

## Cell-Penetrating Metal Complex Optical Probes: Targeted and Responsive Systems Based on Lanthanide Luminescence

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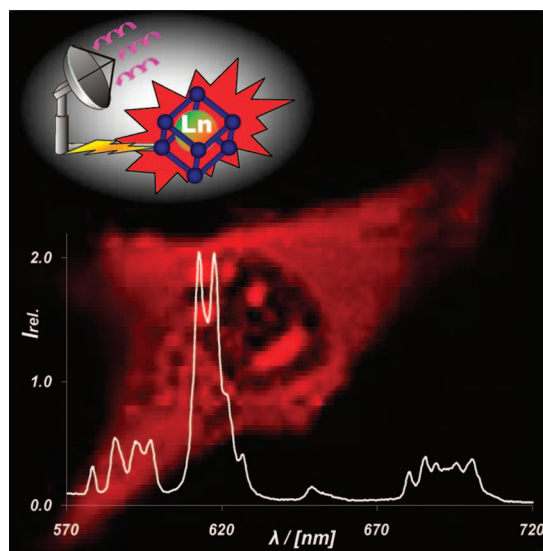
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### CON SPECTUS

To understand better the structure and function of biological systems, cell biologists and biochemists would like to have methods that minimally perturb living systems. The development of emissive optical probes is essential for improving our observation of intracellular signaling and recognition processes. Following excitation of the probe, photons emitted from the probe may be observed by spectroscopy or microscopy and encode information about their environments in their energy, lifetime, and polarization. Such optical probes may be based on organic fluorophores, quantum dots, recombinant proteins, or emissive metal complexes.

In this Account, we trace the emergence of lanthanide coordination complexes as emissive optical probes. These probes benefit from sharp emission bands and long lifetimes. We can design these complexes to report on the concentration of key biochemical variables by modulation of spectral form, lifetime, or circular polarization. These properties allow us to apply ratiometric methods of analysis in spectroscopy or microscopy to report on local pH, pM ( $M = \text{Ca}, \text{Zn}$ ), or the concentration of certain anionic metabolites, such as citrate, lactate, bicarbonate, or urate. For optical microscopy studies in living cells, these probes must be cell-permeable and, ideally, should localize in a given cell organelle. We undertook systematic studies of more than 60 emissive complexes, examining the time dependence of cellular uptake and compartmentalization, cellular toxicity, protein affinity, and quenching sensitivity. These results and their relationship to probe structure have allowed us to identify certain structure–activity relationships. The nature and linkage mode of the integral sensitizing group—introduced to harvest incident light efficiently—is of primary importance in determining protein affinity and cellular uptake and trafficking. In many cases, uptake may occur via macropinocytosis. We have defined three main classes of behavior: complexes exhibit predominant localization profiles in protein-rich regions (nucleoli/ribosomes), in cellular mitochondria, or in endosomes/lysosomes. Therefore, these systems offer considerable promise as intracellular optical probes, amenable to single- or two-photon excitation, that may report on the local ionic composition of living cells subjected to differing environmental stresses.



### 1. Introduction

A key challenge in cell biology and biochemistry research lies in gaining greater understanding about the structure and function of biological sys-

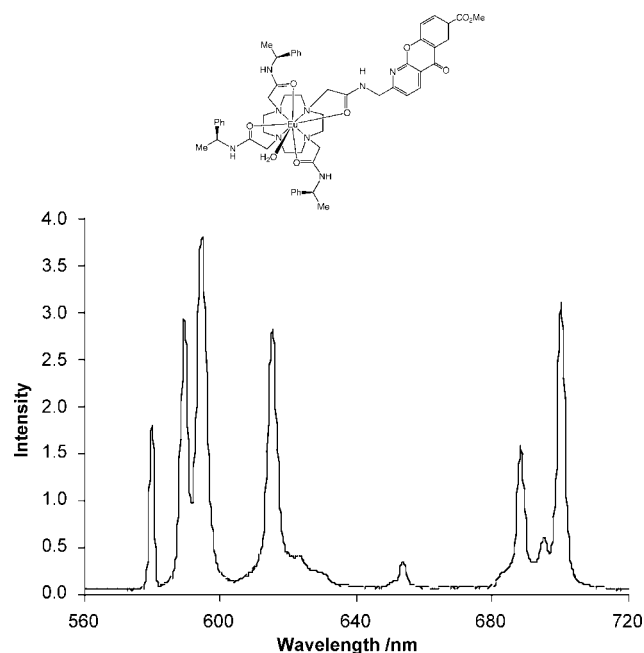
tems through methods that involve minimal perturbation of the system. The development of emissive optical probes is essential to the progression of such research. Following excitation of the

probe, emitted photons may be observed by spectroscopy or microscopy and encode information in their energy, lifetime, and polarization. These optical probes may be based on organic fluorophores, semiconductor nanoparticles (often termed quantum dots), recombinant proteins, or emissive metal complexes. Low MW organic fluorophores are commonly based on rhodamine, fluorescein, cyanine dyes, or dipyrrolylmethane (BODIPY) core structures, and key advances have allowed changes in pH and pM ( $M = \text{Ca}, \text{Zn}$ ) to be tracked.<sup>1–3</sup> Over the past 10 years, quantum dots have been developed, and their utility in cell tracking has been explored, although their large molecular volume may limit their intracellular compartmentalization profile.<sup>4–6</sup> Emissive recombinant proteins (e.g., green fluorescent protein) have proved to be very useful in protein recognition studies, notably when used as components of fluorescence resonance energy transfer (FRET) studies.<sup>7</sup> In this Account, we explore the emerging use of lanthanide coordination complexes as cellular probes.<sup>8–12</sup> In particular, we consider their rational design and discuss how responsive probes can be devised that are able to signal information about the nature of the local environment. We seek to develop responsive optical probes, that can be observed by microscopy and targeted to selective organelles within a living cell and discuss strategies to address this challenging objective.

## 2. Lanthanide Complexes As Responsive Optical Probes

It has long been appreciated that lanthanide(III) complexes ( $\text{Ln} = \text{Sm}, \text{Eu}, \text{Tb}, \text{Dy}, \text{Yb}$ ) have attractive properties as optical probes. They possess large Stokes' shifts (big separation between absorption and emission wavelengths) and long emission lifetimes (range 1  $\mu\text{s}$  to 5 ms) that allow the use of time-gated acquisition methods to enhance signal/noise, minimizing interference from light scattering or autofluorescence.<sup>8,11</sup> The lanthanide excited states are insensitive to quenching by molecular oxygen, and the emission spectral profile consists of a series of well-defined and narrow bands, characteristic of a given ion. The similarity of the solution coordination chemistry of  $\text{Sm(III)}$ ,  $\text{Eu(III)}$ ,  $\text{Tb(III)}$ , and  $\text{Dy(III)}$  ions means that the lanthanide(III) ion may be permuted in a common ligand structure, without significant variation in the chemical stability of the complex.

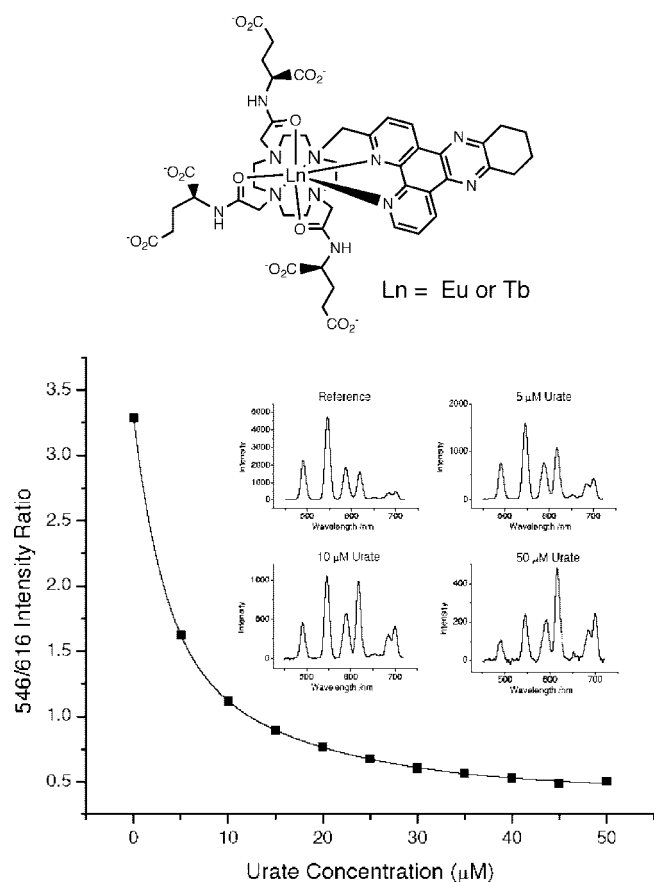
Most work has focused on emissive complexes of  $\text{Eu(III)}$  and  $\text{Tb(III)}$ , because the excited states of these ions are less sensitive to vibrational quenching by energy transfer to OH (i.e., nearby or coordinated water molecules), NH, or CH oscil-



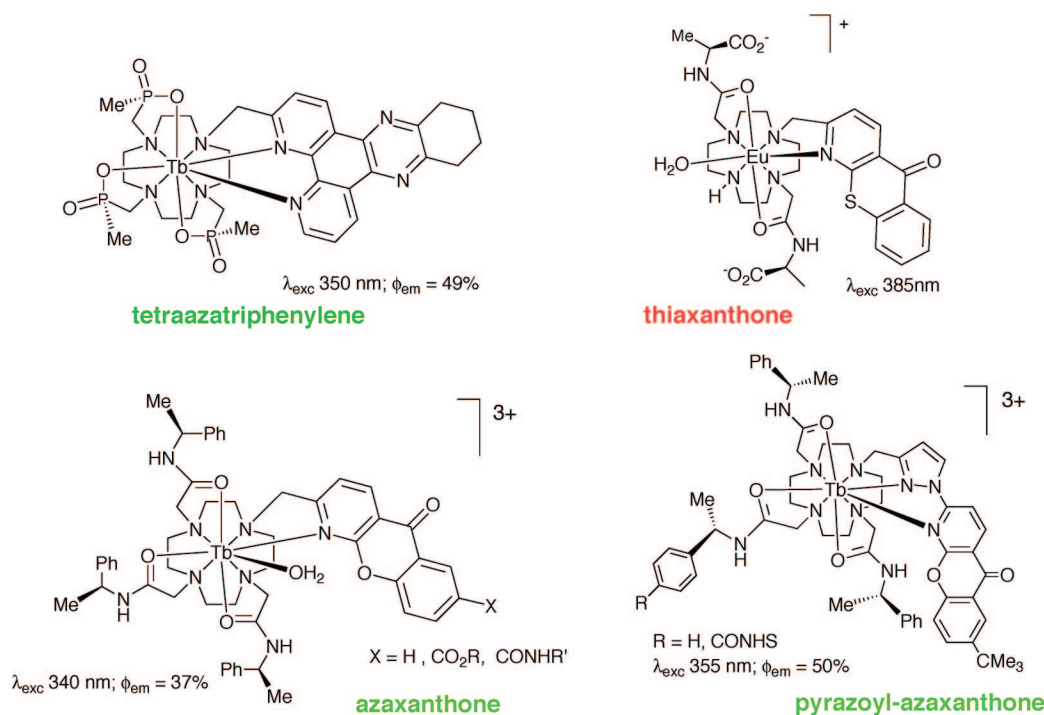
**FIGURE 1.** Emission spectrum of a tetra-amide europium complex showing the transitions between the  $^5\text{D}_0$  excited state and the  $^7\text{F}_n$  ground state (298 K, pH 7.1,  $\lambda_{\text{exc}}$  340 nm).

lators.<sup>13</sup> The absence of degeneracy of the  $\text{Eu(III)}$   $^5\text{D}_0$  excited state means that europium emission spectra are the simplest and the most amenable to interpretation. Reliable correlations have been drawn considering the form and relative intensity of the  $^5\text{D}_0\text{--}^7\text{F}_n$  ( $n = 0\text{--}4$ ) transitions with respect to the speciation and symmetry of the  $\text{Eu(III)}$  center. A typical spectrum (Figure 1) of a nine-coordinate, monocapped square antiprismatic complex, with approximate  $C_4$  symmetry about the  $\text{Eu}\text{--}\text{water}$  bond, shows the single  $\Delta J = 0$  transition at 579 nm and two components of the  $\Delta J = 1$  transition around 590 nm. The polarizability of the ligand donor atoms and the nature of the axial donor profoundly affect the relative intensity of the hypersensitive  $\Delta J = 2$  (around 616 nm) and  $\Delta J = 4$  (around 700 nm) transitions.<sup>14</sup> This property allows ratiometric analyses to be defined in which the intensity ratio of two  $\text{Eu(III)}$  emission bands can be measured; this parameter is independent of the concentration of the probe complex.<sup>15</sup> Alternative ratiometric analyses can be defined by examining changes in the emission intensity of a mixture of  $\text{Eu}$  (616 nm, red) and  $\text{Tb}$  (545 nm, green) complexes of a common ligand. For example, the differential sensitivity of the terbium  $^5\text{D}_4$  (244  $\text{kJ mol}^{-1}$  above ground state) and europium  $^5\text{D}_0$  (206  $\text{kJ mol}^{-1}$ ) excited states to dynamic quenching by the electron-rich urate anion has led to a precise ratiometric assay for uric acid in diluted urine and plasma samples<sup>16,17</sup> (Figure 2).

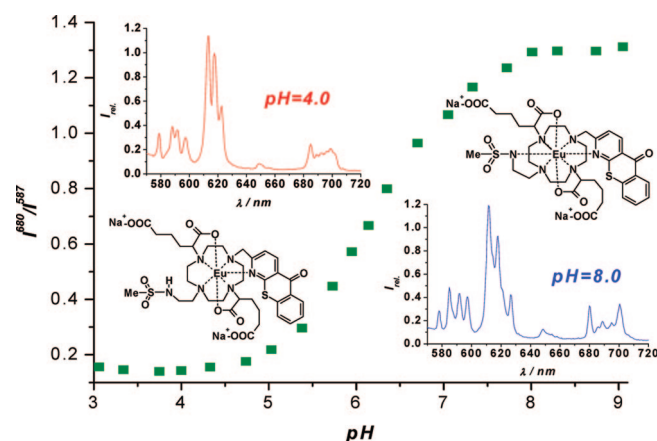
Transitions between the  $f\text{--}f$  electronic states of lanthanide(III) ions are symmetry-forbidden, resulting in extremely low



**FIGURE 2.** Variation of the green Tb (546 nm) to red Eu (616 nm) emission intensity ratio for [Ln·1] (pH 7.4, 0.1 M HEPES, 298 K) with added sodium urate, showing selected spectra recorded (multiwell analyzer).

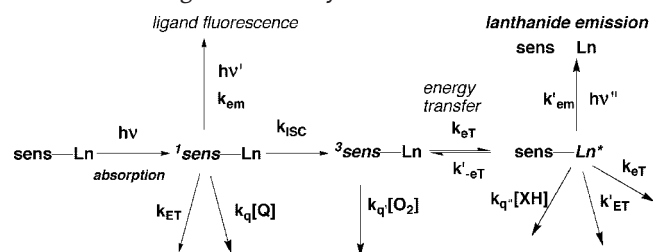
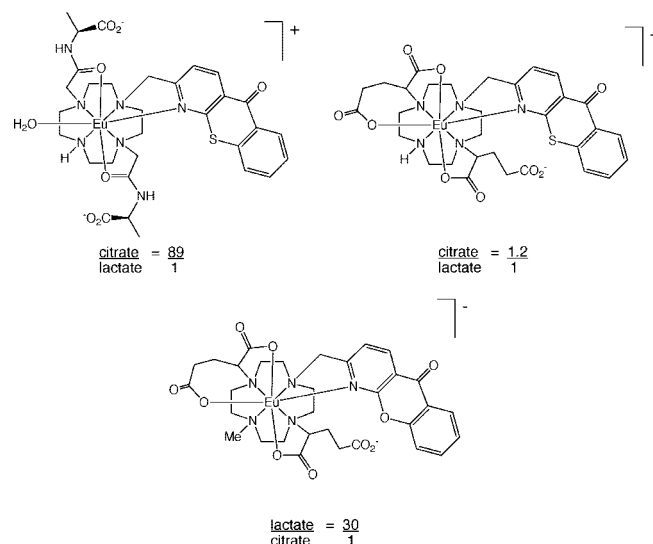


**FIGURE 3.** Structures of selected emissive Eu or Tb complexes with differing heterocyclic sensitizers showing salient chemical or photochemical characteristics.



**FIGURE 4.** pH Dependence of the europium emission spectrum showing the two Eu(III) species ( $\lambda_{\text{exc}}$  380 nm, 298 K, 0.1 M NaCl).

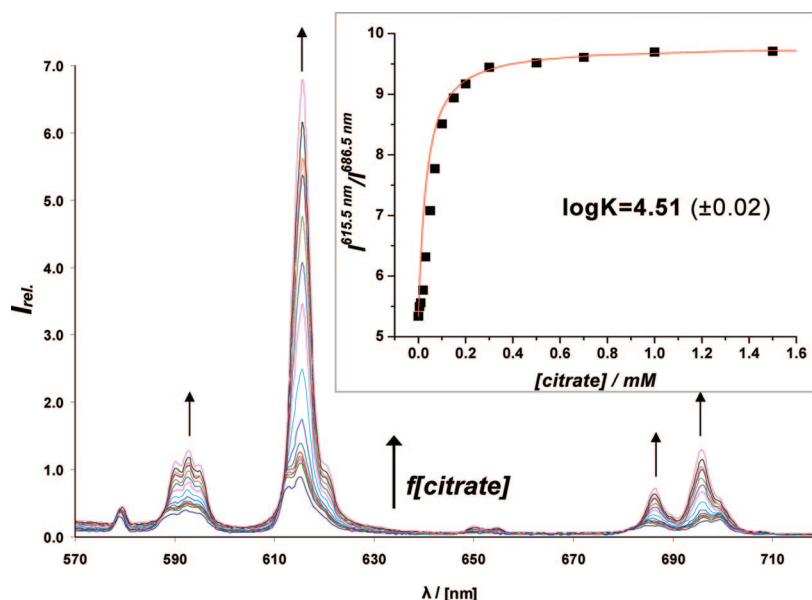
molar extinction coefficients for direct excitation. This problem is averted by incorporating a sensitizing moiety into the ligand structure. This needs to possess a triplet energy at least 2000  $\text{cm}^{-1}$  above the Eu  $^5\text{D}_0$  (17 200  $\text{cm}^{-1}$ ) or Tb  $^5\text{D}_4$  (20 400  $\text{cm}^{-1}$ ) excited states to avoid back energy transfer from the excited lanthanide ion to the triplet state of the sensitizer (Scheme 1). The chromophore must be selected carefully. For Eu or Tb sensitization, this requires a heterocyclic or aromatic moiety that possesses a small singlet–triplet energy gap ( $<7000 \text{ cm}^{-1} \equiv 84 \text{ kJ mol}^{-1}$ ), with an  $\text{S}_1$  excited state lying less than 29 000  $\text{cm}^{-1}$  ( $>340 \text{ nm}$ ) above the ground state. This allows the use of nonquartz optics and minimizes

**SCHEME 1.** Photophysical Pathways in Sensitized Emission Showing Competitive Quenching and Energy and Electron Transfer Processes Involving the Three Key Excited States**SCHEME 2.** Ratio of Anion Binding Constants<sup>a</sup><sup>a</sup> 0.1 M NaCl, pH 6.55, 298 K.

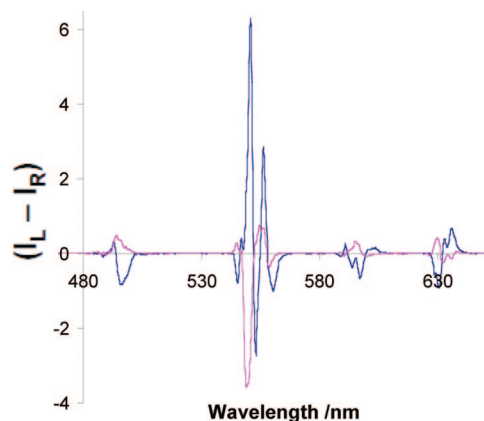
coexcitation of endogenous chromophores in proteins or nucleic acids. Representative examples of complexes with

these features defined in Durham are based on a “privileged” cyclen core structure, (Figure 3). They may be excited in the range 337–410 nm and can be addressed *in vitro*<sup>18</sup> and by microscopy *in cellulo*<sup>19</sup> using two-photon excitation, that is, with an excitation wavelength of 720–810 nm using a Ti–sapphire laser.

Changes in the coordination environment of the Eu(III) ion can cause large changes in spectral form, lifetime, and the circular polarization of emission. The spectral change may be stimulated by variation of pH, pM, or pX<sup>20,21</sup> and can occur in an intramolecular process or via reversible intermolecular association. The former is exemplified by the pH-dependent ligation of a sulfonamide group, giving large changes in the  $\Delta\lambda = 2$  (around 620 nm) and  $\Delta\lambda = 4$  transitions (680–700 nm), (Figure 4).<sup>15</sup> Intermolecular association typically occurs with anionic species. This involves the reversible displacement of coordinated water molecules in complexes with a heptadentate ligand.<sup>22,23</sup> The affinity of the Eu complex for a given anion (e.g.,  $\text{HCO}_3^-$ , lactate, citrate,  $\text{HPO}_4^{2-}$ ) is determined by electrostatics and the steric demand at the metal center.<sup>22,23</sup> Thus, a cationic complex possesses a greater affinity for an anion than its negatively charged analogue possesses. Similarly, selectivity for citrate (triply charged anion at pH 7.4) over the monoanion lactate is most evident with positively charged complexes (Scheme 2).<sup>24,25</sup> The steric demand at the Eu(III) center is determined by the constitution of the polydentate ligand used. This defines the available “space” around the spherical ion allowing either chelation ( $\text{HCO}_3^-$ , 4 ring chelate,<sup>24</sup> lactate/citrate, 5 ring with citrate creating greater steric

**FIGURE 5.** Europium emission spectra (Scheme 1) as a function of added citrate concentration showing (inset) the calibration curve derived from the 614/683 nm intensity ratio and the time-gated spectrum (5  $\mu\text{s}$  delay, pH 6.5, 0.1 M HEPES, 298 K,  $\lambda_{\text{exc}}$  380 nm, [complex] = 10  $\mu\text{M}$ ).

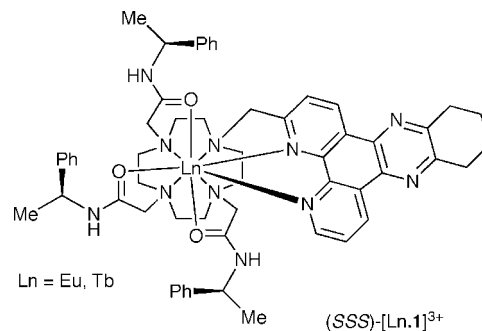




**FIGURE 6.** Circularly polarized emission spectra for (SSS)-[Tb·2]<sup>3+</sup> alone (blue) and in the presence of added human serum albumin (red), showing  $\Delta$  to  $\Lambda$  helicity inversion (295 K,  $\lambda_{\text{exc}}$  355 nm, 15  $\mu$ M complex, 30  $\mu$ M protein).

demand) or a monodentate binding mode (e.g., for  $\text{HPO}_4^{2-}$ ,  $\text{F}^-$ ,  $\text{H}_2\text{O}$ ). An example of spectral changes accompanying citrate binding to a cationic Eu(III) complex (Figure 5) reveals the large variation in the  $\Delta I = 2/I_{\Delta} = 1$  intensity ratio. This permits a selective ratiometric analysis of citrate.

A unique aspect of optical signaling with chiral lanthanide complexes is that the circular polarization of luminescence (CPL) may encode detailed information about the helicity at the metal center, thereby probing the local chiral environment. CPL is the emission analogue of circular dichroism. The excited Ln(III) ions can be considered as “spherical” emitters and avoid the problems associated with anisotropy that can complicate some chiroptical analyses.<sup>26</sup> Strong CPL is observed with enantiopure Eu/Tb complexes, with typical emission dissymmetry factors,  $g_{\text{em}}$ , of between 0.1 and 0.4<sup>27,28</sup> ( $g_{\text{em}} = 2(I_L - I_R)/(I_L + I_R)$ , where  $I_L$  and  $I_R$  are the left- and right-handed vectors of circularly polarized light). The Eu and Tb complexes of the enantiopure ligand (SSS)-1, may exist in two helical ( $\Delta/\Lambda$ ) conformers in solution, with the  $\Delta$ -complex predominant.<sup>29</sup> On binding to serum albumin, the complex inverts helicity, switching the sign of the circular polarization (Figure 6). This process occurs rapidly on the experimental time scale, and competition studies suggest that binding occurs selectively to “drug site II” on the protein. Thus, a unique chiroptical probe for albumin association has been defined.<sup>30</sup> The effect appears to be restricted to complexes of (SSS)-1 and their analogues; no dynamic helicity inversion was observed with complexes of (RRR)-1 nor in the presence of B-DNA, other proteins (e.g.,  $\alpha$ -acid glycoprotein), cyclodextrins or a variety of chiral anions (tartrate/lactate). Such behavior raises the intriguing possibility of using (SSS)-[Ln·1]<sup>3+</sup> as a chiral probe to track protein association *in cellulo* using circularly polarized luminescence microscopy!



### 3. Correlation of Probe Structure with Cell Imaging Behavior

At the outset of this work, we mused about the possibility of using responsive lanthanide probes to investigate spatial and temporal variations inside living cells of the concentration of key bioactive species. Tsien had shown how changes in calcium concentration were critical to cellular signaling by developing cell-permeable fluorescent Ca-selective probes.<sup>31</sup> What about the role of anionic species in a given cell compartment? For example, does modulation of bicarbonate concentration serve only to regulate local pH and cell volume, or might it have a key signaling role in mitochondria? There is evidence that bicarbonate may regulate the release of cyclic-AMP, for example.<sup>32</sup> Also, if every cell contains near millimolar concentrations of the low MW antioxidants urate and ascorbate (operating synergistically to scavenge reactive oxygen species), does their concentration vary in different organelles or when the cell is under oxidative stress? Tracking their changes might be easier than seeking the presence of ephemeral reactive oxygen/nitrogen species, although recent work with probes that may target mitochondria shows some promise to track changes in local singlet oxygen or hydrogen peroxide concentration.<sup>12,33</sup> Finally, citrate and lactate are essential species in cellular respiration. These species are difficult to measure *in situ* noninvasively; only optical probes offer the potential to track their fluctuations with high spatial resolution, provided that signal acquisition can occur quickly. How does their concentration vary with time in mitochondria and between different cell types, for example, cancerous vs healthy cells?

Preliminary cell uptake work with over 60 strongly emissive Eu ( $\phi_{\text{em}} \approx 10\%$ ) and Tb ( $\phi_{\text{em}} \approx 40\%$ ) complexes indicated that these systems (Figure 3) were indeed cell-permeable, and fluorescence microscopy studies revealed an uptake and compartmentalization profile that was dependent upon the structure of the probe.<sup>24,34–36</sup> In seeking to develop a structure–activity relationship that related uptake/egress and intracellular trafficking profiles to com-

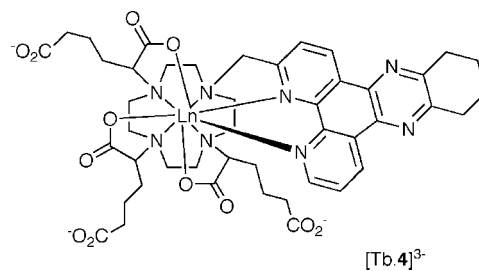
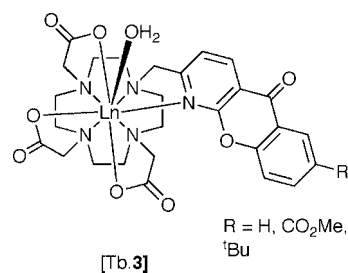
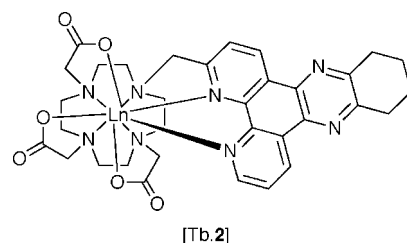
plex constitution, several factors need to be considered: complex charge; lipophilicity/amphipathicity; the nature of the sensitizer and its linkage mode; the number and structure of ligand donor groups attached to the common cyclen ring structure. Other factors relevant to this analysis include the protein affinity of the complex, its sensitivity to excited-state quenching by electron transfer from protein or endogenous reductants (ascorbate, urate, glutathione), its intracellular concentration, and the toxicity profile of the probe. This is commonly assessed via an  $IC_{50}$  value, measuring the concentration needed to kill 50% of a cell population after incubation with the probe for 24 h.<sup>9</sup>

#### 4. Methodology: Fluorescence Microscopy, Dynamic Quenching, Flow Cytometry, and Toxicity Screening

Protein affinity of a lanthanide probe can be measured either by examining the modulation of emission lifetime or intensity as a function of added protein (often serum albumin, because it is most abundant) or by monitoring the change in the relaxivity of analogous Gd complexes with protein concentration. Typical association constant values fall in the range  $10^3$ – $10^5$  M<sup>-1</sup> (Table 1). Excited-state quenching is addressed *in vitro* by measuring Stern–Volmer quenching constants ( $K_{SV}$  m<sup>-1</sup>/mM; this value gives the concentration of quencher that reduces the quenching by 50%) for individual species under controlled conditions in the absence and presence of protein.<sup>10,17,35</sup> Extensive studies revealed that urate was the most effective quencher, ( $K_{SV}$  m<sup>-1</sup> of ca. 20 μM), dynamically quenching Tb complexes most efficiently. The observed ionic strength and temperature dependence of this process were consistent with formation of an excited-state complex between urate and the sensitizing moiety,<sup>17</sup> contrasting with the thermally activated, dynamic quenching process exhibited by iodide.

Quantification of cell uptake requires counting the number of fluorescently labeled cells using a flow cytometer and

measuring the amount of the Eu or Tb in the sorted sample (typically  $10^4$ – $10^6$ ) by inductively coupled plasma mass spectrometry (ICP-MS) or optical emission spectroscopy (ICP-OES). Typical incubations expose the cells to an external concentration of the lanthanide probe on the order of 50 μM. The resultant labeled cells (monitored after 5 min, 1 h, 2 h, 4 h or 8 h) give rise to intracellular concentrations on the same order, that is, about  $10^8$  complexes per cell. It quickly became apparent that certain complexes, for example, [Tb·2], [Tb·3], and [Tb·4]<sup>3-</sup>, were taken up quickly by cells but were difficult to observe by microscopy. These were the very same complexes that were the most sensitive to luminescence quenching by urate<sup>10,17</sup> (and to a lesser extent ascorbate) and also bound weakly to serum albumin (Table 1). Protein binding tends to shield the lanthanide complex from excited-state quenching. Thus, complexes that are more prone to quenching by urate are likely to be those that are difficult to observe by microscopy.



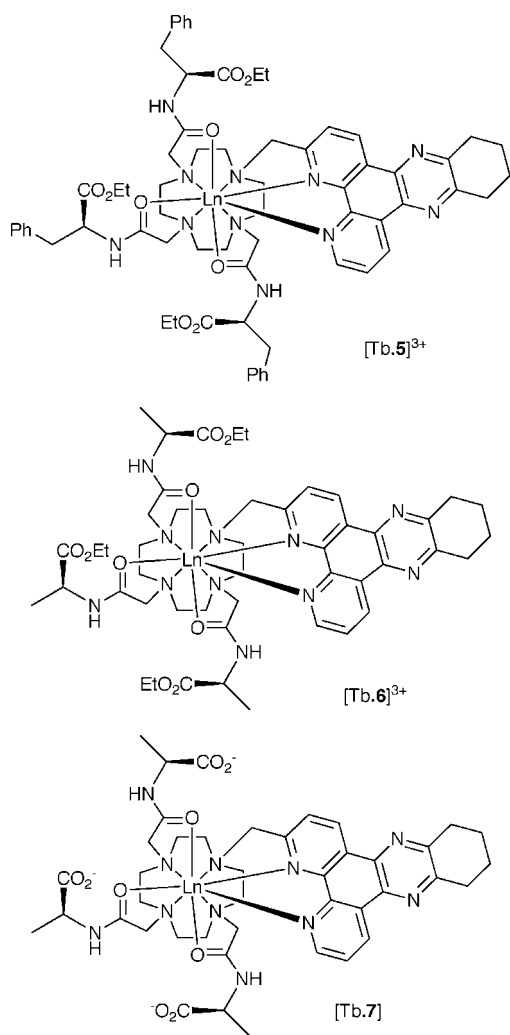
**TABLE 1.** Examples of Serum Albumin Binding Constants Determined by Analyzing Dynamic Quenching of Terbium Emission (pH 7.4, 0.1 M HEPES, 298 K) Following Protein Titration

complex	log <i>K</i>	$\tau_0/\tau$ limit <sup>a</sup>	comment
(SSS)-[Tb·1] <sup>3+</sup>	5.1	1.3	binds to drug site II and inverts helicity
(SSS)-[Tb·5] <sup>3+</sup>	4.2	2.4	amide carbonyl binds in capping apical site with Phe-amide substituent
(SSS)-[Tb·6] <sup>3+</sup>	3.8	3.4	less hydrophobic than [Tb·1] <sup>3+</sup>
(SSS)-[Tb·7]	3.2	7.6	lower steric demand at Tb
(SSS)-[Tb·2]	2.5	7.5	strongly quenched by urate

<sup>a</sup>  $\tau_0$  is the emission lifetime in the absence of added protein; limiting values are estimated by nonlinear least-squares iterative analysis of titration data.

Cell proliferation and viability studies are generally undertaken to examine the toxicity effect of the complex on the given cell type. This involves assessing the perturbation of esterase activity (e.g., using calcein, a probe that is emissive when hydrolyzed by the local enzymes), membrane permeability (e.g., Trypan blue, which only stains dead cells with compromised membranes), or mitochondrial redox. More

detailed analyses of the pathway leading to cell death may be undertaken using flow cytometry, distinguishing necrotic cell death, characterized by loss of outer and nuclear membrane integrity, from apoptotic cell death, signaled by an increase in surface phospho-serine residues, using a fluorescent conjugate of the phospholipid-binding protein annexin-V.<sup>19</sup>



## 5. Classes of Probe Localization Behavior: lysosomal, mitochondrial and nucleoli stains

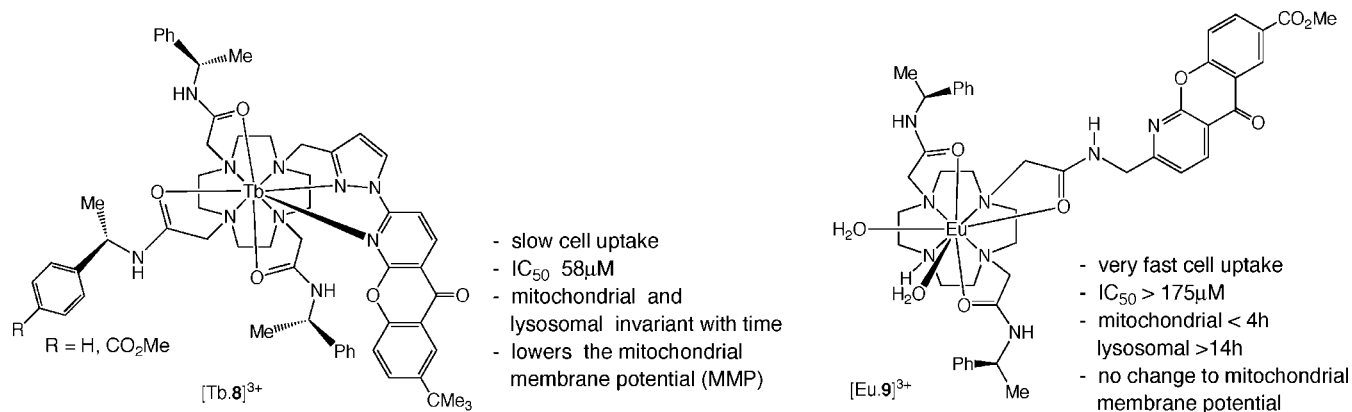
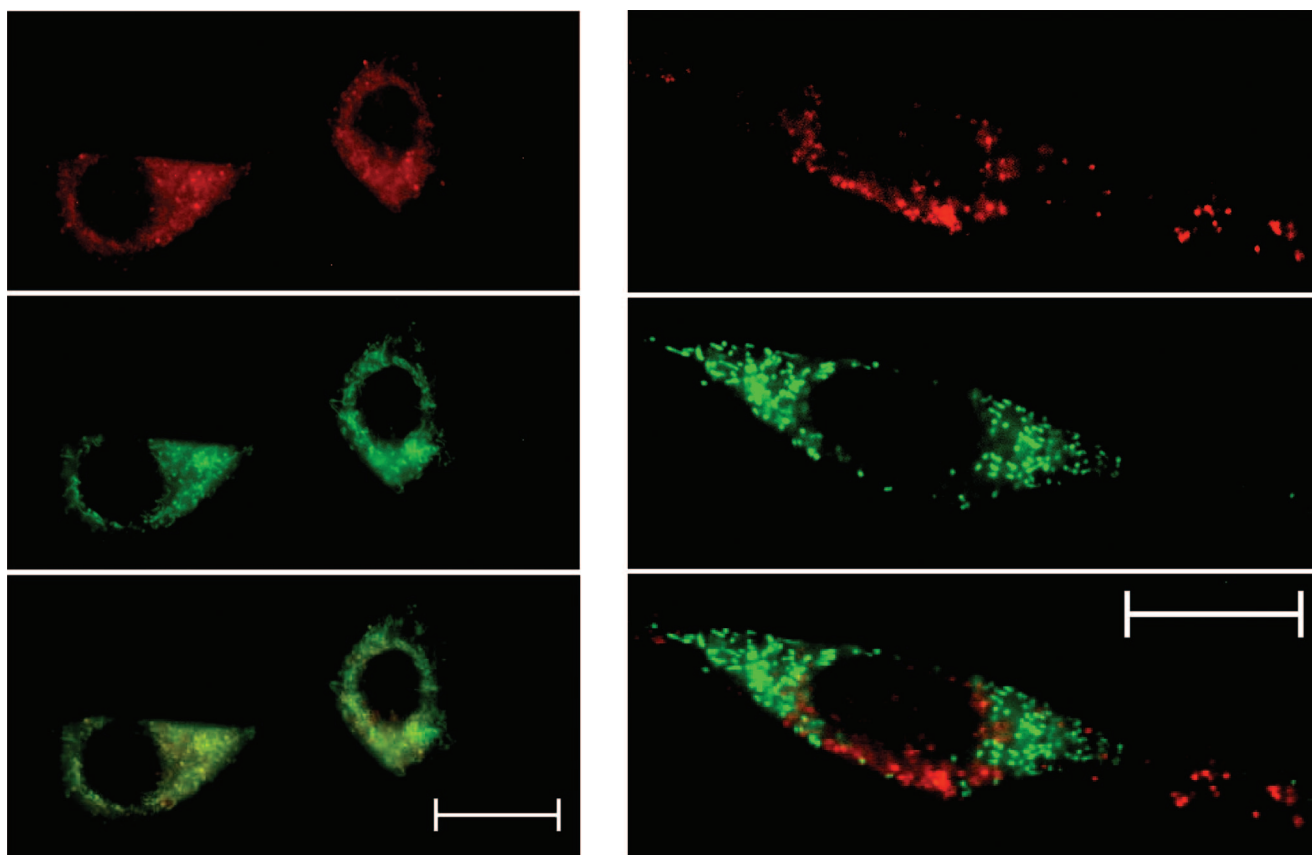
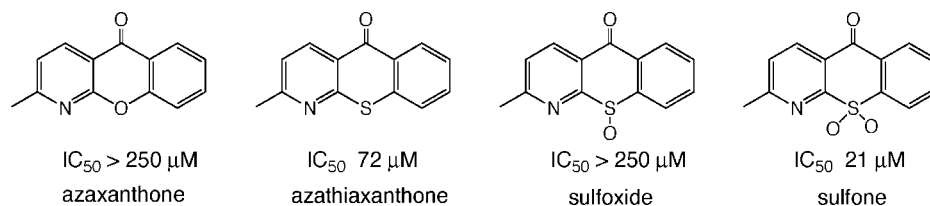
Over 60 different Eu and Tb complexes have been examined using the methods described above. Each either contains a different chromophore with a common ligand core structure or is based on the same chromophore with varying ligand donors. Sensitizers used include tetraazatriphenylenes,<sup>10,29,34,37</sup> acridones,<sup>8,24</sup> azaxanthenes, azathioxanthenes,<sup>9,10,36,38</sup> and pyrazoyl-azaxanthenes,<sup>39</sup> amenable to excitation in the range 337–410 nm. A primary conclusion from examining uptake in mouse skin fibroblasts (NIH-T3 cells), Chinese hamster ovarian (CHO), or carcinoma

cells (HeLa) is that it is the nature and mode of linkage of the sensitizing moiety that primarily determines cell uptake and compartmentalization and not complex charge, lipophilicity, or donor group structure. It is the polycyclic sensitizing moiety that is most likely to be recognized in protein association, which presumably must be a key recognition process in the intracellular trafficking involving recycling vesicles.

Three main classes of behavior have been observed to date. The default localization profile may be termed endosomal–lysosomal<sup>18,24,34</sup> and is exhibited by 80% of the complexes examined; rates of uptake and egress are fast and complexes are usually nontoxic. This pattern of behavior also appears to embrace the cell uptake profile of the helical dinuclear europium complexes reported by Bunzli recently.<sup>39,41</sup> Costaining experiments with Lysotracker Green (for Eu) or Red (Tb) confirm this. A second class of complexes (about six to date) reveal very fast uptake and slow egress and localization involving shuttling between mitochondria and the endosomal/lysosomal compartments, confirmed by costaining with the mitochondrial stain Mitotracker Green. Complexes that remain in the mitochondria for more than about 6–10 h show low IC<sub>50</sub> values and readily induce apoptotic cell death. Certain complexes, for example, [Eu·9]<sup>3+</sup> and its congeners (Scheme 3) show a clear time-dependent localization profile, changing from rapid mitochondrial staining to a lysosomal profile over a number of hours.<sup>9</sup> These complexes are nontoxic and do not perturb the mitochondrial membrane potential. They offer promise as responsive probes, because they bind bicarbonate reversibly and selectively (Figure 7). The third class of complexes stain the protein-dense ribosomes and nucleoli of cells<sup>15,36,42</sup> and show relatively slow uptake and egress. A large proportion of these complexes possess an N-coordinated azathioxanthone moiety (see Scheme 2, above; Figure 8) and show some evidence of toxicity with typical IC<sub>50</sub> values of between 40 and 90 μM. Control experiments examining the heterocyclic sensitizing substructures (Scheme 4) suggest that it may be the products of oxidative metabolism at S that lead to the observed toxicity.<sup>9</sup> The absence of toxicity noted for analogous complexes with an azaxanthone group supports this hypothesis.

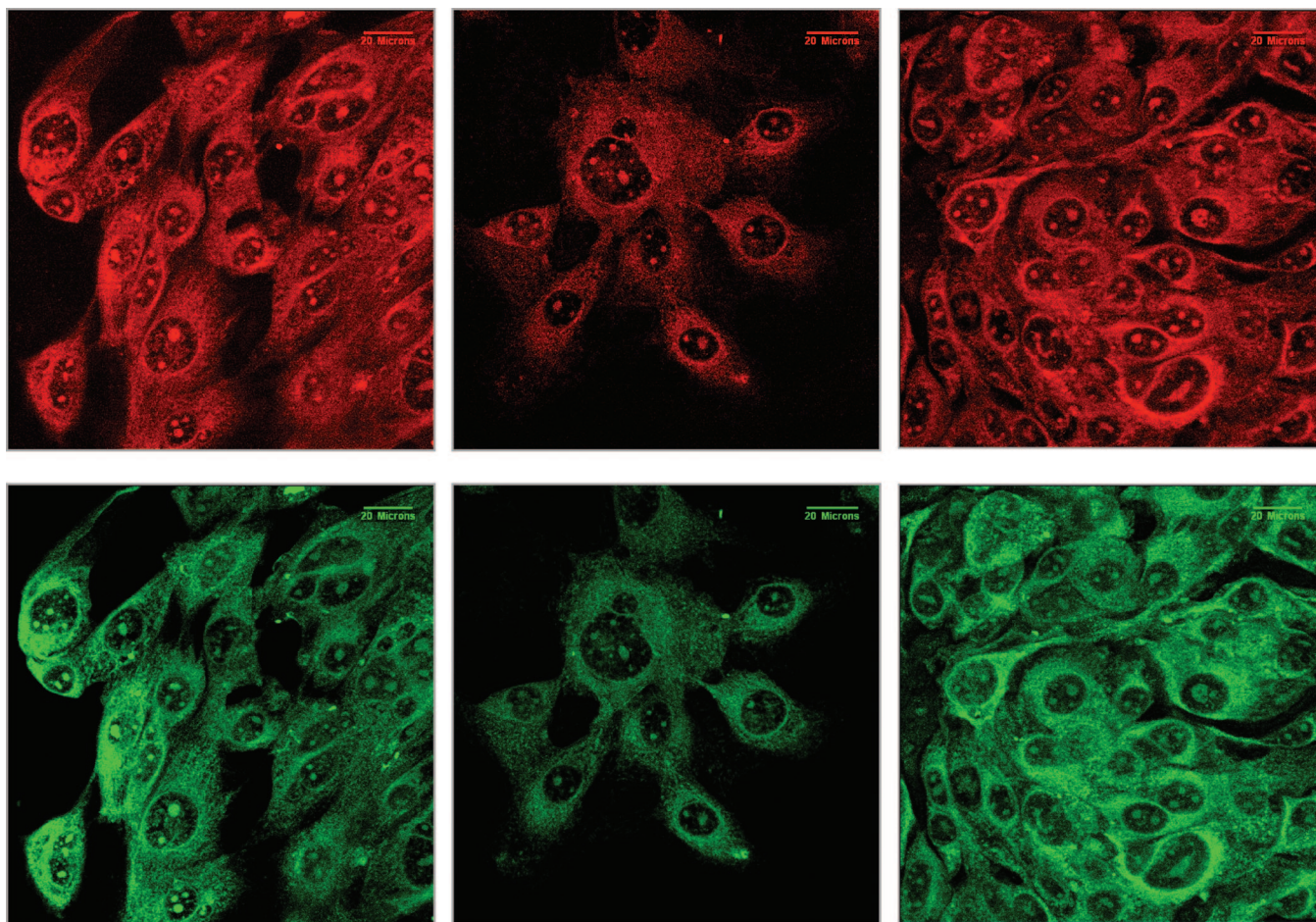
It should be borne in mind that the more lipophilic and surface-active probe complexes may themselves give rise to enhanced membrane permeability (similar to the effect of

## SCHEME 3

SCHEME 4.  $IC_{50}$  Values for Selected Sensitising Moieties

**FIGURE 7.** Epifluorescence microscopy images for  $[Eu.9]^{3+}$  (left, 4 h incubation; right 24 h; 50  $\mu M$  complex) showing (left) at 4 h a mitochondrial localization profile (upper, Eu emission; center, Mitotracker Green; lower, merged imaged; scale bar 20  $\mu m$ ) and (right) at 24 h a lysosomal profile.





**FIGURE 8.** Confocal microscopy images ( $\lambda_{\text{exc}}$  405 nm) showing the intracellular localization profile of a typical europium–azathioxanthone complex (green, ligand fluorescence; red, Eu emission), highlighting its distribution in the protein-dense nucleoli (bright spots, center) and ribosomes.

adding the surfactant Triton-X or 70% ethanol), and hence show an enhanced uptake. Such behavior is typically associated with a low  $\text{IC}_{50}$  value. Colocalization experiments with propidium iodide shed further light: this DNA-intercalating dye only penetrates compromised (i.e., more permeable) cell membranes and localizes within the nucleus at the nuclear membrane, highlighting chromosomal DNA. This aspect and the key question of why these patterns of cell uptake are exhibited require a better understanding of the mechanism of cell uptake.

## 6. Mechanistic Features: Targeting Vectors or Systematic Structural Perturbation of the Sensitizer?

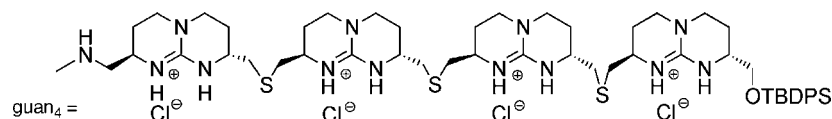
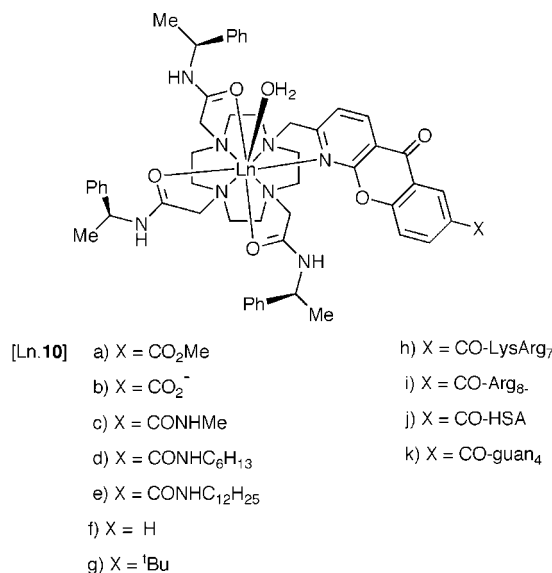
The rational development of emissive optical probes that can penetrate cell membranes and localize in a given organelle ultimately requires an understanding of the mechanism of cell entry and subsequent trafficking. The current understanding of such interlinked processes is crit-

ical to the development of current imaging and therapeutic entities. Recent work has revealed several well-defined cell uptake processes, leading to the definition of low MW inhibitors and promoters for a given uptake type.<sup>43</sup> Thus, for relatively low MW probes, the major pathway for cell entry is endocytosis, involving the vesicular uptake of extracellular species, following invagination of the cell membrane. This may be clathrin-mediated or involve caveolae. There are two other morphologically distinct pathways, termed macropinocytosis and clathrin/caveolae-independent cytos. Clathrin-mediated endocytosis is most common and is inhibited by chlorpromazine or  $\text{K}^+$  depletion; the presence of filipin, nystatin, and genestein inhibits caveolae-dependent pathways, while wortmannin and amiloride suppress macropinocytosis, and phorbol esters promote it. Preliminary microscopy experiments in the absence and presence of these inhibitors/promoters in different cell types with several Eu/Tb complexes strongly suggest that the dominant mechanism of cell uptake is

macropinocytosis.<sup>44</sup> Macropinocytosis accompanies cell-surface ruffling and involves formation of large endocytotic vesicles of irregular shape and size generated by invagination of the plasma membrane. Macropinosomes are regarded as leakier vesicles than other endosomes and constitute a unique population that may recycle their contents back to the surface of the cell.<sup>43</sup>

Given that it is the constitution of the sensitizing moiety that seems to primarily determine cell uptake and localization, a recent systematic study has compared cell uptake and toxicity with variations in sensitizer substitution<sup>19,45</sup> for 11 derivatives with a common core structure, [Ln·**10**]. The par-

chondrial membrane potential and induced apoptotic cell death (IC<sub>50</sub> 12 μM). Both the methyl ester [Tb·**10a**] and the peptide conjugates were nontoxic and internalized very rapidly (<5 min), the latter into perinuclear endosomes/lysosomes, while the former showed a more punctuate cytosolic compartmentalization, consistent with its predominant localization in macropinosomes. The locally hydrophilic carboxylate and carboxamide derivatives [Tb·**10b/10c**] were both very slow to enter the cell, while the <sup>t</sup>Bu-substituted complex, [Tb·**10g**] was rather toxic (IC<sub>50</sub> 58 μM), localized in mitochondria and endosomes, and caused the HeLa cells, in particular, to markedly increase in volume after several hours,



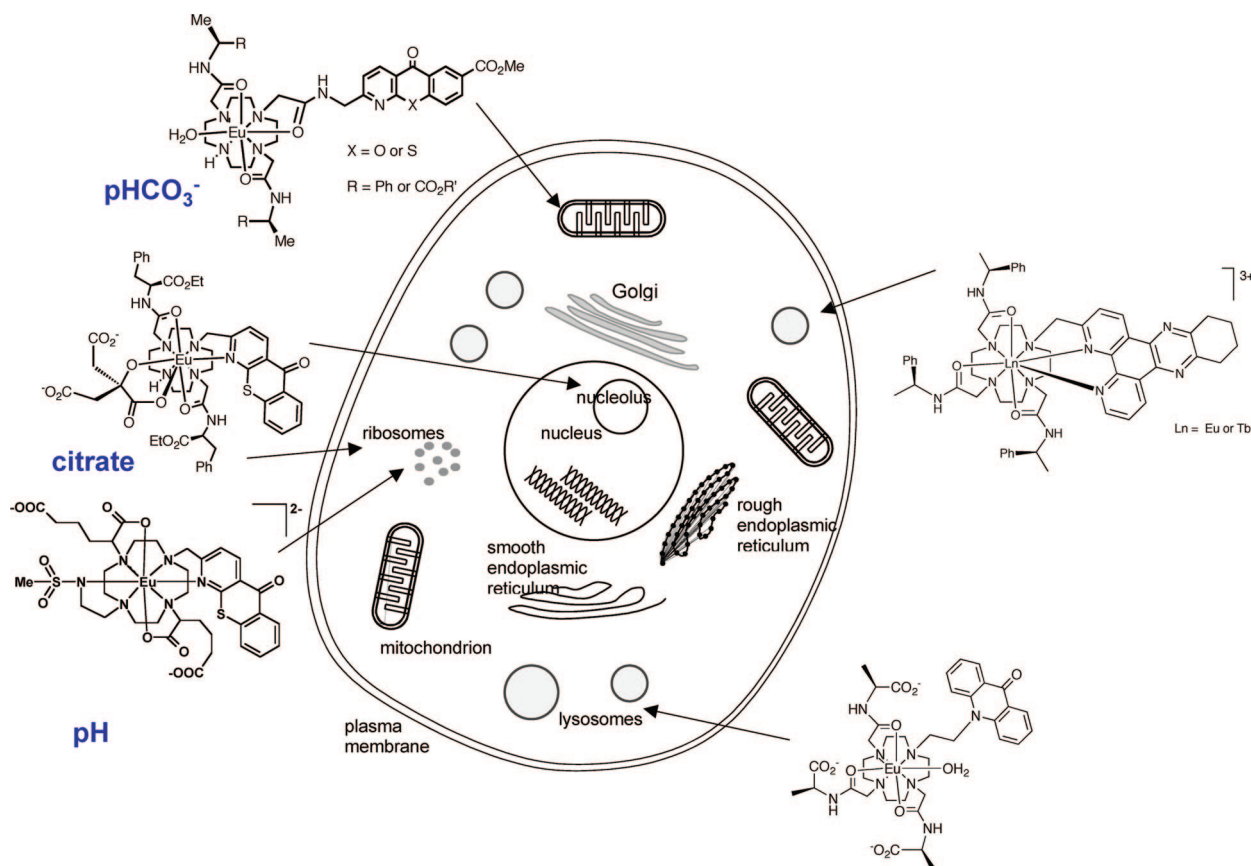
ent complex [Tb·**10f**] ( $\phi_{em} \approx 20\%$ ) for this study incorporates an N-coordinated azaxanthone group and possesses three bulky, lipophilic  $\alpha$ -phenylethylamide arms that suppress intermolecular excited-state quenching by urate/ascorbate and by proteins.<sup>10,17</sup> The 7-substituent on the sensitizer was varied systematically. Examples included polycationic conjugates of Lys-Arg<sub>7</sub> or Arg<sub>8</sub><sup>46</sup> and a lipophilic oligoguanidinium vector<sup>47</sup> that targets mitochondria. The covalent conjugate [Tb·**10**] with human serum albumin (HSA) showed no evidence for cell uptake in either fibroblasts, ovarian, or breast carcinoma cells. Combined single and two-photon ( $\lambda_{exc}$  720 nm) microscopy, flow cytometry and toxicity studies showed that the C<sub>12</sub>-amide derivative rapidly led to necrotic cell death (IC<sub>50</sub> 8 μM) by destabilizing the cell membranes. The oligo-guanidinium conjugate rapidly localized in mitochondria but lowered the mito-

suggesting some membrane destabilization. Very clearly, this study indicates that the nature of the chromophore substituent dramatically influences uptake/trafficking behavior, consistent with the hypothesis that protein binding to the sensitizing moiety regulates these processes.

## 7. Conclusions and Challenges

Luminescence from judiciously designed europium and terbium complexes is long-lived, typical lifetimes are on the order of 1 ms, so either time-gated spectroscopy or microscopy allows the selective detection of this optical signal. With responsive probes, the lifetime, circular polarization, and emission spectral form encodes information that allows an assessment of key biochemical parameters. For exam-





**FIGURE 9.** Schematic illustration of the dominant intracellular localization profiles of the emissive Eu and Tb macrocyclic complexes described in this Account.

ple, the ratio of the intensities of two europium emission bands can be designed to be a function of local pH or the concentration of key metabolites such as citrate, lactate, or urate. These lanthanide(III) complexes exhibit well-defined cell uptake behavior, allowing the use of luminescence microscopy to track their fate *in cellulo*. A first proof-of-principle study, for example, has allowed the intracellular pH of nucleoli to be estimated to be 7.4.<sup>15,42</sup> It is the nature and linkage mode of the sensitizing moiety that primarily defines the extent of protein interaction, in turn defining the cellular uptake/egress rates and the compartmentalization profile. So we are poised to uncover a family of emissive probes, targeted to given organelles (Figure 9), that can report on the ionic composition of the local environment in real time and in living cells. These responsive probes must be chemically reversible and photostable, otherwise they function simply as dosimeters, losing the ability to track changes with time.

Further developments will require improvements to instrumentation making high-resolution time-resolved microscopy and spectroscopy cheaper and more commonplace. The advent of two-photon excitation makes it possi-

ble to excite the probe using lower energy photons (720–820 nm). In this spectral range, biological media, and even tissue, is most transparent. Optical imaging information may also be combined with lower resolution (200  $\mu\text{m}$ ) magnetic resonance imaging (MRI) studies, especially using paramagnetic gadolinium(III) complexes as contrast agents, analogous to the Eu/Tb complexes defined herein. These MRI experiments for *in vivo* studies require specific cell loadings on the order of  $10^8$  complexes/cell, well within the grasp of this approach.<sup>48</sup>

In conclusion, there remains a great deal to be done - or as the Italian poet Ungaretti put it, albeit in a rather different context, "Mi illumino d'immenso".

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**Craig P. Montgomery** was born in Steeton, West Yorkshire, in 1983. He was awarded a First Class M.Chem. (Industrial) degree in Chemistry from Durham University in 2005. He recently was awarded a Ph.D. working with David Parker on luminescent lanthanide complexes as cellular imaging agents or HTRF assay components.

**Benjamin S Murray** was born in Kendal, Cumbria in 1983. He was awarded a first class M.Chem. degree by Durham University in 2005, including a final year project on nucleic acid bio-conjugation strategies working with Dr. David Hodgson. He recently completed his Ph.D. thesis on anion binding studies with responsive lanthanide complexes.

**Elizabeth J. New** was born in Sydney, Australia, in 1984. She has a B.Sc. (Advanced, Hons 1M) from the University of Sydney, from where she also obtained her M.Sc. in 2007 with Trevor Hambley. She is currently a Ph.D. student with David Parker in the Department of Chemistry at Durham University.

**Robert Pal** was born in Eger, Hungary, in 1980. He was awarded a M.Sc. with highest achievement award in Chemistry from KLTE (University of Debrecen), Hungary. He received his Ph.D. in 2007 working with David Parker on responsive luminescent lanthanide complexes. Currently he is a postdoctoral research associate in Durham examining the application of responsive emissive probes.

**David Parker** was born in Leadgate, County Durham, in 1956. He gained a D.Phil. working with John M. Brown on asymmetric catalysis. Following a NATO fellowship with Jean-Marie Lehn, he joined the Department of Chemistry of Durham University in 1982. Since 1992, he has been a Professor of Chemistry, serving twice as Head of Department. His research interests embrace complexation phenomena in solution and their relation to analysis, imaging, and therapy. He is the recipient of numerous awards and prizes and was elected a Fellow of the Royal Society in 2002.

## FOOTNOTES

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