9

Lanthanide Ions as Luminescent Probes of Proteins and Nucleic Acids

Claudia Turro, Patty K.-L. Fu, and Patricia M. Bradley

Department of Chemistry, The Ohio State University,
Columbus, OH 43210, USA

1. INTRODUCTION
   1.1 General Description of Applications
2. PHOTOPHYSICAL PROPERTIES OF LANTHANIDES
   2.1. General Photophysical Properties
   2.2. Europium
   2.3. Terbium
   2.4. Other Lanthanides
   2.5. Complexes for Biological Applications
3. NUCLEIC ACIDS
   3.1. Nucleobases, Nucleotides, and DNA
   3.2. Probes of RNA and DNA Structure
   3.3. Binding of Proteins and Drugs to DNA and RNA
   3.4. Oligonucleotide Hybridization and Gene Analysis
4. PROTEINS AND ENZYMES
   4.1. Probes of Coordination Environment in Active Sites
   4.2. Distance Measurements with FRET
   4.3. Fluoroimmunoassays

5. CONCLUSIONS
ACKNOWLEDGMENTS
ABBREVIATIONS
REFERENCES

1. INTRODUCTION

1.1. General Description of Applications

The emission from lanthanide ions has been utilized extensively in biological systems. The applications range from luminescent labels of biologically-relevant molecules and the detection of cellular functions *in vivo* to the elucidation of structure and function of enzymes and proteins. Various techniques have been employed, including enhancement or quenching of the lanthanide emission, energy transfer, and delayed luminescence. The luminescence of aqueous solutions of Tb(III) and Eu(III) ions is weak owing to the low absorption cross section of the ions in the UV-visible region. In addition, there is significant nonradiative deactivation of the emissive states of various lanthanide ions through the O–H vibrations of coordinated water molecules. It has been shown that the addition of chelating agents or encapsulation of the lanthanide leads to longer emission lifetimes and higher quantum yields. Significantly greater emission intensities can be obtained upon chelation of the ion by ligands that, when excited with light, can transfer energy to the emissive state of the lanthanide. The photophysical properties of lanthanides typically used and recent applications to biological systems will be discussed in the sections to follow.

The emission from lanthanides has proven useful as a sensitive detection method in biological systems and has facilitated their understanding. The changes in the intensity of the Eu(III) luminescence upon binding to proteins and enzymes have been utilized to examine the ligation sphere within the active site, whereas distance and conformational information under physiological conditions has been obtained from energy transfer studies either between two lanthanide ions or from the protein's residues to Eu(III) or Tb(III) bound to the active site.

The sensitive detection of single-stranded regions of DNA, including mutations and mismatches, is critical in nucleic acid hybridization assays with applications that range from the determination of genetic and infectious diseases to providing accurate personal identification. Luminescence enhancement of a given probe in the presence of nucleic acids can in principle yield such detection, with marked safety and environmental advantages over radioactive labeling. Owing to the emissive properties of Tb(III) and Eu(III), including their luminescence enhancement through energy transfer, and their ability to bind single-stranded regions of DNA, these ions are potentially valuable for the selective detection of base mismatches.

Single-stranded oligonucleotides are known to enhance the emission of Eu(III) and Tb(III) ions in solution. This feature has been utilized in the detection of distorted DNA regions, and to probe the interactions of DNA and RNA with enzymes, proteins, and drugs. Lanthanide chelating agents tethered to oligonucleotides have proven important in luminescence energy transfer experiments, as well as in the sequence specific detection of DNA.

2. PHOTOPHYSICAL PROPERTIES OF LANTHANIDES

2.1. General Photophysical Properties

The valence 4*f* electrons of trivalent lanthanide ions, Ln(III), are well shielded from the environment by the outer core 5*s* and 5*p* electrons and are minimally involved in bonding. Because of this shielding, the atomic properties of these ions are typically retained after complexation. The total ligand-field (LF) splitting of an *f*-electron term is rarely more than a few hundred cm^{-1}, while for transition metal complexes, *d*-orbital splittings of 25,000 cm^{-1} are not uncommon [1]. As a result of the small LF splitting for the Ln(III) complexes, radiationless decay processes are relatively inefficient and emission from these complexes is common.

The absorption and emission spectra of Ln(III) ions consist of sharp, narrow bands corresponding to the *ff* transitions of the metal ion. Figure 1 shows the important energy levels for several lanthanide ions. The *ff* transitions are Laporte forbidden, resulting in low molar absorption coefficients in the near-UV and visible ranges (<10 M^{-1} cm^{-1}) and long,

millisecond, luminescence lifetimes [2]. The number of bands depends on the particular lanthanide ion and its arrangement of electronic states. Upon complexation with ligands, only minimal changes are observed in the electronic absorption and emission spectra, such as small displacements in the peak positions, usually toward longer wavelengths, and changes in the relative intensities of some of the emission peaks [3].

Direct excitation with UV radiation into the Ln(III) *ff* transitions leads to weak emission, whereas the sensitized emission in chelate complexes can result in an increase in the lanthanide-based emission by several orders of magnitude. In this case the ligand excitation is followed by intramolecular energy transfer from the ligand triplet state to the lower lying emissive lanthanide excited state.

Emission from Ln(III) ions can often be quenched by high frequency vibrations of solvents or ligands such as those of OH, CH, or NH bonds [4]. There is also the possibility of thermal quenching by the ligand triplet state in chelate complexes. Both of these types of quenching will be discussed below as they apply to a particular ion.

2.2. Europium

The photophysical properties of europium(III) ions are perhaps the most studied of all of the lanthanide ions. Since both of the levels involved in the luminescent transition are nondegenerate, only one emission line is observed at ~580 nm when a single chemical species is present [1,5,6]. As shown in Figure 1, the lowest energy emissive state, $^5D_0$, is 17,250 cm^{-1} above the $^7F_0$ ground state.

In coordinating solvents the emission of the Eu(III) ion is quenched by OH, NH and CH groups at or near the coordination site, and it has been shown that this quenching can be reduced by deuterium substitution [7-9]. The number of coordinated OH groups from water or methanol can be estimated from changes in the lifetime by the Horrocks equation, although this relation is not very accurate when the number of OH groups in the first coordination sphere is small [10]. Encapsulation of the ion by various chelators can increase the emission quantum yield of a complex compared to the free ion. For example, the complex $Eu(TTA)_3{\cdot}2DMSO$ (TTA = thenoyltrifluoroacetonate) in DMSO showed Eu(III) based emission with

a lifetime of 0.72 ms, whereas the hydrated complex exhibits only weak emission from the ligand triplet state [11].

An increase in the lifetime from 0.26 ms in $EuCl_3$ to 0.68 ms was reported for CH_3OH solutions of the europium(III) complex with the terphenyl-based ligand shown in Figure 2a [12]. In addition, the use of methanol-d_1 (CH_3OD) as the solvent led to a further increase in the lifetime of the complex to 0.97 ms. In each case, an increase in the emission lifetime was accompanied by an increase in the quantum yield (ϕ).

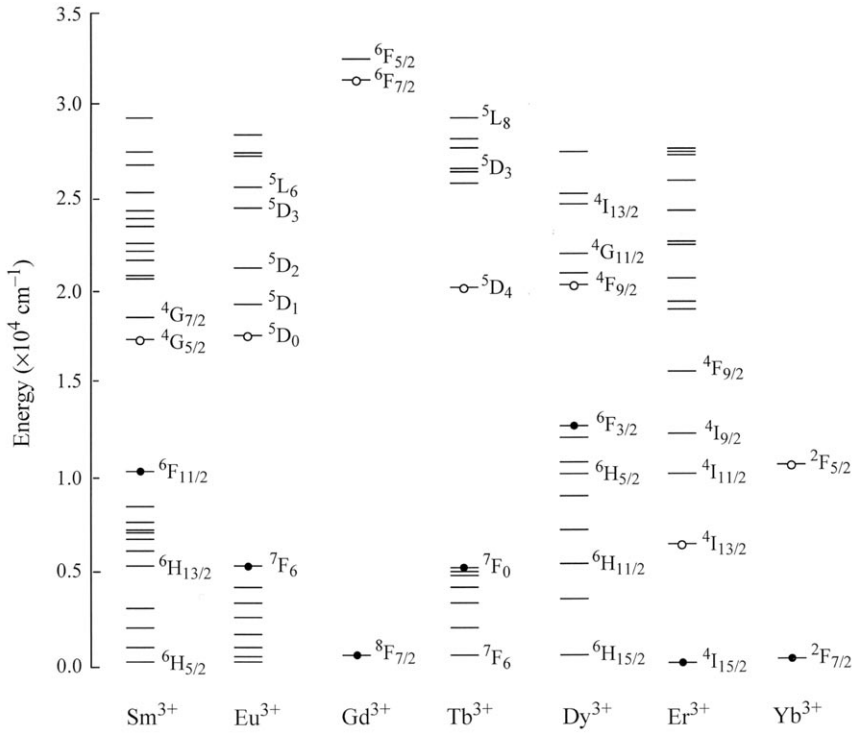

FIG. 1. Energy levels of various Ln(III) ions (closed circles = HOMO, open circles = LUMO).

Energy transfer (EnT) from $^3\pi\pi^*$ states of aromatic ligands to the Eu(III) emissive $^5D_0$ state can also result in enhanced lanthanide emission. This type of sensitization is very common for Eu(III) complexes

because many ligand triplet states lie just above the $^5D_0$ emissive state and it has been reported for many different types of ligands including, diketonates [11,13], tetraphenyl imidodiphosphinate [14], Schiff base ligands [15], multidentate pyridine [16], bipyridine [17], terpyridine [18], polyaminopolycarboylates [19–20,21,22], cryptands [23,24], calixarenes [25,26], cyclodextrins [27], and crown-ether derivatives [28].

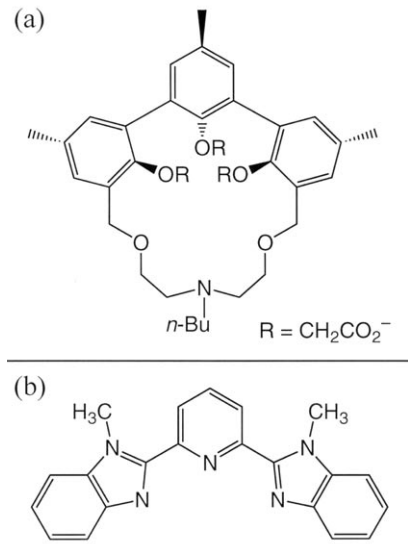

FIG. 2. Structures of typical ligands utilized in luminescent Ln(III) complexes (see text).

When the $^3\pi\pi^*$ state of the ligand lies just above the $^5D_1$ state of the Eu(III) ion (see Figure 1), as in the case of tris(dibenzoylmethide), luminescence from both the $^5D_1$ and $^5D_0$ states is observed upon excitation of the ligand [29]. Emission from the $^5D_1$ level exhibits a rapid initial rise and a lifetime, τ, of ~2 μs. Concurrently, the $^5D_0$ emission exhibits an initial fast rise followed by a slow rise of ~2 μs, then a characteristic decay of ~ 0.5 ms. These observations have been attributed to energy transfer from the $^3\pi\pi^*$ of the ligand to the $^5D_1$ state of the Eu(III) ion, followed by

relaxation to the $^5D_0$ state. Quantum yields for this type of sensitization tend to be lower than when the energy transfer is directly from the ligand to the $^5D_0$ state because of nonradiative deactivation from the $^5D_1$ state.

Thermal quenching of the Eu(III) based emission is possible if the energy of the triplet state of the ligand is close enough to the emissive $^5D_0$ state for there to be significant back energy transfer. This phenomenon was reported for Eu(III) complexes of the tridentate aromatic ligand shown in Figure 2b [30]. A dramatic decrease in the emission quantum yield was observed upon going from $Eu(NO_3)_3L(MeOH)$ (2.8%) to $[Eu(L)_3](ClO_4)_3$ (8.2×10^{-5}%).

2.3. Terbium

Tb(III) emission has also been utilized extensively in biological systems. The luminescence from the Tb(III) ions is quenched by OH groups of coordinated water molecules to a much lesser extent than Eu(III) owing to the larger energy gap between the ground state and the emissive state for Tb(III) (20,500 cm^{-1}) compared to that in Eu(III) (17,250 cm^{-1}). The nonradiative deactivation through vibrational levels of coordinated water molecules becomes less efficient as the gap between the emissive state and the ground state increases [31,32].

Sensitized luminescence is common in Tb(III) complexes resulting from energy transfer from the lowest-lying ligand triplet excited state to the emissive $^5D_4$ state. It was shown that for strong chelators the energy of the donor triplet state, which must lie above the $^5D_4$ excited state of Tb(III), is the most important parameter for efficient energy transfer to take place [27]. Sensitized luminescence has been observed for ligands such as phenyl phosphonic and phenyl phosphinic acids [33], imidophosphinate [13], calixarenes [34], terpyridines [35], and terphenyl based ligands [36]. When anionic transition metal complexes such as $[Au(CN)_2]^-$ and $[Ag(CN)_2]^-$ are used as counter ions for Tb(III) in solution, sensitized emission is also observed [37]. In general, the sensitized luminescence from Tb(III) is temperature dependent and results in activation energies consistent with the predicted energy separation between the $^5D_4$ state of the Tb(III) ion and the triplet state of the ligand or transition metal complex.

2.4. Other Lanthanides

Pr(III), Ho(III), Er(III), Tm(III), and Yb(III) ions are only weakly emissive because the energy gap between the ground state and emissive states is small in each case, thus making non-radiative decay an important factor in the luminescence intensities of these ions. The f^0 and f^{14} electron configurations of La(III) and Lu(III), respectively, result in no ff transitions for these ions. Complexes of Gd(III) generally exhibit only ligand centered emission, since the lowest-lying excited state of the ion is very high in energy at ~32,000 cm^{-1} (Figure 1) [1,38,39]. As a result, these complexes are often used to determine the energy of ligand triplet states.

Yb(III), Nd(III), and Er(III) display near-infared emission centered at 880 nm, 1060 nm, and 1330 nm, respectively, with lifetimes in the millisecond range [40]. The smaller energy gap between the ground state and emissive state of these ions compared to Eu(III) and Tb(III) (see Figure 1) results in more efficient nonradiative decay through solvent or ligand vibrations [1]. However, sensitized emission is still observed in complexes of these ions [40].

The energy gaps between the emissive excited states and the ground states for Sm(III) and Dy(III) are 7400 cm^{-1} and 7850 cm^{-1}, respectively, also significantly smaller than those of Eu(III) and Tb(III) (see Figure 1) [1]. These ions, therefore, commonly exhibit emission in the near-infrared region both from direct excitation into the emissive states and from intramolecular energy transfer from ligand triplet states [41-43].

2.5. Complexes for Biological Applications

As discussed above, complexes of Tb(III) and Eu(III) can in some cases be highly luminescent and typically exhibit emission lifetimes in the millisecond range. These long lifetimes provide a means to detect the biomolecules labeled with lanthanide ions with high sensitivity, since the measurement of delayed luminescence from these systems permits a zero-background measurement, thus eliminating the short-lived background emission from biosystems. Recently, lanthanide ion complexed to a variety of ligands have been developed, which permits the tuning of the

emission wavelenghts from 520 nm to 680 nm and lifetimes from 50 to 500 μs [44]. Complexes of Tb(III) and Eu(III) that appear promising for biological applications are abundant. Some prominent recent examples will be discussed here.

Dipicolinic acid (DPA) and various derivatives have been shown to greatly enhance the Tb(III) emission upon complexation owing to favorable energy transfer from the ligand's $^3\pi\pi^*$ excited state to the $^5D_4$ excited state of the ion. In the 4-X-DPA ligands shown in Figure 3a, variation in the substituent, X, resulted in the following order of the emission intensity: NH_2 > OH > NHAc > H > Cl > Br [45]. For 4-NH_2-DPA the quantum yield of sensitized Tb(III) emission was 13.6 and the lifetime 1.58 ms. The DPA ligand with the –$NHCOCH_2$– linker in the 4-position has been utilized as a covalent label for goat immunoglobulin and may prove useful as a highly sensitive luminescent probe in immunoassays. The labeled protein exhibited strong emission upon treatment with Tb(III), and the strong binding of the lanthanide is evident by its ability to remain bound to the DPA ligand upon soaking in EDTA.

Calixaranes, such as those shown in Figure 3b, have also been shown to bind lanthanide ions upon deprotonation of three hydroxyl groups and to result in the formation of dimeric structures of the type $[Ln(H_2L)_2(DMSO)_x]$, where H_5L_5 = calixa-5-rane and Ln = Gd^{3+}, Tb^{3+} with x = 4 or Eu^{3+} with x = 3 [46]. Although the Eu(III) calixarane complexes are non-emissive due to a low-lying ligand-to-metal (LMCT) state, the Tb(III) complex is emissive upon ligand excitation. However, the enhancement is modest due to back energy transfer deactivation to the $^3\pi\pi^*$ state of the ligand. In order to achieve greater Tb(III) and Eu(III) luminescence enhancement with calixaranes, the ligand was functionalized with crown ethers and related moieties that are able to tightly bind lanthanide ions [47]. The structures of crown-modified calixa-4-anes are shown in Figure 3c (**3** and **4**), along with the control compounds (**1** and **2**). The greatest luminescence enhancement was measured for the Tb(III) complex of **3**, which was ~15 times greater than that observed for **1**, **2** and **4**. It is believed that **3** binds Tb(III) more strongly than the other ligands. In contrast, Eu(III) complexes of these ligands are only weakly emissive owing to low-lying LMCT states.

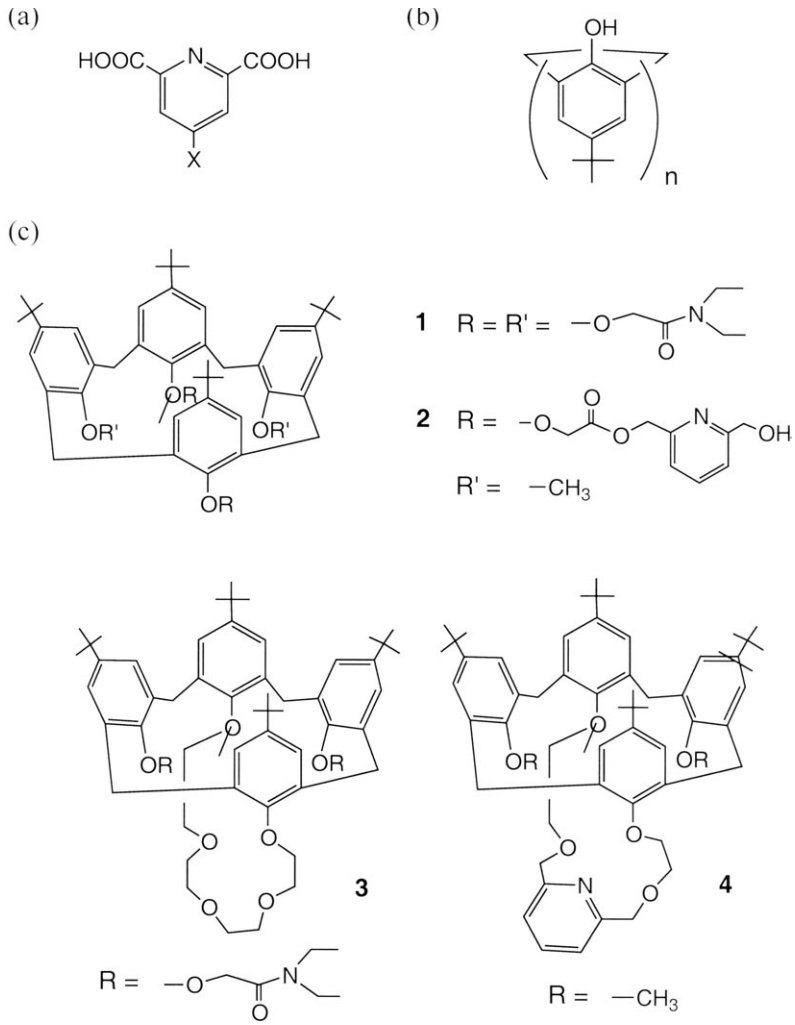

FIG. 3. Structures of (a) 4-X-DPA, (b) p-tert-butylcalix[n]arane, and (c) ligands based on p-tert-butylcalix[4]arane utilized in emission enhancement studies (see text).

3. NUCLEIC ACIDS

3.1. Nucleobases, Nucleotides, and DNA

The enhancement of Tb(III) emissision typically occurs through energy transfer from a coordinated ligand excited state to the emissive $^5D_4$ state of the ion. Although energy transfer is also observed for Eu(III), the luminescence enhancement mechanism typically exploited in aqueous environment arises from the decrease in the number of coordinated H_2O molecules upon ligand binding. Owing to this intrinsic difference in the mechanisms of emission enhancement that are typically employed for each ion, the Tb(III) and Eu(III) will usually be discussed separately in the sections to follow.

The enhancement of Tb(III) luminescence by nucleic acids through energy transfer requires the efficient production of the long-lived $^3\pi\pi^*$ state of the base upon its absorption of light, that the $^3\pi\pi^*$ lies above the $^5D_4$ state of Tb(III), and strong binding of the ligand to the ion. The $^5D_4$ state of Tb(III) is located 20,500 cm^{-1} above the ground state, and the $^3\pi\pi^*$ states of all nucleobases in the 26,300 to 27,900 cm^{-1} range. It has been shown that for ligands with similar $^3\pi\pi^*$ energies as those of the nucleobases, strong emission enhancement of Tb(III) through energy transfer was observed. It is expected that intersystem crossing from the initially produced $^1\pi\pi^*$ of the base to the $^3\pi\pi^*$ should be efficient for systems in which Tb(III) is strongly bound to the base owing to spin-orbit coupling induced by the lanthanide ion (heavy atom effect). Therefore, when discussing systems composed of DNA bases, the greatest consideration should be the strength of the binding of Tb(III) to the ligand, as well as the distance from the bound ion to the energy transfer donor.

The luminescence enhancement of Eu(III) from the decrease in coordinated H_2O molecules upon complexation is modest compared to sensitized Tb(III) emission. In the Eu(III) experiments the excitation wavelengths are consistent with transitions of the ion in the visible region. Comparisons of the emission lifetime of the Eu(III) ion in H_2O, D_2O, and in the presence of a given ligand, such as nucleotides, can be utilized to calculate the number of coordinated water molecules assuming a coordination number of 8 or 9 around the ion. In turn, this information

can be used to calculate the number of atoms from the ligand bound to the each Eu(III) ion in solution. In addition, species with different lifetimes can be assigned to Eu(III) ions in different coordination environments (or binding modes), which differ from one another in the number of coordinated H_2O molecules.

The enhancement of Tb(III) luminescence through energy transfer was measured for the four DNA nucleobases whose structures and numbering scheme are shown in Figure 4 [48]. The greatest Tb(III) emission enhancement monitored at λ_{em} = 545 nm was observed upon addition of cytosine (C) with λ_{exc} = 260 nm. An overall decrease in the Tb(III) luminescence was observed upon addition of guanine (G), adenine (A), and thymine (T) due to an inner-filter effect resulting from absorption of 260 nm photons by the bases with small or no subsequent energy transfer to the lanthanide. However, the decrease in emission observed for G was not as pronounced as those measured for A and T, indicative of some enhancement of Tb(III) luminescence by G. With 25 μM Tb(III) and 50 μM bases, the emission intensities were enhanced by factors of 12 and 2 in the presence of C and G, respectively, relative to A and T.

These results indicate that in the absence of the phosphate group available for electrostatic binding to the lanthanide ion, C is able to coordinate to Tb(III) such that energy transfer can take place upon excitation of the base. To a lesser extent, this is also the case for G, but not for A or T. From these observations it may be postulated that nearby O and N atoms available for coordination in each nucleobase are necessary for binding to Tb(III). As shown in Figure 4, such sites are only found in C (O2 and N3) and in G (O6 and N7).

Although cytosine alone enhances the Tb(III) emission, the deoxynucleotide dCMP does not, indicating that the lanthanide bound to the phosphate group is too far away from the base for efficient energy transfer. Conversely, the enhancement of the Tb(III) luminescence by dGMP is greater than that of G, where the phosphate appears to aid in the binding of the ion to the base. The enhancement of Tb(III) luminescence by GMP and dGMP has long been known [49]. Enhacement of Tb(III) luminescence with λ_{exc} = 260 nm was not observed for dAMP or dTMP.

A possible explanation for the markedly different Tb(III) luminescence enhancement in G and dGMP is that the latter is able to fold over and

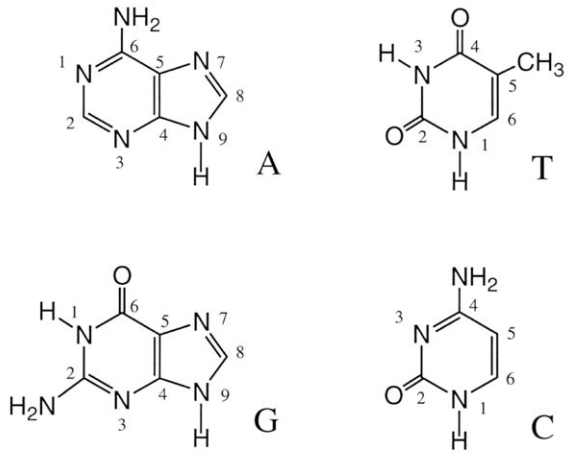

FIG. 4. Molecular structures and numbering schemes of the nucleobases. adenine (A), thymine (T), gnanine (G), and cytosine (C).

permit coordination of the ion to the O6 and/or N7 atoms of the base while still bound to the anionic phosphate oxygens, thus increasing the binding affinity and promoting efficient energy transfer. Evidence for such foldover of nucleotides upon binding La(III) and Tb(III) was observed with ^{1}H NMR and FTIR spectroscopies, showing interaction of the ions with both the phosphate group and N7 from the base [50]. In addition, it has been shown that the luminescence enhancement of the ion is greater for GMP than GTP, indicating that proximity of Tb(III) to the base is important [51]. Evidence for interaction of Tb(III) to N7 of guanine was also found in guanine tetrads [52].

The displacement of H_2O molecules from the coordination sphere of Eu(III) also results in the enhancement of emission of the ion in the presence of nucleotides. The emission lifetime in solutions containing dCMP, dAMP, and dTMP exhibit biexponential decays, assigned to two different binding modes of the Eu(III) ion to the nucleotides [53]. The short lifetime is likely free Eu(III) with ~9 bound H_2O molecules, whereas the number of waters coordinated to the ion in the presence of dAMP, dCMP, and dTMP are 3, 2, and 6, respectively, and is consistent with a strong binding mode. However, the stoichiometry of these complexes is

unclear. A more careful study revealed that the strong binding of dGMP to Eu(III) ion in the 1:1 complex corresponded to 3 or 4 atoms from the nucleotide bound to each ion, indicative of simultaneous coordination from the phosphate and the base [50].

3.2. Probes of RNA and DNA Structure

Single-stranded oligonucleotides greatly enhance the Tb(III) emission, but duplexes do not [48]. Single mismatches in the sequence of a duplex lead to selective luminescence enhancement in the presence of Tb(III). The largest enhancement was observed for the GG mismatch, followed by CA, GA, and CC, and the smallest emission intensity was measured for TT and TG mismatches. It was concluded that A is able to transfer energy to Tb(III) when bound to the ion, but in the absence of the supramolecular assembly it cannot coordinate strongly enough to the lanthanide to effect energy transfer. The low emission enhancement by the TG mismatch has been explained in terms of wobble pair formation. These findings show that the enhanced emission of lanthanides can be successfully utilized to selectively detect single mismatches in duplex DNA.

The ability of single-stranded DNA to enhance Tb(III) emission has been utilized in the detection of single-stranded regions of duplex DNA and to study DNA conformation. Luminescence enhancement was observed upon denaturation of ds-DNA, but not following single- or double-strand breaks [54]. It was also shown that the emission enhancement observed for Tb(III) by single-stranded regions of DNA requires coordination of the ion to the bases in addition to binding to the phosphate backbone, since it competes for Cu(II) binding sites [55]. In addition to denatured duplex, an increase in Tb(III) emission intensity was observed in the presence of poly(G) and poly(C), and excitation spectra showed that energy transfer was operative [49b]. In contrast, no enhancement was observed for Dy(III), Lu(III), Eu(III), or Sm(III) under similar conditions. The changes in the emission lifetime of Eu(III) were utilized to monitor the conformational change from B- to Z-DNA, showing that on average there are fewer H_2O molecules coordinated to the ion when bound to Z-DNA than to B-DNA [56].

3.3. Binding of Proteins and Drugs to DNA and RNA

The luminescence of lanthanide ions and various experiments utilizing their energy transfer properties have provided information on the binding of proteins, drugs, and transition metal ions and complexes to DNA and RNA, as well as resulting distortions. For example, the emission of Eu(III) ions was recently utilized to determine the metal-binding domains of RNA hairpin loops, indicating it binds at the same nonpolar site as Mg(II) ions with ~7 water molecules being displaced [57].

It was shown that lanthanide ions could be utilized in long-range EnT for distance measurements in labeled oligonucleotides, where an Eu(III) chelate donor and an organic acceptor, such as Cy5, result in a 50-100 fold improvement in signal-to-background [58,59]. Furthermore, the enhancement of EnT signal has been utilized for the rapid detection of sequence-specific DNA binding proteins by bringing together two labeled DNA fragments that make up approximately half of the DNA sequence corresponding to the protein's binding site, thus producing EnT signal [60]. In other work, the distortion of DNA was determined in solution under standard transcription conditions upon binding of catabolite activator protein (CAP) to form the CAP-DNA complex utilizing EnT between a donor in the protein and a complementary probe placed at various locations on the DNA, leading to results similar to those measured in the crystal structure [61]. It has also been reported that binding of HMG1 to a 30-bp duplex DNA sequence results in ~150° bend angle, measured utilizing EnT labels covalently tethered to the ends of the oligonucleotide [62]. Similarly, EnT measurements with Eu-DTPA-AMCA (DTPA = di ethylenetriaminepentaacetic acid, AMCA = 7-amino-4-methylcoumarin) and Cy5 donor-acceptor couple were utilized to elucidate the bend angle in B-DNA were determined upon the binding of HMG1 proteins from evolutionary distant organisms, showing that they distort DNA in a similar manner [63]. The protein-bound Eu-DTPA-AMCA system was recently utilized in EnT experiments designed to elucidate the structure of the polymerase-bound fork junction DNA, where various positions of the DNA were labeled with Cy5. The results show little perturbation of the duplex part of the fork, however, a sharp kink of the DNA was observed in the ds/ss boundary upstream of the transcription bubble [64].

3.4. Oligonucleotide Hybridization and Gene Analysis

The luminescence from lanthanide ions or energy transfer signal from or to lanthanide complexes has been utilized extensively in hybridization assays to detect certain DNA or RNA sequences [65]. For example, the delayed luminescence from an Eu(III) chelate labeled ss-oligonucleotide was detected when it bound to the corresponding complementary sequence of DNA attached to a microparticle. After washing, luminescence is only observed from particles to which the correct sequence was attached, and those with a single mismatch resulted in 2 orders of magnitude lower emission from the Eu(III) chelate [66]. In addition, methods utilizing particle-bound oligonucleotide sequences tagged with combinations of various organic dyes have been utilized to simultaneously detect six oligonucleotides labeled with an Eu(III) chelate, showing that it is possible to screen mutations and deletions related to cystic fibrosis in the same sample [67].

DNA hybridization techniques that require immobilization of the target DNA on a solid support can be time consuming, therefore the use of EnT techniques that permit the utilization of homogeneous solutions to greatly simplify the measurement are desirable. One approach, shown schematically in Figure 5a, is to attach a Tb(III)-DTPA energy transfer donor complex to approximately half of the complementary strand of the target DNA sequence and salicylate energy transfer donor to the other, such that when both complementary strands bind to the template the donor and acceptor are in close proximity resulting in strong energy transfer [68]. Similarly, direct coordination form a β-diketone tethered to half of the complementary strand to an Eu(III)-EDTA complex linked to the other half results in enhanced Eu(III) emission owing to energy transfer from the diketone upon hybridization with the trarget sequence, resulting in a detection limit of 6 pM (0.6 fmol per assay) [69]. Tb(III) chelates whose emission is sensitive to the environment have been employed for high throughput screening of genetic markers combining the enhanced luminescence from the Tb(III) complex upon cleavage during PCR [70].

Related homogeneous assays utilizing a biotin label with its high affinity binding partner, streptavidin (SA), in addition to the lanthanide chelate and organic dyes that effect EnT have been reported, thus resulting

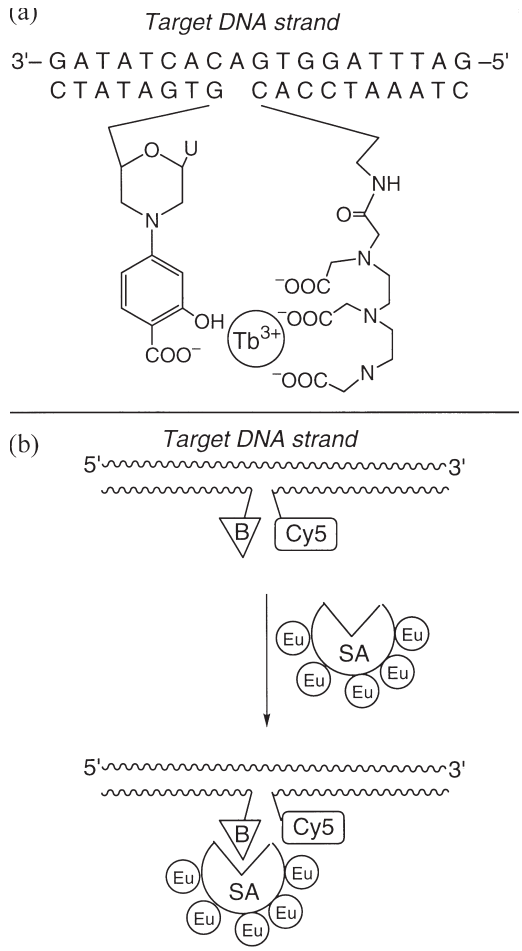

FIG. 5. Examples of two schemes utilized in hybridization assays, showing (a) the labeling of the two halves of the complementary strand for energy transfer and (b) the additional use of biotin (B) and stepavidin (SA) for stronger binding.

the detection of small concentrations of a given target oligonucleotide sequence. In the assay shown in Figure 5b, half of the complementary strand is labeled with biotin and the other with Cy5, such that upon hybridization with the target sequence the biotin and dye would be brought into close proximity [71]. In the presence of SA labeled with

an Eu(III) chelate, efficient EnT takes place, thus resulting in increased Cy5 emission which can be monitored by steady-state or time-resolved techniques. A similar method was recently reported utilizing a Tb(III) chelate and Cy3 dye, resulting in a detection limit of 30 pM of taget DNA [72]. In addition, a related method utilizing the biotin-SA couple along with various chelates of distinct combinations of Eu(III), Tb(III), and Sm(III) tethered to oligonucleotides was utilized to detect seven target sequences simultaneously [73]. In a related method, UTP covalently labeled with an Eu(III) complex can be incorporated directly into RNA, along with biotin-labeled CTP, thus providing a means to selectively bind the RNA to an SA conjugate labeled with an appropriate organic dye for EnT [74].

4. PROTEINS AND ENZYMES

4.1. Probes of Coordination Environment in Active Sites

Lanthanides are known to form complexes with many functional groups found in biological molecules, especially with donor groups that posses a lone pair of electrons, such as oxygen atoms in the carboxylic groups of amino acids. Lanthanides can bind at the active sites of biomolecules, replacing various ions that include Ca(II), Zn(II), Mg(II), Mn(II), Fe(II) and Fe(III). The ability of lanthanide ions to luminesce at room temperature and their specific spectroscopic characteristics made them a widely used probe for the study of the biochemical processes that take place in metal binding proteins and metalloenzymes.

Information regarding the composition and structure of the metal binding site can be obtained from the emission and excitation spectra of Tb(III) and Eu(III)-protein complexes. Furthermore, binding constants of metal ions such as Ca(II) and Zn(II) to proteins can also be calculated using Eu(III)-metal competition titration method [75]. The binding constant of Eu(III) is first determined using the titration curve of the intensity of the $^7F_0 - {}^5D_0$ (λ_{exc} = 579 nm) emission as a function of total equivalents of Eu(III) added to the protein. The intensity of the Eu(III) luminescence decreases as a known quantity of the competing metal ion is added, which is proportional to the amount of replaced Eu(III), making

the relative binding constant measurement possible [75,76].

The hydration number (the number of water molecules) in the inner coordination sphere of the metal can also be determined by measuring the differences of emission lifetime of Tb(III) and Eu(III) in H_2O and D_2O [76,77]. As discussed earlier, the excited state lifetime of Eu(III) is very sensitive to the number of water molecules coordinated to the ion [78], thus replacing H_2O by D_2O can cause lifetimes to increase dramatically (by a factor of ~9) [76]. For example, two water molecules were found at the Ca^{2+} binding site in the Satellite tobacco necrosis virus [77], and four waters were found to coordinate at the catalytic Mg^{2+} site in the ATP dependent enzyme glutamine synthase [79]. Knowledge of the number of water molecules leads to the direct estimation of the total number of coordinating atoms supplied by the protein, since Ln(III) ions usually possess total coordination numbers of 7 to 9.

Laser-excited Ln(III) ion has been widely used to identify the metal binding site in a variety of proteins and metalloenzymes [75–78,80]. Analysis of the $^5D_0$–$^7F_0$ band in the excitation spectrum and luminescence titration of Eu(III) in a complex with bovin α-lactalbumin, at least two different calcium binding sites and three kinds of ligands have been determined [78,81]. Studies were also performed of the emission intensity and lifetime of the excited state of Tb(III) in bovine prothrombin protein, which revealed the functional structural fragment containing γ-carboxyglutamic acid [82]. Furthermore, the measurement of the decay kinetics of the enhanced luminescence by Tb(III) in metalloenzymes, such as alkaline phosphatase, has provided information on the amino acid residues present at the catalytic site [80,83].

4.2. Distance Measurements with FRET

Fluorescence resonance energy transfer (FRET) has been widely used for distance measurements in biological molecules and in their complexes in solution [84–85,86,87]. The advantage of FRET is its ability to determine both inter- and intramolecular distances in solution under many different conditions. Its major limitation is that the largest measurable distances are ~90 Å. Energy transfer distance measurements using luminescent Eu(III) and Tb(III) ions have been reported for some time, where the lanthanide

ion is usually introduced in specific protein or enzyme sites via chemically modified reactive residues. Several amino- and thio-reactive fluorescent chelates derived from AMCA and DTPA have been designed primarily for FRET measurements [88]. The lanthanide chelates can then be used either as energy donors or acceptors in these distance measurements. These energy transfer processes usually take place from aromatic amino acids (donor) to Tb(III) (acceptor) or from Tb(III) donor to Fe(III) (acceptor) in the metal binding proteins [81].

The Ln(III) ions have been used extensively to study calcium-binding proteins since Ca(II) and Ln(III) are very similar in size, with ionic radii of 0.100-0.118 nm for Ca(II) and 0.086-0.122 nm for Ln(III) [76,77,80,89]. Their bonding and coordination geometry in biological environments of both ions are also alike, with coordination numbers of 6 to 9, and both have similar rate constants of ligand exchange ($\sim 10^8$ s^{-1}) [76]. Furthermore, both ions show the same preference of certain donor atoms, oxygen > nitrogen > sulfur. These similarities make the trivalent lanthanide ions isomorphous replacement probes for Ca(II) in a variety of calcium binding proteins [81,82,90–91,92,93,94].

Protein-bound Tb(III) ions exhibit a significant luminescence enhancement upon irradiation with 280 nm excitation, a wavelength where the aromatic amino acids such as phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) absorb light. Nonradioactive energy transfer between an intrinsic fluorescent amino acid (energy-donor) and protein-bound Tb(III) or Eu(III) ions (energy-acceptor) has been well investigated to measure the distance between these intrinsic fluorophores and bound metal ions [88].

Calmodulin (CaM) is a small (Mr = 16,790), heat-stable, acidic (pI = 3.9-4.3) protein found in both prokaryotes and eukaryotes [92]. One of its functions is to mediate the rapid, transient increases in intracellular calcium concentration and thereby modulate the action of various enzymes. There are four calcium binding sites. The binding of calcium to CaM is a sequential process, with binding occurring initially at two high-affinity sites (domains III and IV), followed by binding at two low-affinity sites (domains I and II). An examination of the amino acid sequences of CaM from numerous sources reveals high homology and strongly suggests a common ancestor. Domains I and II are typically conserved,

such that the variability in the primary sequence occurs in domain III and IV [94]. The effects of minor differences in the amino acid sequences between vertebrates and invertebrates on metal binding were extensively investigated using laser-induced Eu(III) and Tb(III) luminescence [75,78,84,85,95–96,97,98,99].

4.3. Fluoroimmunoassays

Time-resolved fluoroimmunoassay is a technique based on the long-lived lanthanide fluorescence [77,79,100–101,102,103]. Detection of the delayed emission from the lanthanide ions increases the signal-to-noise ratio of the probe, thus improving the detection limit (usually on the order of μg to pg/mL) of the probe [99]. The chelates of Eu(III), Tb(III), Dy(III), and Sm(III) have been successfully used as labels of antigens and antibodies for the immunological analysis. The method has widespread applications in the field of immunoassays in both clinical and diagnostic studies, such as the determination of hormones and viral antigens in humans, herbicides in water, and steroids in animals [104–105,106]. The technique is based on the binding of an antigen (analyte) with an appropriate antibody, thus forming an immunological complex. Usually the immunoassay is carried out on a solid support, such as a plastic surface, microtitration strip wells, microbeads, or electrophoretic gel, to which antibody is covalently linked.

In order to measure the amount of analyte in the solid phase, the emissive label is attached to one of the reactants of the immunological reaction. Fluoroimmunoassays are safer, more sensitive, and relatively fast and simple compared to radioimmunoassays [81,89]. The large Stokes shifts and long excited state lifetimes are some of the advantages of using lanthanides instead of fluorescent organic compounds as labels [98]. Quantification of the laser induced luminescence of an appropriate lanthanide complex is usually performed using the time-resolved method with a decay of the order of 200-400 μs [81,103–105]. These characteristics make it possible to avoid background interference from other sample constituents, such as emissive proteins and light-scattering particles, thus ensuring that only the lanthanide emission is measured.

A lanthanide chelate suitable for fluoroimmunoassays should be

water soluble and should also contain a high absorbance ligand (to ensure a good excitation spectrum), as well as a high yield of energy transfer from the ligand to the lanthanide ion. Furthermore, it should contain functional groups capable of covalent binding with the immunological active compounds such as antigens or antibodies. There are two different commercially available fluoroimmunoassays, DELFIA [77,79] and FIAgen [100,101].

Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) contains two different lanthanide complexes and the assay is carried out in two steps. In the first step, the lanthanide ions form a complex with the isothiocyanate derivative of EDTA. The thiocyanate group of this complex then covalently binds with the free amino group of the protein that is coated on the solid phase. After the immunological reaction, the antigen-antibody conjugate labeled with lanthanide is then treated with an enhancement solution, which results in the dissociation of the lanthanide ions from the primary complex, forming a chelate with 2-naphthoyltrif luoroacetone present in the enhancement solution. The latter complex exhibits very intense luminescence.

The FIAgen fluoroimmunoassay labels the antigen (analyte) in solution instead of the antibody in the solid phase. It utilizes Tb(III) or Eu(III) chelates with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) as the emissive label, since the two sulphonyl chloride groups in BCPDA can react with the free amino groups of the analyte proteins (antigen). This label is more effective when the analyte is also covalently tethered to streptavidin and is used against the solid phase (antibody) tagged with biotin for more sensitive and stronger binding [101,103,104]. The presence of an immunological complex was detected for fetoprotein and thyroxin hormone in human serum. In addition, the emission from alkaline phosphatase bound to streptavidin-europium or terbium chelate labels was detected upon the formation of the corresponding antigen-antibody immunological complex [100,101]. Measurement of the resulting luminescence can be utilized to accurately determine the amount of analyte bound to the appropriate antibody [81,100,101].

Other lanthanide complexes have also been developed for immunoassays over the years. Terbium complexed with transferrin

has been used to detect the antibiotic gentamycin and europium-4-iodoacetamidodipicolinic acid was used to label immunoglobulin G [107]. Multidentate chelating agents such as bipyridine and terpyridine derivatives have also been explored for further development of fluoroimmunological assays [81,102].

5. CONCLUSIONS

The physical and chemical properties of the lanthanide ions and its chelates have provided a means to investigate various biological systems utilizing their luminescence. Their coordination characteristics and long lifetimes provide a means to detect small quantities of sample accurately. In addition, the ease in the techniques make lanthanide luminescence a valuable tool in biological assays. Although much research has been conducted in this area to date, new techniques are currently being developed to further understand new biological systems or to selectively detect biomolecules.

ACKNOWLEDGMENTS

The authors thank for the partial support from The National Science Foundation (CHE-9733000), National Institutes of Health (RO1 GM64040-01), and The Arnold and Mabel Beckman Foundation Young Investigator Award.

ABBREVIATIONS

A	adenine
AMCA	7-amino-4-methylcoumarin
ATP	adenosine 5’-triphosphate
BCPDA	4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid

C	cytosine
CaM	calmodulin
CAP	catabolite activator protein
CTP	cytidine 5’-monophosphate
Cy3	cyanine dye 3
Cy5	cyanine dye 5
dAMP	2’-deoxyadenosine 5’-monophosphate
dCMP	2’-deoxycytidine 5’-monophosphate
DELFIA	dissociation-enhanced lanthanide fluoroimmunoassay
dGMP	2’-deoxyguanosine 5’-monophosphate
DMSO	dimethylsulfoxide
DPA	dipicolinic acid
dTMP	thymidine 5’-monophosphate
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediamine-N,N,N’,N’-tetraacetate
EnT	energy transfer
FRET	fluorescence resonance energy transfer
FTIR	Fourier transform infrared
G	guanine
GMP	guanosine 5’-monophosphate
GTP	guanosine 5’-triphosphate
HMG1	high mobility globulin 1
HOMO	highest occupied molecular orbital
LF	ligand field
LUMO	lowest unoccupied molecular orbital
LMCT	ligand-to-metal charge transfer
Phe	phenylalanine
PCR	polymerase chain reaction
SA	streptavidin
T	thymine
Trp	tryptophan
TTA	thenoyltrifluoroacetonate
Tyr	tyrosine
UTP	uridine 5’-monophosphate

REFRENCES

1. W. DeW. Horrocks, Jr. and M. Albin, *Prog. Inorg. Chem.*, *31*, 1 (1984).
2. K. A. Gschneider and L. R. Eyring, *Handbook on the Physics and Chemistry of Rare Earths*; North-Holland Company, Amsterdam, 1978, vol. 1.
3. W. T. Carnall, K. A. Gschneider, Jr., and L. Eyring, *Handbook on the Physics and Chemistry of Rare Earths*; North-Holland Company, Amsterdam, 1979, vol. 3, pp. 171.
4. V. L. Ermolaev and E. B. Sveshnikova, *Russ. Chem. Rev.*, *63*, 905 (1994) and references therein.
5. J.-C. G. Bünzli, and C. R. Choppin, Lanthanide Probes in Life, Chemical and Earth Sciences, Elsevier, Amsterdam, 1989.
6. G. E. Buono-Core, H. Li, and B. Marciniak, Coord. Chem. Rev., 99, 55 (1990).
7. M. P. Oude Wolbers, F. C. J. M. van Veggel, B. H. M. Snellink-Ruël, J. W. Hofstraat, F. A. J. Geurts, and D. M. Reinhoudt, *J. Am. Chem. Soc.*, *119*, 138 (1997).
8. I. Hemmilä, V.-M. Mukkala, and H. J. Takalo, *Fluorescence*, *5*, 159 (1995).
9. R. S. Dickins, D. Parke, A. S. de Sousa, and J. A. G. Williams, *Chem. Commun.*, 697 (1996).
10. (a) W. DeW. Horrocks, Jr. and D. R. Sudnick, *Acc. Chem. Res.*, *14*, 384 (1981). (b) R. C. Holz, C. A. Chang, and W. DeW. Horrocks, Jr., Inorg. Chem., 30, 3270 (1991).
11. H. F. Brito, O. L. Malta, and J. F. S. Menezes, *J. Alloy Cmpds.*, *303-304*, 336 (2000).
12. F. C. J. M. van Veggel, M. P. Oude Wolbers, and D. N. Reinhoudt, *J. Phys. Chem. A*, *102*, 3060 (1998).
13. I. Hemmilä, M.-V. Mukkala, M. Latva, and P. J. Kiilholma, *Biochem. Biophys. Methods*, *26*, 283 (1993).
14. S. W. Magennis, S. Parsons, A. Corval, J. D. Woollins, and Z. Pikramenou, *J. Chem. Soc, Chem. Commun*, 61 (1999).

15. R. D. Archer and H. Chen, *Inorg. Chem.*, *37*, 2089 (1998).
16. H. Takalo, V.-M. Mukkala, H. Mikola, P. Liiti, and I. Hemmila, *Bioconjugate Chem.*, *5*, 278 (1994).
17. V.-M. Mukkala and J. Kankare, *Helv. Chim. Acta*, *75*, 1578 (1992).
18. A. Saha, K. Kross, E. Kloszewski, D. Upson, J. Toner, R. Snow, C. Black, and V. Desai, *J. Am. Chem. Soc.*, *115*, 11032 (1993).
19. K. Mitrunen, K. Petersson, T. Piironen, T. Björk, H. Lilja, and T. Lövgren, *Clin. Chem.*, *41*, 1115 (1995).
20. V.-M. Mukkala, P. Litti, I. Hemmila, H. Takalo, C. Matachescu, and J. Kankare, *Helv. Chim. Acta*, *79*, 295 (1996).
21. M. Remuiñán, H. Román, M. Alonso, and J. Rodríguez-Ubis, *J Chem. Soc., Perkin Trans. 2*, 1099 (1993).
22. H. Takalo, and V.-M. Mukkala, PTC Appl. /FI91/00373, 1991.
23. B. Alpha, J.-M. Lehn, and G. Mathis, *Angew. Chem. Int. Ed. Engl.*, *26*, 266 (1987).
24. G. Blasse, G. Dirksen, N. Sabbatini, S. Perathorner, J.-M. Lehn, J.-M. and B. Alpha, *J. Phys. Chem.*, *92*, 2419 (1998).
25. N. Sato, N. and S. Shinkai *J. Chem. Soc. Perkin Trans. 2*, 621 (1993).
26. N. Sabbatini, M. Guardigli, A. Mecati, V. Balzani, R. Ungaro, E. Ghidini, A. Casnati, and A. Pochini, *J. Chem. Soc., Chem. Commun.*, 818 (1990).
27. a) Z. Pikarmenou and D. Nocera, *Inorg. Chem.*, *31*, 532 (1992);

    b) C. M. Rudzinski, W. K. Hartman, and D. G. Nocera, Coord. Chem. Rev., 171, 115 (1998).
28. N. Sabbatini, S. Dellonte, and G. Blasse, *Chem. Phys. Lett.*, *129*, 541 (1986).
29. W. M. Watson, R. P. Zerger, J. T. Yardley, and G. D. Stucky, *Inorg. Chem.*, *14*, 2675 (1975).
30. F. R. Gonçalves de Silva, R. Longo, O. L. Malta, C. Piguet, and J.-C. G. Bünzli, Phys. Chem. Chem. Phys., 2, 5400 (2000).
31. J. L. Kropp and M. W. Windsor, *J. Chem. Phys.*, *39*, 2769 (1963).
32. J. L. Kropp and M. W. Windsor, *J. Chem. Phys.*, *42*, 1599 (1965).

33. B. S. Panigrahi, *J. Luminescence*, *82*, 121 (1999).
34. N. Iki, T. Horiuchi, H. Oka, K. Koyama, N. Morohashi, C. Kabuto, and S. Miyano, *J. Chem. Soc., Perkin Trans. 2*, *11*, 2219 (2001).
35. (a) H.-R. Murner, E. Chassat, R. P. Thummel, and J.-C. G. Bünzli, *J. Chem. Soc. Dalton Trans.*, 2809 (2000). (b) A. P. de Silva, H. Q. N. Gunaratne, and T. E. Rice, Angew. Chem. Int. Ed. Engl., 35, 2116 (1996).
36. S. I. Klink, G. A. Hebbink, L. Grave, F. G. A. Peters, F. C. J. M. van Veggel, D. N. Reinhoudt, and J. W. Hofstraat, *Eur. J. Org. Chem.*, *10*, 1923 (2000).
37. M. A. Rawashdeh-Omary, C. L. Larochelle, and H. H. Patterson, *Inorg. Chem.*, *39*, 4527 (2000).
38. B. Yan, H. Zhang, S. Wang, and J. Ni, *Mon. Chem.*, *129*, 567 (1998).
39. B. S. Santos, C. de Mello Donega, and G. F. de Sa, *J. Luminescence*, *72-74*, 535 (1997).
40. G. A. Hebbink, S. I. Klink, P. G. B. Oude Alink, and F. C. J. M. van Veggel, *Inorg. Chim. Acta*, *317*, 114 (2001).
41. M. P. Oude Wolbers, F. C. J. M. van Veggel, B. H. M. Snellink-Ruel, J. W. Hofstraat, F. A. J. Geurts, and D. N. Reinhoudt, *J. Chem. Soc., Perkin Trans. 2*, 2141 (1998).
42. D. J. Qian, H. Nakahara, K. Fukuda, and K.-Z. Yang, *Langmuir*, *11*, 4491 (1995).
43. R. Ludwig, H. Matsumoto, M. Takeshita, K. Ueda, and S. Shinkai, *Supramol. Chem.*, *4*, 319 (1995).
44. J. Chen and P. R. Selvin, *J. Am. Chem. Soc.*, *122*, 657 (2000).
45. (a) J. B. Lamture, Z. H. Zhou, A. S. Kumar, and T. G. Wensel, *Inorg. Chem.*, *34*, 864 (1995). (b) J. B. Lamture and T. G. Wensel, Tet. Lett., 34, 4141 (1993).
46. L. J. Charbonniere, C. Balsiger, K. J. Schenk, and J.-C. G. Bünzli, *J. Chem. Soc., Dalton Trans*., 505 (1998).
47. N. Sabbatini, A. Casnati, C. Fischer, R. Girardini, M. Guardigli, I. Manet, G. Sarti, and R. Ungaro, *Inorg. Chim. Acta*, *252*, 19 (1996).

48. P. K.-L. Fu and C. Turro, *J. Am. Chem. Soc.*, *121*, 1 (1999).
49. (a) C. Formoso, *Biochem. Biophys. Res. Commun.*, *53*, 1083 (1973). (b) M. D. Topal and J. R. Fresco, Biochemistry, 19, 5531 (1980). (c) D. P. Ringer, B. A. Howell, and D. E. Kizer, Anal. Biochem., 103, 337 (1980).
50. H.-A. Tajmir-Riahi, *Biopolymers*, *31*, 1065 (1991).
51. S. L. Klakamp and W. D. Horrocks, Jr., *J. Inorg. Biochem.*, *46*, 175 (1992).
52. N. Nagesh, P. Bhargava, and D. Chatterji, *Biopolymers*, *32*, 1421 (1992).
53. S. L. Klakamp and W. D. Horrocks, Jr., *J. Inorg. Biochem.*, *46*, 193 (1992).
54. Z. Balcarova and V. Brabec, *Biophys. Chem.*, *33*, 55 (1989).
55. D. S. Gross and H. Simpkins, *J. Biol. Chem.*, *256*, 9593 (1981).
56. S. Klakamp and W. D. Horrocks, Jr., *Biopolymers*, *30*, 33 (1990).
57. N. L. Greenbaum, C. Mundoma, and D. R. Peterman, *Biochemistry*, *40*, 1124 (2001).
58. E. Heyduk and T. Heyduk, *Anal. Biochem.*, *248*, 216 (1997).
59. (a) M. Li and P. R. Selvin, *Bioconjugate Chem.*, *8*, 127 (1997). (b) P. R. Selvin, T. M. Rana, and J. E. Hearst, J. Am. Chem. Soc., 116, 6029 (1994). (c) P. R. Selvin and J. E. Hearst, Proc. Natl. Acad. Sci. U. S. A., 91, 10024 (1994).
60. T. Heyduk and E. Heyduk, *Nat. Biotechnol.*, *20*, 171 (2002).
61. A. N. Kapanidis, Y. W. Ebright, R. S. Ludescher, S. Chan, and R. H. Ebright, *J. Mol. Biol.*, *312*, 453 (2001).
62. E. Heyduk, T. Heyduk, P. Claus, and J. R. Wisniewski, *J. Biol. Chem.*, *272*, 19763 (1997).
63. J. R. Wisniewski, N. M. Krohn, E. Heyduk, K. Grasser, and T. Heyduk, *Biochim. Biophys. Acta*, *1447*, 25 (1999).
64. (a) E. Heyduk and T. Heyduk, *Biochemistry*, *41*, 2876 (2002). (b) D. L. Matlock and T. Heyduk, Biochemistry, 39, 12274 (2000). (c) E. Heyduk and T. Heyduk, J. Biol. Chem., 274, 3315 (1999).

65. M. Elbanowski and B. Makowska, *J. Photochem. Photobiol. A: Chemistry*, *99*, 85 (1999).

66. H. Hakala, E. Mäki, and H. Lönnberg, *Bioconjugate Chem.*, *9*, 316 (1998).

67. H. Hakala, P. Virta, H. Salo, and H. Lönnberg, *Nucleic Acids Res.*, *26*, 5581 (1998).

68. A. Oser and G. Valet, *Angew. Chem. Int. Ed.*, *29*,1167 (1990).

69. G. Wang, J. Yuan, K. Matsumoto, and Z. Hu, *Anal. Biochem.*, *299*, 169 (2001).

70. (a) J. Nurmi, M. Kiviniemi, M. Kujanpää, M. Sjöroos, J. Ilonen, and T. Lövgren, *Anal. Biochem.*, *299*, 211 (2001). (b) J. Nurmi, H. Lilja, and A. Ylikoski, Luminescence, 15, 381 (2000).

71. S. Sueda, J. Yuan, and K. Matsumoto, *Bioconj. Chem.*, *11*, 827 (2000).

72. S. Sueda, J. Yuan, and K. Matsumoto, *Bioconj. Chem.*, *13*, 200 (2002).

73. M. Samiotaki, M. Kwiatkowski, N. Ylitalo, and U. Landegren, *Anal. Biochem.*, *253*, 156 (1997).

74. (a) B. Alpha-Bazin, H. Bazin, L. Boissy, and G. Mathis, *Anal. Biochem.*, *286*, 17 (2000). (b) B. Alpha-Bazin, H. Bazin, S. Guillemer, S. Sauvaigo, and G. Mathis, Nucl. Nucl. Nucl. Acids, 19, 1463 (2000).

75. J. A. Bruno, W. D. Horrocks, Jr., and R. J. Zauhar, *Biochemistry*, *31*, 7016 (1992).

76. (a) S. E. Burroughs, G. Eisenman, and W. D. Horrocks, Jr., *Biophys. Chem*., *42*, 249 (1992). (b) W. D. Horrocks, Jr., Method Enzymol., 226, 495 (1993)

77. (a) E. Soini and T. Lövgren, *CRC Crit. Rev. Anal. Chem.*, *18*, 105 (1987). (b) E. Soini, Trends Anal. Chem., 9, 90 (1990).

78. (a) W. D. Horrocks, Jr. and D. R. Sudnick, *J. Am. Chem. Soc*., *101*, 334 (1979). (b) W. D. Horrocks, Jr. and W. E. Collier, *J. Am. Chem. Soc*., *103*, 2856 (1981).

79. H. Harma, P. Aronkyto, and T. Lövgren, *Anal. Chim. Acta,*, *410*, 85 (2000).

80. D. D. Root, S. Shewart, and J. Xu, *Biochemistry*, *41*, 1786 (2002).
81. M. Elbanowski and B. Makowska, *J. Photochem. Photobiol. A*, *99*, 85 (1996).
82. H. C. Marsh, Jr, R. A. Hoke, D. W. Deerfield, L. G. Peterson, R. G. Hiskey, and K. A. Koehler, *Inor. Chem.*, *25*, 4503 (1986).
83. R. Bertrand, J. Derancourt, and R. Kassab, *Biochemistry*, *39*, 14626 (2000).
84. E. Pidcock and G. R. Moore, *J. Biol. Inorg. Chem.,*, *6*, 479 (2001).
85. C. I. MacManus, J. P. Banville, and A. G. Szabo, *Anal. Biochem*., *210*, 1 (1993)
86. C. R. MacKenzie, I. D. Clark, S. V. Evans, I. E. Hill, J. P. MacManus, D. Dubuc, D. R. Bundle, S. A. Narang, and N. M. Young, *Immunotechnol.*, *1*, 139 (1995).
87. J. D. Moore, M. A. Skinner, D. R. Swatman, A. R. Hawkins, and K. A. Brown, *J. Am*. *Chem. Soc*., *120*, 7105 (1998).
88. E. Heyduk and T. Heyduk, *Anal. Biochem.*, *248*, 216 (1997).
89. J. H. Howanitz, *Arch. Pathology Lab. Med*., *112*, 775 (1998).
90. D. Chaudhuri, W. D. Horrocks, Jr., J. C. Amburgey, and D. Weber, *Biochemistry*, *36,* 9674 (1997).
91. R. M. Supkowski, J. P. Bolender, W. D. Smith, L. E. L. Reynold, and W. D. Horrocks, Jr., *Coord. Chem. Rev.*,, *185-185*, 307 (1999).
92. B. D. Schlyer, D. G. Steel, and A. Gafni, *J. Biol. Chem.*, *270*, 22890 (1995).
93. F. S. Richardson and J. P. Riehl, *Chem. Rev*., *12*, 86 (1986).
94. M. Elbanowski, J. Paetz, J. Slawminski, and L. Ciesla, *Photochem. Photobiol*., *47*, 463 (1988).
95. C. S. Cierniewski, T. A. Haas, J. W. Smith, and E. F. Plow, *Biochemistry*, *33*, 12238 (1994).
96. H. Ren, C. B. Klee, S. E. Burroughs, and W. D. Horrocks, Jr., *Biochemistry*, *33*, 10428 (1994).
97. I. Bertini, M. B. L. Janik, Y.-M. Lee, C. Luchinat, and A. Rosato, *J. Am. Chem. Soc*., *123*, 4148 (2001).

98. Y. C. Lee, *Anal. Biochem.*, *297*, 123 (2001).

99. E. F. G. Dickson, A. Pollak, and E. P. Diamandis, *J. Photochem. Photobiol. B*, *27*, 3 (1995).

100. (a) T. K. Christopoulos and E. P. Diamandis, *Analyst*, *116*, 627 (1991). (b) T. K. Christopoulos and E. P. Diamandis, *Anal. Chem*., *64*, 342 (1992).

101. (a) E. P. Diamandis, P. C. Morton, E. Reichstein, and M. J. Hosravi, *Anal. Chem*., *61*, 48 (1989). (b) E. P. Diamandis, *Analyst*, *117*, 1879 (1992),

102. M. Li and P. R. Selvin, *J. Am. Chem. Soc*., *117*, 8132 (1995).

103. G. Marriot, M. Heidecker, E. P. Diamandis, and Y. Y. Marriot, *Biophys. Journal*, *67*, 957 (1994).

104. H. Zijlmans, J. Bonnet, J. Burton, K. Kardos, T. Vail, R. S. Niesbala, and H. J. Tanke, *Anal. Bioche*m., *267*, 30 (1999).

105. M. Wortberg, C. Middendorf, A. Katerkamp, T. Rump, J. Krause, and K. Cammann, *Anal. Chim. Acta*., *289*, 177 (1994).

106. C. T. Elliott, K. S. Francis, and W. J. McCaughey, *Analyst*, *119*, 2565 (1994).

107. H. H. Shi and Y. S. Yang, *Alloys Compounds*, *29*, 207 (1994).