Highlight Review

Lanthanide Luminescent Bioprobes (LLBs)

Jean-Claude G. Bünzli

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Abstract

Lanthanide luminescent bioprobes (LLBs) are amongst the most sensitive luminescent probes because their excited states are long-lived, which allows time-resolved detection (TRD) of their luminescence, boosting the signal-to-noise ratio and the sensitivity; in addition they are fairly insensitive to photobleaching since lanthanide ions are good quenchers of triplet states. Applications of LLBs to immunoassays, DNA analysis, ligand binding assays, analyte sensing, and cellular imaging are reviewed. Future developments in immunohistochemistry, targeting of specific organelles, lab-on-a-chip technology, and instrumental design are evoked.

♦ Luminescence and Bioanalyses

Luminescence and time-resolved luminescence have grown during the past 20 years into major methodologies in biochemistry, biophysics, biotechnology, and medicine. They are central research tools in noninvasive medical diagnostics, flow cytometry, genetic analysis, DNA sequencing, microscopy, and cellular imaging, only to name a few. The reason is simple. Light travels almost instantaneously through a sample (about 30 cm per nanosecond) and can reach regions of complex molecular edifices not accessible to other molecular probes. In addition, light is easily detected by highly sensitive devices (e.g., charge-coupled device cameras) and techniques, including single-photon detection, so that analyses based on luminescence are the most sensitive available, while being environmentally clean, as opposed to methods using radiolabels.

Luminescence is defined as emission of light from an electronically excited state usually produced by excitation with light (photoluminescence), electric current or electric field (electroluminescence), or by a chemical or biochemical reaction (chemi- or bioluminescence), to name a few. Depending on the spin of the initial (emitting) and final (usually ground) states, two categories of emission are distinguished: *fluorescence* for transitions without spin change ($\Delta S = 0$) and *phosphorescence* for transitions with spin change ($\Delta S > 0$). In the case of organic luminophores, which usually bear aromatic residues, absorption of light leads to a singlet excited state, and the return to the ground state is allowed, so that the emission rate is fast, in the range 10^7–$10^9\ s^{-1}$, corresponding to a lifetime of the excited state of 100–1 ns. Due to this short timescale, time-resolved detection (TRD) of fluorescence necessitates sophisticated optics and detection systems but is quite feasible nowadays. On the other hand, spectral discrimination is often impossible to achieve because the Stokes' shifts of the organic chromophores are small. Quinine sulfate (blue), fluorescein (green), acridine orange (AO, yellow), Rhodamine (orange), and pyridine-1 (red) are common fluorescent analytical probes covering the entire visible range; cyanine (CY), Alexa Fluor®, and BODIPY® dyes are examples of more recently proposed luminescent tags.[1]

In some cases the excited organic luminophore relaxes to a triplet state in which one electron has changed its spin. Transitions from this state to the (singlet) ground state are forbidden by selection rules so that emission of light is slow with rates in the range $1–10^3\ s^{-1}$, corresponding to lifetimes of 1 s to 1 ms. TRD experiments are easier to conduct, but the fraction of molecules reaching the triplet state is usually small, at least at room temperature, and due to their long lifetime, triplet states may give rise to photochemical reactions which destroy the luminophore, a phenomenon known as photobleaching.

The need for TRD (or, alternatively, spectral discrimination) is considerable when it comes to bioanalyses because the samples contain a large variety of aromatic substances and when illuminated with UV or blue light, they generate a highly unwanted and intense luminescence background. Given the limitations discussed above, lanthanide ions are increasingly being used as alternatives to organic bioprobes for TRD bioanalyses.

♦ Lanthanide Bioprobes

The lanthanides (Ln = La–Lu, $Z = 57$–71) are a fascinating series of elements which, in aqueous solution, exist essentially under the +3 oxidation state, the only exceptions being cerium(IV) and europium(II). The $4f^n$ electronic configurations of Ln^{III} ions are protected from outside interactions by the more external $5s^25p^6$ subshells of the xenon core. This generates hard and spherical metal ions with large and variable coordination numbers, which can be inserted into a variety of chemical environments. Since the 4f wave functions are fairly pure the numerous electronic transitions (the $4f^6$ configuration of Eu^{III} generates 3003 electronic levels for instance) are narrow and easily recognizable, their energy being fairly insensitive to the chemical environment. Furthermore, most of the ions are luminescent and their emission spans a broad spectral range (Figure 1), from UV (Gd^{III}) to visible (e.g., Sm^{III}, Eu^{III}, Tb^{III}, Dy^{III}, and Tm^{III}) and near infrared (NIR, e.g., Nd^{III}, Ho^{III}, Er^{III}, and Yb^{III}). Some ions are fluorescent, others are phosphorescent, and some are both. Intraconfigurational 4f–4f transitions are forbidden so that the lifetime of excited states is long (μs–ms) allowing easy TRD experiments. The inherent drawback of these ions, namely, the very weak absorbance of the f–f transitions can be circumvented by exciting the Ln-containing chromophores into their surroundings through a process termed "luminescence sensitization."

Prof. Jean-Claude G. Bünzli
Laboratory of Lanthanide Supramolecular Chemistry, Swiss Federal Institute of Technology Lausanne (EPFL), BCH 1402, CH-1015 Lausanne, Switzerland
E-mail: jean-claude.bunzli@epfl.ch

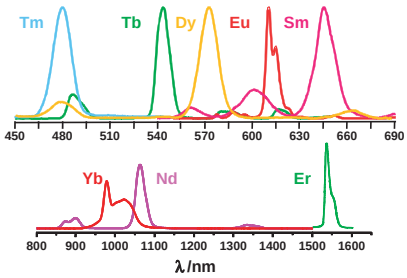

Figure 1. Luminescence of some lanthanide β-diketonates. Redrawn from ref 2.

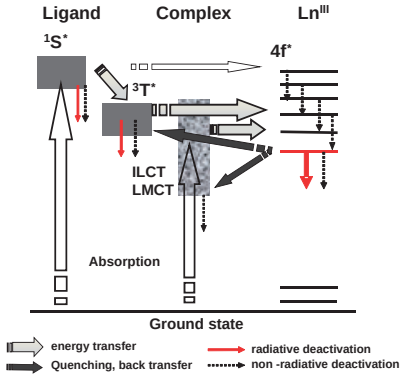

Figure 2. Simplified sketch of the energy-transfer process from an organic antenna to a lanthanide ion; key: $^1S^*$ singlet state, $^3T^*$ triplet state, ILCT intraligand charge transfer state, MLCT metal-to-ligand charge transfer state.

The energy donor is either the coordinating units or one of its substituents (the "antenna"). The energy-transfer process is rather complex and summarily sketched in Figure 2.

As a consequence of this complexity, optimization of this process in lanthanide luminescent bioprobes (LLBs) remains difficult although improvements have been achieved during the past 10 years in modeling energy transfers.[3–5] The luminescence efficiency η(LLB) of a LLB is given by:

$$\eta(\mathrm{LLB}) = \varepsilon_{\max} \cdot Q^{\mathrm{L}}_{\mathrm{Ln}} \quad (1)$$

$$Q^{\mathrm{L}}_{\mathrm{Ln}} = \eta_{\mathrm{sens}} \cdot Q^{\mathrm{Ln}}_{\mathrm{Ln}} = \eta_{\mathrm{sens}} \cdot \frac{\tau}{\tau_{\mathrm{rad}}} \quad (2)$$

where $\varepsilon_{\max}$ is the molar absorption coefficient of the lanthanide-containing edifice at the excitation wavelength, $Q^{\mathrm{L}}_{\mathrm{Ln}}$ is the quantum yield of the lanthanide emission upon excitation into the ligand electronic bands, η_{sens} is the efficiency with which the lanthanide surroundings transfer energy onto the metal ion, $Q^{\mathrm{Ln}}_{\mathrm{Ln}}$ is the intrinsic quantum yield, i.e., the quantum yield measured upon direct metal ion excitation, τ the observed lifetime of the Ln excited state and τ_{rad} its radiative lifetime, i.e., the lifetime in absence of nonradiative deactivation which can be calculated from Einstein spontaneous emission coefficients. In the case of Eu^{III}, a simplified equation gives access to τ_{rad}:[6]

$$A(\mathrm{Eu}) = \frac{1}{\tau_{\mathrm{rad}}} = 15.3 \cdot n^3 \left(\frac{I_{\mathrm{tot}}}{I_{\mathrm{MD}}}\right) \quad [\mathrm{s}^{-1}] \quad (3)$$

in which I_{tot} is the total integrated emission intensity (transitions $^5D_0 \rightarrow {}^7F_J$, $J = 0$–6, i.e., 580–830 nm), I_{MD} the intensity of the magnetic dipole transition $^5D_0 \rightarrow {}^7F_1$ occurring in the range 590–595 nm, and n the refractive index. The values of τ_{rad}(Eu) range typically between 3 and 8 ms.

The intrinsic quantum yield can be optimized by controlling the inner coordination sphere of the metal ion, building a rather rigid environment devoid of high-energy vibrators such as OH or NH groups. On the other hand, η_{sens} is much more difficult to predict in view of the intricate situation prevailing for energy transfer. A sizeable overlap between the emission spectrum of the donor state and the absorption spectrum of the Ln ion is essential, while the overlap with the emitting state should be minimized to avoid back transfer. Since the donor state is usually quite broad while f–f transitions are very narrow, a very small change in the ligand structure may lead to large differences in the sensitization efficiency.

A LLB may be used is several different ways. Basic applications have long been (i) simple substitution of Ca^{II} or Zn^{II} by Ln^{III} in proteins to obtain information on the composition of metal-binding sites (e.g., the number of coordinated water molecules) or metal-to-metal and/or metal-to-chromophore distances by Förster resonant energy-transfer experiments (FRET),[7] or (ii) titration of a biocompound with Ln^{III} salts to determine the number of metal-binding sites.[8,9] Nowadays more subtle applications have arisen for which the lanthanide ion is embedded into a suitable coordinating cavity; the resulting chelate is either used directly, e.g., in analytical responsive probes and simple cellular imaging experiments without specific targeting, or conjugated to a protein or to an antibody which specifically couples with a targeted biomolecule. Requirements for an efficient LLB are numerous and demanding: (i) water solubility, (ii) large thermodynamic stability, (iii) kinetic inertness, (iv) intense absorption above 330 nm, (v) efficient sensitization of the metal luminescence, (v) embedding of the emitting ion into a rigid and protective cavity minimizing nonradiative deactivation, (vi) long excited state lifetime, (vii) when relevant, ability to couple to bioactive molecules while retaining its photophysical properties and not altering the bioaffinity of the host. In view of the wealth of molecules developed to date, the design of LLBs is nevertheless becoming well understood, at least from the chemical and biochemical points of view.[6,10–14]

Generally speaking an LLB can be used directly, luminescence being detected (in TRD mode) after a suitable and specific reaction with the analyte, or indirectly by transferring the excitation energy onto an organic acceptor by a FRET process. An example of direct measurement is depicted in Figure 3 (top) for an homogeneous immunoassay. FRET occurs when the donor (D) and the acceptor (A) lie at distances larger than 40 pm (4 Å), the corresponding mechanism being dipole–dipolar (through space), as opposed to through bond (Dexter mechanism). In this case, the yield of the transfer is given by:

$$\eta_{\mathrm{et}} = 1 - \frac{\tau_{\mathrm{obs}}}{\tau_0} = \frac{1}{1 + \left(\frac{R_{\mathrm{DA}}}{R_0}\right)^6} = \frac{{R_0}^6}{{R_0}^6 + {R_{\mathrm{DA}}}^6} \quad (4)$$

where τ_{obs} and τ_0 are the lifetimes of the donor in presence and in absence of acceptor, respectively, R_{DA} is the D–A distance and R_0 the D–A distance for which the yield of transfer is 50%.

The advantage of FRET is to eliminate the need for washing

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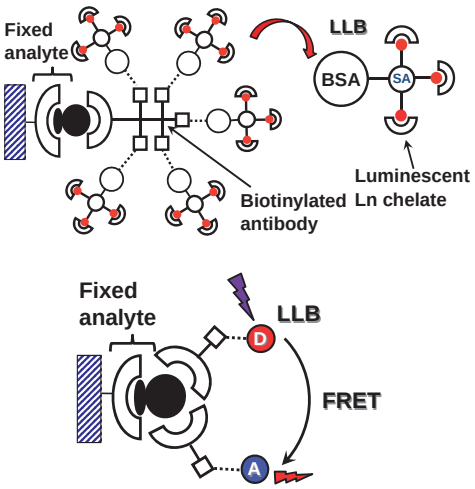

Figure 3. Direct (top) and indirect (bottom) use of a LLB.

unreacted reagents since the transfer only occurs when the two entities, the LLB donor and the organic acceptor, are linked together via some kind of conjugation. The major point here is that the organic fluorophore emits light with an apparent lifetime equal to the lifetime of the donor since its excited state is populated via the LLB. The latter performs better than organic dyes in that the distance at which the transfer may be detected is larger (typically up to 800–1000 pm, as opposed to 500–600 pm). Applications of FRET are far reaching, ranging from homogeneous immunoassays (see below) to DNA hybridization assays, or analyte imaging in cells (such as calcium or phosphorylated molecules).[15]

♦ Bioconjugation

Covalent coupling of a lanthanide luminescent chelate to bioactive molecules such as peptides, proteins, or nucleic acids relies on the presence of chemically reactive groups on these molecules. The most common ones are aliphatic α- or ε-amines. The latter is typical of the amino acid lysine, the pK_a of which is 9.2 so that lysine reacts easily and cleanly above pH 8 to yield stable covalent binding. Moreover, there are usually several lysine groups per protein (e.g., avidin which bears 36 of them). The α-amino group is more acidic with pK_a around 7, and each protein bears at least one such group per subunit or peptide chain. Thiol residues are other common reactive groups. The free thiol group (e.g., in cysteine) is more nucleophilic than amines and generally is the more reactive group in proteins, even at neutral pH. Phenol (e.g., in tyrosine) or carboxylic acids (e.g., in aspartic and glutamic acids) are other potential candidates. To couple with these functionalities, the lanthanide chelate is first activated. Here again, several routes using different functions are possible, the main ones being depicted in Figure 4. In immunoassays, coupling between proteinic amines and LLBs fitted with isothiocyanato, chlorosulfonyl (particularly arenesulfonyl), or 2,4-dichloro-1,3,5-triazinyl groups proved to be the most successful, the latter being more efficient than isothiocyanate.[13] Another very convenient coupling group is *N*-hydroxysuccinimide (NHS, or its sulfo derivative, sulfo-NHS) which can be easily generated by direct reaction of a carboxylic acid with *N*-hydroxysuccinimide in presence of the dehydrating agent

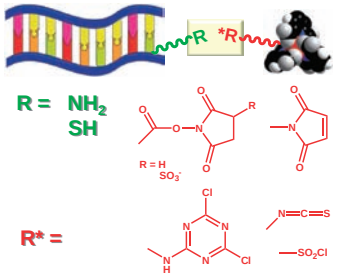

Figure 4. Bioconjugation: typical reacting functional groups.

(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC, or EDCI). Since proteins bear several coupling functions, the number of attached LLBs may be large (up to 46),[16] especially if the bioconjugate is further attached to a protein such as BSA which effortlessly reacts with biotinylated biomolecules as shown in Figure 3 (top); this results in more sensitive analyses.

Since bioaffinity assays for diagnostics are gaining in interest, numerous lanthanide bioconjugates have been synthesized. However, a large fraction of them are described in patent literature.[17] Bioconjugation of LLBs incorporated into nanoparticles, quantum dots, or up-converting phosphors is also feasible, for instance with EDC technology.[18]

♦ Analytical Applications: Time-resolved Luminescence Detection in HPLC

In order to improve the detection limit of HPLC, special instruments have been designed which rely on TRD of lanthanide chelates, particularly Eu^{III} and Tb^{III}, reaching detection limits in the range 10^{-10}–10^{-12} M. Lanthanide labels are used in several different ways: (i) if the analyte has α,β-unsaturated carbonyl groups, it sensitizes the luminescence of the probe in protective micelles, (ii) a nonluminescent lanthanide chelate is covalently bound to the analyte and converted into a luminescent entity by postcolumn treatment with an enhancement solution, (iii) as a combination of the previous two techniques, the nonluminescent label is bound to silica gel surface, and the analytes are detected as they move along the column because they transfer energy onto the metal ion, and (iv) direct labeling of the analyte by a highly luminescent lanthanide tag. The latter system has been applied to the determination of estrogens in drinking water and detection limits are as low as 2 ng/L.[19]

♦ Analytical Applications: Time-resolved Luminescent Immunoassays

Time-resolved luminescent immunoassays have been a standard practice for more than 20 years for quantitative determination of clinical compounds that are in very low concentration and for which no specific chemical methods are at hand. These include proteins (antigens and enzymes), hormones, and drugs. These commercially available assays, which compete quite favorably with ELISA (enzyme-linked immunosorbent assays) have replaced the waste-generating radioimmunoassays, with comparable or better sensitivity and they are often less time-consuming.

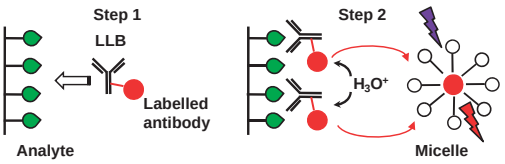

Figure 5. Principle of a heterogeneous immunoassay (DELFIA®, dissociation-enhanced fluoroimmunoassay).

The first assays proposed were so-called heteroimmunoassays, conducted in two steps (Figure 5). Firstly, a labeled antibody is coupled with the analyte and excess reagents are washed. The lanthanide chelate (Ln = Eu, Sm, Tb, or Dy) is then dissociated by lowering the pH, and an enhancement solution is added which contains a chelating agent (usually a beta-diketonate) and Triton X-100 to form a protective micelle around the luminescent chelate. The emitted light is detected in TRD mode.[20–23]

Homogeneous immunoassays have the advantage of being performed in one step since they use the FRET technology: luminescence from the organic acceptor (Figure 3, bottom) is only detected in TRD mode when effective transfer between the donor LLB and the acceptor occurs; that is, there is no need to eliminate excess reagents. Both Eu and Tb LLBs are used in these assays which also apply to drug screening, hybridization assays, receptor–ligand interactions or real-time PCR technology, to name a few.[13,14,24,25]

Interesting developments are the use of nanoparticles,[26,27] quantum dots,[28] and of up-converting phosphors (UCPs).[18,29] Inserting the LLBs into submicron-sized particles contributes to protect them against nonradiative deactivation, which increases the sensitivity. As an example, the detection limit of free prostate-specific antigen (PSA) can be lowered to 0.1 ng/mL.[26] In the case of UCPs, since near-infrared light (NIR) is much less absorbed by biological tissues and fluids than visible light, excitation is more efficient, especially that this also avoids exciting the autofluorescence of the samples.

♦ Analytical Applications: Responsive Probes

A series of successful lanthanide-responsive probes for the analysis of various analytes of biological relevance are based on the cyclen framework. The latter usually features three coordinating units (carboxylates, substituted amides, or phosphinates) and one sensitizing pendant which usually also coordinates the central metal ion (Figure 6). Signaling is achieved either by enhancing or quenching the metal-centered luminescence. The three main modes of action of the LLB can be understood by reference to Figure 2: (A) the analyte partially quenches the $^1S^*$ state of the sensitizer, resulting in a decrease in Ln luminescence; this may be caused by electron or charge transfer, as well as by protonation or metal binding of the sensitizer; (B) the triplet state of the sensitizer is perturbed by interaction with the analyte: metal ion binding will alter the energy of this state, while collisional quenching, for instance by molecular oxygen, will quench the triplet state if its rate, $k_q[O_2]$, is competitive with k_{et}; (C) the Ln excited state is quenched by interaction with an analyte featuring high-energy vibrations or by energy transfer onto the analyte; alternatively, the analyte may replace a quenching water molecule in the first coordination sphere and light up the Ln luminescence.

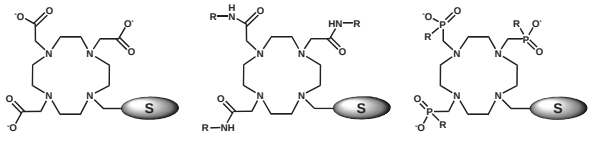

Figure 6. Cyclen core structures used for lanthanide luminescent responsive probes (S = sensitizing unit).

Typical parameters which can be determined are pH, $p(O_2)$, and the concentration of anions (halides, oxyanions such as HPO_4^{2-} or SO_4^{2-}, acetate, oxalate, malonate, succinate) or aminoacids.[30] Alternate substrates have been used for the same purpose, for instance, chiral tripodal ligands for the analysis of anions,[31] polyaminocarboxylates immobilized on sensory chips for detection and separation of histidine-tagged ubiquitin proteins,[32] or dendrimeric complexes for NIR detection of anions.[33] Eu- and Tb-based luminescent probes responsive to nucleotides and phosphates are also commonly used to determine enzyme activities.[34]

♦ Proteins and Nucleic Acid Staining

Separation and quantitative analysis of complex protein mixtures are commonly performed with gel electrophoresis. Proteins are directly detected in the polyacrylamide gels or after electro blotting onto membranes by staining with inorganic (e.g., silver and gold) or organic (e.g., Coomassie blue) dye or by fluorescence labeling techniques (e.g., coumarin). Recently, a europium chelate, Eu^{3+}–BHHT–SO_3^- was shown to be at least as sensitive as other available techniques while detected in TRD mode (BHHT stands for 4,4′-bis(1″,1″,1″,2″,2″,3″,3″,3″-heptafluoro-4″,6″-hexanedione-6″-yl)chlorosulfo-*o*-terphenyl). This LLB can also be used in 2D-polyacrylamide gel analyses.[12]

In order to understand the fate of proteins in vivo, Imperiali and co-workers have developed a series of LLBs in which the emissive lanthanide ion is linked to a small, genetically encoded protein fusion partner. The latter contains sensitizer moieties strategically located on the residue for efficient energy transfer. Typically, tryptophan (trp) is used for sensitizing Tb upon excitation at 280 nm, while acridone (acd) is an efficient chromophore for Eu under excitation at 390 nm. Carbostyril 124 (cs124) sensitizes the luminescence of both Eu and Tb upon excitation at 337 nm.[35] A typical sequence of peptides developed for strong Ln binding is H_2N–FITDNNDGXIEGDELLLEEG–$CONH_2$ in which the amino acid labeled X bears the sensitizing unit (trp, acd, or cs124). With standard laboratory equipment, these tags display comparable sensitivity as Coomassie blue, but application of TRD detection could largely lower the detection limit of the LLB-tagged proteins.

Nucleic acid diagnostics are a cornerstone of biological applications. Several analytical methods using LLBs have been proposed with sensitivity in the subnanomolar range, taking advantage of either FRET processes in hybridization probes[36] or of LLB-doped nanoparticles.[37] We have also recently proposed a versatile method, for both DNA and low molecular weight PCR products.[38]

♦ Time-resolved Cellular Imaging

Cellular and tissue imaging is another tool essential to diag-

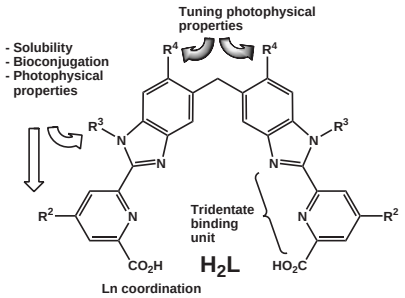

Figure 7. Ligand framework used for assembling bimetallic helical LLBs.

nostics and therapy. Bornhop and collaborators have proposed brightly emitting Tb macrocyclic labels for in vivo and in vitro analysis of abnormal tissues.[5,39] Europium and terbium chelates with the ligands depicted in Figure 6 have been shown to stain either the nucleoli or the cytoplasm of various cells lines. A systematic study of several such complexes has revealed interesting behaviors with respect to localization in the cell (nucleoli, mitochondria, or endosomes/lysosomes) and cytotoxicity, but a precise structure/activity profile remains to be found.[40] In our laboratory we have concentrated on producing bimetallic LLBs by self-assembly at room temperature under physiological conditions. In this context, hexadentate ditopic ligands derived from a pyridine substituted in the 2-position by a strongly coordinating group, e.g., a carboxylic acid, and in position 6 by a derivatised benzimidazole moiety have proved to be an entire class of novel and versatile receptors (Figure 7). Homobimetallic[41] triple-stranded helicates are obtained in which the nine-coordinated metal ion environment has a tight pseudo-tricapped trigonal prismatic geometry resulting in remarkable luminescent properties. In addition, the presence of two emissive centers, could lead to bimodal probes, either dual luminescent or luminescent and magnetic,[42] and the inherent chirality to chiral probes. Sufficient hydrophilicity is achieved through the grafting of a short polyoxyethylene chain in R^2 or R^3 positions which by the same token is used for bioconjugation.

Thermodynamic, photophysical, and biochemical properties of the homobimetallic helicates[41] are adequate for cellular imaging. The helicates $[Ln_2L_3]$ are the major species in solution (>95% at total ligand concentration of 100 μM), the quantum yields are large (10–24% for Eu^{III}), the luminescence of several other ions (e.g., Sm^{III}, Tb^{III}, and Yb^{III}) is sensitized, and lifetimes are long, e.g., 2.3–2.4 ms for $Eu(^5D_0)$. These LLBs are taken up into the cytoplasm of several lines of cancerous (HeLa, MCF-7, HaCat) and noncancerous (Jurkat) cells by endocytosis while being noncytotoxic (IC_{50} > 500 μM) and showing very slow egress.[41,43–46] The helicates are localized in lysosomes and later liposomes of the endoplasmatic reticulum as shown on Figure 8, in which one clearly sees the localization of the LLB. With the best bimetallic helical LLB designed so far, TRD luminescence microscopy images can be obtained for incubation concentration as low as 5 μM.[47] Extending the excitation wavelength in the range 350–400 nm is also at hand.[48]

♦ *Perspectives and Challenges*

As shown in the sections above, several key biological parameters may be extracted from complex systems thanks to responsive lanthanide luminescent bioprobes, in view of their very specific photophysical properties; in particular, time-resolved detection offers an edge over conventional measurement techniques. After the large breakthroughs achieved in time-resolved luminescent immunoassays twenty years ago, present efforts focus on even more elaborate analyses and on cellular imaging. For instance, proof of principle has been established for measuring the pH in the nucleoli[49] and elaborate bioconjugation should soon lead to the targeting of given organelles and to the measurements of other key analytes in them. Further developments currently include immunohistochemical imaging, detection of cell apoptosis,[13] and lab-on-a-chip technology.[50] Multicolor labelling for bioaffinity assays and imaging, dual labelling combining magnetic resonance and optical imaging, as well as NIR-emitting bioprobes are further tracks along which many interesting phenomena will be discovered. Furthermore, one of the main limitations of LLBs, namely, their excitation wavelength in the range 320–400 nm is being lifted with the advent of multiphoton excitation between 700–980 nm. Interesting instrumental developments should also take place to fully take advantage of the time-resolved detection potential in microscopy with multicolor labels and of the range of available laser diodes as excitation sources.

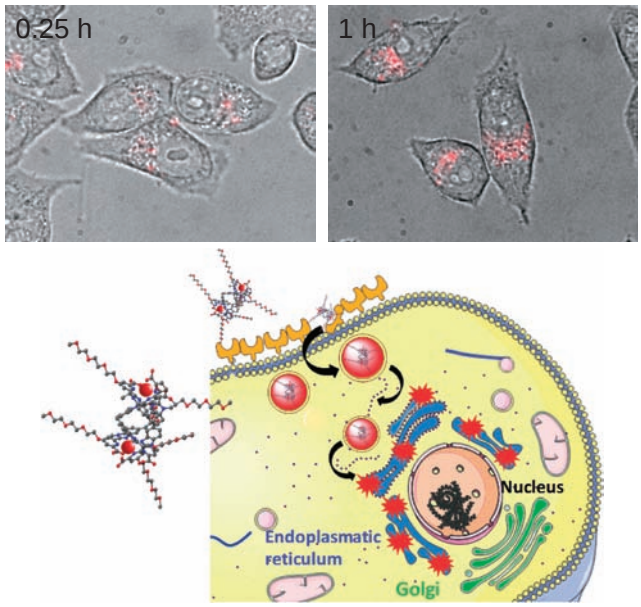

Figure 8. (Top) Time-resolved luminescence microscopy image of a bimetallic triple helicate LLB in live HeLa (cervix cancer) cells incubated at 37 °C (5% CO_2) in RPMI 1640 containing 100 μM of the LLB after 15 and 60 min. (Bottom) Schematic representation of the uptake of $[Ln_2L_3]$ helicates and their migration into the endoplasmatic reticulum.

With all these advantages at hand, there is no doubt that LLBs will have a larger share in bioanalyses, medical diagnostics and therapy in the near future.

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