Dalton Trans 48–54
Blair S, Lowe MP, Mathieu CE, Parker D, Senanayake KP (2001) Optical pH sensors based on luminescent europium and terbium complexes immobilised in a sol-gel glass. Inorg Chem 40:5860–5867
Blair S, Kataky R, Parker D (2002) Sol gel immobilised terbium complexes for luminescence sensing of dissolved oxygen in aqueous solution by analysis of lanthanide emission decay. New J Chem 26:530–535
Bobba G, Frias J-C, Parker D (2002) Highly emissive, nine-coordinate enantiopure lanthanide complexes incorporating tetraazatriphenylenes as probes for DNA. Chem Commun Camb 8:890–891
Bretonniere Y, Cann MJ, Parker D, Slater R (2002) Ratiometric probes for hydrogen carbonate analysis in intracellular or extracellular environments using europium luminescence. Chem Commun Camb 17:1930–1931
Bruce JI, Dickins RS, Govenlock LJ, Gunnlaugsson T, Lopinski S, Lowe MP, Parker D, Peacock RD, Perry JJB, Aime S, Botta M (2000) The selectivity of reversible oxy-anion binding in aqueous solution at a europium and terbium centre; signalling of carbonate chelation by changes in the form and circular polarisation of luminescence emission. J Am Chem Soc 122:9674–9684
Bruce JI, Dickins RS, Parker D, Tozer DJ (2003) Correlation of optical and ^{1}H NMR spectral information with coordination variation for axially symmetric europium and ytterbium(III) complexes: importance of axial donor polarisability in determining affinity and ligand field. Dalton Trans, pp 1264–1271
Cann MJ, Chen Y, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J (2000) Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289:625–628
Caravan P, Ellison J, McMurry TJ, Lauffer RB (1999) Gadolinium (III) chelates as MRI contrast agents: structure, dynamics and applications. Chem Rev 99:2293–2345
Chipperfield AR, Harper AA (2000) Chloride in smooth muscle. Progr Biophys Mol Biol 74:175–221
Demas JN, DeGraff BA (2001) Applications of luminescent platinum group metal complexes to sensor technology and molecular probes. Coord Chem Rev 211:317–351
Demas JN, DeGraff BA, Coleman PB (1999) Oxygen sensors based on luminescence quenching. Anal Chem 71:793A–800A
Dickins RS, Aime S, Batsanov AS, Beeby A, Botta M, Bruce JI, Howard JAK, Love CS, Parker D, Peacock RD, Puschmann H (2002) Structural, luminescence and NMR studies of the reversible binding of acetate, lac-

tate, citrate and selected amino-acids to chiral di-aqua ytterbium and europium complexes. J Am Chem Soc 124:12697–12705
Frias JC, Bobba G, Cann MJ, Parker D, Hutchison CJ (2003) Luminescent nonacoordinate cationic lanthanide complexes as potential cellular imaging and reactive probes. Org Biomol Chem 1:905–907
Haugland RP (2002) Handbook of fluorescent probes and research chemicals, 9th edn. Molecular Probes, Eugene, Oregon
Imao Y, Okura I, Miyashita T (2000) Optical oxygen sensing based on quenching of europium(III) complex immobilised in fluoropolymer film. Bull Chem Soc Jpn 73:2663–2668
Jones SG, Lee DY, Wright JF, Jones CN, Tee ME, Gregory SY, Burns DD (2001) Improvements in the sensitivity of time-resolved fluorescence energy transfer. J Fluoresc 11:13–18
Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Kluwer Academic/Plenum, New York
Lopinski S, Bruce JI, Parker D, Peacock RD (2002) Survey of factors determining the circularly polarised luminescence of macrocyclic lanthanide complexes in solution. Chirality 14:562–567
Lowe MP, Parker D, Reany O, Aime S, Botta M, Castellano G, Gianolio E, Pagliarin R (2001) pH Dependent modulation of relaxivity in macrocyclic gadolinium complexes based on reversible intramolecular sulfonamide ligation. J Am Chem Soc 123:7601–7609
Mathis G (1995) Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. Clin Chem 41:1391–1397
Parker D, Williams JAG (1996) Getting excited about lanthanide complexation chemistry. J Chem Soc Dalton Trans, pp 3613–3628
Parker D, Williams JAG (2003) Responsive luminescent lanthanide complexes. In: Sigel H, Sigel A (eds) Metal ions in biological systems: the lanthanides and their interrelations with biosystems. Marcel Decker, New York, pp 233–280
Parker D, Senanayake PK, Williams JAG (1998) Luminescent sensors for pH, pO_2, halide and hydroxide ions using phenanthridine as a photosensitiser in macrocyclic europium and terbium complexes. J Chem Soc Perkin Trans 2:2129–2141
Parker D, Dickins RS, Puschmann H, Crossland C, Howard JAK (2002) Being excited by lanthanide coordination chemistry: aqua species, chirality, excited state chemistry and exchange dynamics. Chem Rev 102: 1977–2010
Sabbatini N, Guardigli M, Manet I, Ungaro R, Casnati R, Fischer C, Ziessel R, Ulrich G (1995) Synthesis and luminescence of Eu and Tb complexes with novel calyx[4]arene ligands carrying 2,2′-bipyridine subunits. New J Chem 19:137–140

Sammes PG, Dadabhoy A, Faulkner S (2000) Small singlet-triplet energy gap of acridone enables longer wavelength sensitisation of europium(III) luminescence. J Chem Soc Perkin Trans 2:2359–2360

Toner JL, Saha AK, Kross K, Kloszewski ED, Upson DA, Snow RA, Black CDV, Desai VC (1993) Time resolved fluorescence of a new europium chelate complex – demonstration of highly sensitive detection of protein and DNA samples. J Am Chem Soc 115:11032–11033

Werts MHV, Verhoeven JW, Hofstraat JW (2000a) Efficient visible light sensitisation of water soluble near infra-red luminescent lanthanide complexes. J Chem Soc Perkin Trans 2:433–439

Werts MHV, Wondenberg RH, Emmerink PG, van Gassel R, Hofstraat JW, Verhoeven JW (2000b) A near infra-red luminescent label based on Yb(III) ions and its application to a fluoroimmunoassay. Angew Chem Int Ed Engl 39:4542–4544