DOI: 10.1002/ejic.200800332

# Lanthanide Complexes and Quantum Dots: A Bright Wedding for Resonance Energy Transfer

**Loïc J. Charbonnière*[a] and Niko Hildebrandt[b]**

**Keywords:** Lanthanides / Quantum dots / Immunoassays / Luminescence / FRET (Fluorescence Resonance Energy Transfer)

In this microreview we describe the principle of Förster resonance energy transfer (FRET) occurring between closely spaced energy-donor and -acceptor molecules. The theoretical treatment is depicted in relation with the data extractable from spectroscopic measurements. We present the specific case of semiconductor nanocrystals (or quantum dots – QDs) as energy donors in FRET experiments and a particular emphasis is put on the specific advantages of these fluorophores with regard to both their exceptional photophysical properties and their nanoscopic morphology. In a following section, the special attributes of luminescent lanthanide complexes (LLCs) are outlined with illustrations of properties such as their characteristic emission spectra, long-lived luminescence, and large "Stokes shift". Finally, the successful combination of LLCs and QDs in FRET experiments is demonstrated, showing the unrivaled benefits of this singular marriage, opening doors for energy transfer at very large distances and excellent sensitivity of detection within the frame of time-resolved fluoroimmunoassays.



## Introduction

The second half of the last century has witnessed tremendous discovery in molecular and supramolecular phenomena, and the nanoworld has half-opened numerous doors with their nanoquestions behind them. Although technological issues, such as the incoming of electronic microscopy techniques, allowed for a deep look inside the submicrometer dimensions, there still remain black boxes where the basic understanding of the events occurring at that scale have to be elucidated. Among those, most biological events involving the smallest molecules, multiple large proteins, or elongated D(R)NA strands in complex matrices of organic compounds are challenges for researchers who possess only few analytical tools to meet them.

At distances shorter than ca. 10 nm, Förster resonance energy transfer (FRET) spectroscopy is an important technique used to study encountering and recognition events of molecules. FRET can occur between an excited energy donor and an acceptor molecule, provided that they are in

[a] Laboratoire de Chimie Moléculaire associé au CNRS, ECPM, 25 rue Becquerel, 67087 Strasbourg Cedex 02, France
Fax: +33-3-90-24-27-42
E-mail: charbonn@chimie.u-strasbg.fr
[b] Physikalische Chemie, Institut für Chemie und Interdisziplinäres Zentrum für Photonik, Universität Potsdam, Karl-Liebknecht-Strasse 24-25, 14476 Potsdam-Golm, Germany
E-mail: hildebr@uni-potsdam.de
www.chem.uni-potsdam.de/pc

*Loïc Charbonnière was born in Lyon (France) in 1968. From 1988 to 1991 he studied chemistry at the European Higher Institute of Chemistry of Strasbourg and received a MS in organic chemistry at the University Louis Pasteur of Strasbourg in 1993. He then joined the group of Prof. Alan Williams at the University of Geneva (Switzerland), where he obtained his PhD in 1996 for his work on dinuclear triple stranded helicates. After working as an assistant at the University of Lausanne (Switzerland) with Prof. Jean-Claude Bünzli, he returned to Strasbourg at the end of 1997, in the group of physical chemistry working with Dr. Françoise Arnaud. Since 1998, he has been working as a researcher at the Centre National de la Recherche Scientifique in the Laboratory of Molecular Chemistry directed by Dr. Raymond Ziessel. His main research interests are the design and synthesis of lanthanide complexes applied to labeling and sensing, and more recently he has focused on the development of lanthanide-based nanoscopic luminescent scaffolds.*

*Niko Hildebrandt was born in Bad Segeberg (Germany) in 1975. From 1997 to 2001 he studied Medical Physics at the University of Applied Sciences in Berlin (Germany). In 2001 he joined the group of Prof. Hans-Gerd Löhmannsröben in the Department of Physical Chemistry at the University of Potsdam (Germany), where he obtained his PhD in 2007 for his work on laser spectroscopy of biochemical FRET systems by using quantum dots and lanthanide complexes. Since 2007 he has been working as a researcher in the same group working on the integration of nanophotonic FRET systems into in vitro diagnostics and point-of-care analysis.*

   

close spatial proximity and that there is significant spectral overlap of donor emission and acceptor absorption. FRET applications provide insight into the spatial arrangement of the donor–acceptor couple such as their intermolecular distance, their relative orientation, or their distribution. Thus, FRET has become an outstanding tool for the study of intermolecular interactions with many applications in biological technologies, sensing, and conformational studies. Nevertheless, there are still opportunities to further enlarge the scope of FRET applications, in particular by adapting FRET to newly developed luminescent compounds as energy donors or acceptors, and also by the design of nanometric scaffolds for improved energy transfer.

This microreview aims to highlight the exceptional marriage of two such luminescent partners, namely, semiconductor nanocrystals (quantum dots, QDs) and luminescent lanthanide complexes (LLCs). After a brief summary of the main FRET principles important for spectroscopic applications, the specific advantages of QDs and LLCs will be emphasized separately before concluding on the advances attainable by their combination in FRET experiments.

## 1. The Principle of FRET

The theory of FRET defines a $1/r^6$ distance-dependent, nonradiative transfer of energy from an excited donor (D) to an acceptor molecule (A). First experimental results of energy transfer over distances larger than collision radii date back to 1922.[1–3] Important contributions to the development of FRET were made by Hartmut Kallmann and Fritz London[4] as well as by Jean[5,6] and Francis[7,8] Perrin. However, the relationship between easily accessible spectroscopic data and theoretical equations (that are still valid today) was the achievement of Theodor Förster,[9–15] thus enabling the possibility of many FRET applications in all kinds of natural sciences. A very interesting summary concerning the history of FRET can be found in a recent publication by Robert Clegg,[16] whereas detailed theory and various modern aspects of FRET development are compiled in several excellent books and reviews.[17–23] Here, we would like to give a short overview of the important theoretical and practical aspects necessary for FRET applications.

The bottom line of spectroscopic properties for a successful FRET application is the so-called Förster radius, $R_0$, the distance between D and A where the energy transfer is 50% efficient. $R_0$ can be calculated from spectroscopic data of donor luminescence and acceptor absorption, which are both relatively easy to measure.

The equation derived by Förster is Equation (1):

$$R_0^6 = \frac{9000(\ln 10)\Phi_D\kappa^2}{128\pi^5 n_r^4 N_{Av}} J(\lambda) \tag{1}$$

in which $n_r$ is the refractive index of the surrounding medium (e.g., $n_r$ = 1.33 for water), $N_{Av}$ is Avogadro's number ($6.022\times10^{23}$ mol$^{-1}$), $\Phi_D$ is the luminescence quantum yield of D, and $\kappa^2$ is an orientation factor taking into account the relative orientations of the D and A dipoles, which can range from 0 to 4. The value of $\kappa^2$ is often assumed to be 2/3, as for a random orientation of D and A, but discussions about this orientation factor could fill several books and we advise the interested reader to peruse the literature cited above. The last term in Equation (1) defines the overlap integral of D emission and A absorption [Equation (2)]:

$$J(\lambda) = \int_0^\infty f_D(\lambda)\ \varepsilon_A(\lambda)\ \lambda^4\ \mathrm{d}\lambda \tag{2}$$

with the acceptor absorbance $\varepsilon_A(\lambda)$ and the normalized donor luminescence $f_D(\lambda)$ at wavelength $\lambda$ for which [Equation (3)]

$$f_D(\lambda) = \frac{F_D(\lambda)}{\int_0^\infty F_D(\lambda)\mathrm{d}\lambda} \tag{3}$$

where $F_D(\lambda)$ is the donor luminescence intensity at the wavelength $\lambda$. If $f_D(\lambda)$ is in nm$^{-1}$, $\varepsilon_A(\lambda)$ in M$^{-1}$ cm$^{-1}$, and $\lambda$ in nm, then $J(\lambda)$ is in M$^{-1}$ cm$^{-1}$nm$^4$ and the Förster radius (in Å) can be calculated by Equation (4):

$$R_0^6 = 8.79\times10^{-5} n_r^{-4}\Phi_D\kappa^2 J(\lambda) \tag{4}$$

The FRET rate constant $k_{\mathrm{FRET}}$ is defined by Equation (5):

$$k_{FRET} = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{5}$$

where $r$ is the D–A distance and $\tau_D$ is the D luminescence decay time (in the absence of A).

FRET efficiency $E_{FRET}$ can be defined as the fraction of donor excited states leading to acceptor excited states [expressed in rate constants with subscript "$L$" for luminescence and "$NR$" for nonradiative; Equation (6)]:

$$E_{FRET} = \frac{k_{FRET}}{k_{FRET} + k_L + k_{NR}} \tag{6}$$

$E_{FRET}$ can be calculated by distances ($R_0$ and $r$) or by the luminescence quantum yields ($\Phi$), decay times ($\tau$), or intensities ($I$) of D in the absence (subscript "$D$") and in the presence (subscript "$DA$") of A [Equation (7)]:

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{\Phi_{DA}}{\Phi_D} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{I_{DA}}{I_D} \tag{7}$$

Because of higher measurable accuracy, luminescence decay times should be preferentially used instead of quantum

  

yields (quantum yield equals decay time multiplied by rate constant: $\Phi = \tau k_L$). Often, the experimentally easily accessible donor steady-state luminescence intensities $I_D$ and $I_{DA}$ are used. These values must be normalized to their respective concentrations of D. As a result of possible experimental errors such as trivial reabsorption; secondary fluorescence; incomplete labeling of the molecules of interest; concentration differences of labeled, unlabeled, bound, and unbound Ds and As; and changes in the microenvironment, determination of both luminescence decay times and intensities is advisable. In order to be able to clearly distinguish between FRET and other quenching mechanisms, one should work with both luminescent Ds and As. In this case, an increased luminescence of A together with a decreased luminescence of D would be strong evidence for D to A energy transfer.

Determination of $E_{FRET}$ from sensitized acceptor luminescence can be difficult due to donor background emission and other experimental errors (vide supra). Assuming no luminescence of D at the emission wavelength of A, complete donor labeling, and low absorbances, $E_{FRET}$ can be calculated by Equation (8):

$$E_{FRET} = \frac{A_A(\lambda_D)}{A_D(\lambda_D)}\left[\frac{I_{AD}(\lambda_D)}{I_A(\lambda_D)} - 1\right] \quad (8)$$

where $\lambda_D$ is an absorption wavelength of D, $A_A(\lambda_D)$ and $A_D(\lambda_D)$ are the absorbances of A and D at $\lambda_D$, respectively, and $I_{AD}(\lambda_D)$ and $I_A(\lambda_D)$ are the emission intensities of A in the presence and in the absence of D (both excited at $\lambda_D$), respectively.

Measuring acceptor luminescence decay times is another possibility to calculate $E_{FRET}$. As an excited acceptor (A*) is generated by FRET from an excited donor (D*), the concentration of A* after pulsed excitation can be expressed by a differential equation with rate constants (superscript "*A*" stands for acceptor) and concentrations of D* and A* [Equation (9)]:

$$\frac{d[A^*]}{dt} = k_{FRET}[D^*] - (k_L^A + k_{NR}^A)[A^*] \quad (9)$$

The first part of the equation describes the increase in the acceptor excited-state population owing to FRET from D* and the right part of the equation represents radiative and nonradiative deactivation of A*. Equation (10) results by solving the differential equation and by assuming no direct excitation of A:[21]

$$[A^*] = \frac{E_{FRET}[D^*]_0}{1-(\tau_{DA}/\tau_A)}\left[\exp\left(-\frac{t}{\tau_A}\right) - \exp\left(-\frac{t}{\tau_{DA}}\right)\right] \quad (10)$$

where $[D^*]_0$ is the initial concentration of D* and $\tau_A$ is the luminescence decay time of A in the absence of D.

Figure 1 represents the temporal evolutions of the concentrations of the D and A excited states [calculated from Equation (10)] for varying values of $\tau_A$ and $E_{FRET}$ ($\tau_D$ = 1 ms and $[D^*]_0$ = 1).

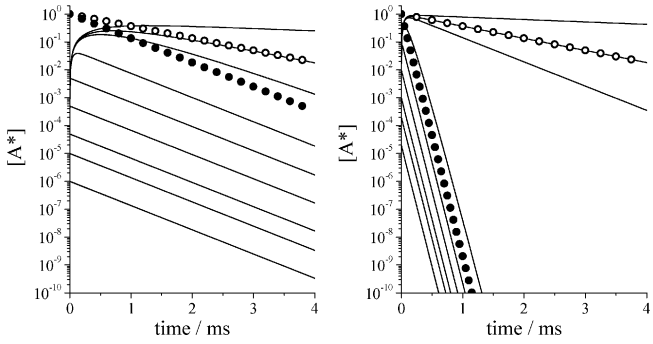


Figure 1. Concentration decay curves of D* (pure donor – open circles), $D^*_A$ (D in the presence of A – filled circles), and $A^*_D$ (A in the presence of D – solid lines) calculated with Equation (10) for $\tau_A$ = 1 ns, 10 ns, 50 ns, 500 ns, 5 µs, 50 us, 500 µs, 1 ms, and 5 ms (from bottom to top), $\tau_D$ = 1 ms, and $[D^*]_0$ = 1. Left: $E_{FRET}$ = 0.5; right: $E_{FRET}$ = 0.95.

It becomes obvious that the decay times $\tau_{AD}$ (slopes of the solid-line curves) and $\tau_{DA}$ (slope of the filled-circle curve) are equal when $\tau_A << \tau_{DA}$. In the case of a very large difference between the luminescence decay times of D and A, such as in the case of LLCs as Ds and QDs or fluorescent compounds as As, $\tau_{DA}$ can be replaced by $\tau_{AD}$ in Equation (7) and measurement of the luminescence decay time of A becomes a comfortable way to gain insight into FRET efficiency.

Interestingly, it can also be seen that for lower values of $E_{FRET}$ and high values of $\tau_A$, a transitory rise of A* is clearly present and should be detectable in the luminescence evolutions of such systems.

The transfer efficiency is also influenced by the number of Ds and As. Many different oligomeric D–A arrangements are conceivable, and in a recent publication, V. Raicu developed a FRET theory for these cases.[24] Finding a simple equation for $E_{FRET}$ considering all these cases is difficult, as the efficiency will be influenced by several other factors (besides the number of Ds and As) such as the excited-state lifetimes of D and A, the amount of D* and A* after excitation, and the distances between the different Ds and As. A qualitative view on the influence of $E_{FRET}$ for particular cases of multiple D and A arrangements will be provided.

It is obvious that $E_{FRET}$ will increase with the number of As ($n$) around a single D, as the probability for D to transfer its energy will increase with the number of possible energy pathways. An arrangement with a single QD donor to several As[27] leading to an equation for $E_{FRET}$ will be described in Section 2. In contrast, it was also shown that a high D to A ratio can be unfavorable,[25,26] as simultaneous FRET from several Ds ($m$) to A cannot occur (one A can be excited by only one D). If the $m$ Ds and A have similar luminescence lifetimes, the probability of each D to transfer its energy to A will decrease with the number of Ds (due to

  

the competition between the different Ds). However, if the Ds have long-lived excited states (e.g., LLCs with ms lifetimes), one A with a short-lived excited state (e.g., fluorophores or QDs with ns lifetimes) can accept the energy from several Ds successively (nonsimultaneously). In this case, the competition between the different Ds can be neglected.

Concerning spectroscopic applications, the number of Ds and As is very important for the brightness of the complete FRET system. This can be defined as the number of photons created by FRET (emission intensity of A as a result of FRET from D) divided by all photons used for excitation. Assuming plenty of excitation photons in the case of D and A having approximately the same excited state lifetimes, the brightness will increase with $E_{FRET}$ when $n$ is increased as a result of more excited As. Increasing the number of Ds will not change the brightness (although $E_{FRET}$ decreases due to competition), as only the D luminescence intensity will increase but that of A will remain the same. For long-lived excited state Ds (although $E_{FRET}$ is not influenced), the brightness linearly increases with $m$ because of more FRET processes. In this case, the number of As does not influence the brightness because a single A can already accept energy from several Ds.

The multiple donor influence on brightness is very important, for example, in immunoassays, where very low analyte concentrations have to be detected. The more analyte-specific light is produced, the higher the signal-to-background ratio. The use of several donors and one acceptor was already found to improve sensitivity in FRET assays.[28]

In summary, FRET allows precise and sensitive measurements on a nanometer scale by using steady-state and/or time-resolved spectroscopic techniques. QDs as well as LLCs are theoretically very well suited for FRET. In the following sections we want to outline that this theoretical hypothesis also holds for practical applications.

## 2. Properties of QDs and Their Particular Advantages in FRET Experiments

QDs appeared in the recent years as particularly interesting probes for FRET experiments. They are composed of a nanometer-sized core of a semiconductor material, often coated by a passivating shell consisting of a larger bandgap semiconductor, and an external layer of surface ligands introduced during the synthesis or after an exchange step. In many aspects, their physicochemical properties surpass those of fluorescent compounds.[29] Depending on both the semiconductor material and the size of the nanocrystals,[30] the absorption and emission properties can be tuned at will in almost all of the spectral domain from UV to near infrared. QDs display extremely large one- and two-photon absorption cross sections, the possibility of large spectral separations between excitation and emission, and narrow emission bands. First developed as suspensions in organic solvents,[31] the interest in understanding the synthesis of nanocrystals in general[32] and of QDs in particular, led to the availability of water solubilization of QDs by using various strategies based on their surface capping with hydrophilic molecules.[30,33] Recent examples even described the direct synthesis of highly luminescent CdTe QDs and quantum rods in aqueous solutions.[34,35] QDs display good-to-excellent luminescence quantum yields and are far more resistant to photobleaching than organic dyes.[36] From all the above mentioned properties, QDs appear as excellent probes for FRET experiments, and following the pioneering works in the solid state[37] and in solution,[38] demonstration was achieved in numerous analytical applications. Much of this work has been reviewed in excellent monographs[20,29,30,33,39–42] and rather than recalling them again, we will put the emphasis on the particular advantages of using QDs in solution based FRET experiments. It is already to be noticed that a large part of these contributions deals with QDs as energy donors in FRET. The case of QDs as energy acceptors will be discussed in more detail in Section 4, as almost all examples deal with the use of LLCs.

### QD Size Adjustment for FRET Efficiency Optimization

During the synthesis of the QDs, the nucleation step is followed by a growing step, in which the core size gradually increases, with a concomitant bathochromic shift in the absorption and emission spectra.[43,44] As $E_{FRET}$ increases with $R_0$ [Equation (7)], optimization of the energy transfer can be obtained by fine tuning the Förster radius within a D–A pair. This could be achieved by a controlled synthesis of QDs, defining the spectral QD properties. In a set of FRET experiments consisting of the surface attachment of different Alexa Fluor acceptors to amino-functionalized CdSe/ZnS core/shell QD donors, such an optimization was nicely illustrated by Nikiforov et al.[45] Three kinds of QDs emitting at 565, 605, and 655 nm were labeled with Alexa Fluor 680, which strongly absorbs at 679 nm ($\varepsilon$ = 184000 $M^{-1}$ $cm^{-1}$) and emits at 702 nm. $R_0$ values for the three kinds of labeled QDs were calculated, and $E_{FRET}$ was determined by monitoring the fluorescence intensity of labeled and unlabeled QDs. Thus, the average D–A distances were accessible according to Equation (7). The measured values are collected in Table 1.

Table 1. Calculated $R_0$, measured $E_{FRET}$, and interchromophoric distances ($r$) between QDs and Alexa Fluor 680.[45] The CdSe core sizes were estimated from spectroscopic data.[43,44]

| QD emission wavelength / nm | $R_0$ / Å | $E_{FRET}$ | $r$ / Å | Estimated CdSe core size / Å |
|---|---|---|---|---|
| 565 | 55.2 | 0.35 | 95.8 | 34–40 |
| 605 | 67.5 | 0.61 | 97.1 | 46–48 |
| 655 | 84.0 | 0.79 | 102.2 | 83 |

Interestingly, one can notice that despite an increase in the D–A distance $r$, $E_{FRET}$ increases significantly due to the larger $R_0$ values. Unfortunately, a direct correlation between QD size and $E_{FRET}$ can hardly be obtained. The commercially available QDs are protected by a passivating shell and further covered by a water-solubilizing polymer layer. $R_0$ and $E_{FRET}$ are dependent on the luminescence quantum

  

yield of D, which, in the case of QDs, may vary as a function of the core size,[34] of the thickness of the passivating shell,[46,47] or of the organic layer.[47] Therefore, the overall size of the QDs cannot be directly linked to the spectroscopic properties. This is illustrated by the comparison with the estimated core sizes (Table 1) that one would obtain from literature data.[43,44] The increase from QDs 565 to 655 should double the core size, whereas only a 6.4 Å increase in $r$ was observed, probably as a result of the different thicknesses of the outer layers of the different QDs. However, as the Alexa Fluor acceptor is covalently linked to the surface of the nanocrystals with a short spacer, $r$ is a good estimate of the overall radii of the QDs.

Other examples of size matching have been reported,[27,48] and further perspectives in this field may emerge from smart control of the QD spectroscopic properties, as demonstrated by the bandgap engineering of CdTe nanocrystals through chemical surface modifications.[49]

## Multiple FRET within a Single QD

Conventional FRET systems usually consist of a single donor molecule and a single acceptor one. As mentioned in Section 1, $E_{FRET}$ can be increased if more than one acceptor molecule is present in the vicinity of the donor. The nanometer-sized spherically (or ellipsoidally) shaped QDs present a particular advantage in this approach. The large surface can be functionalized to introduce multiple interaction sites, even with large molecules such as proteins, while keeping the whole system in a relative compact volume.

Following this approach, Mattoussi and coworkers designed an elegant system in which CdSe/ZnS core/shell QDs, water solubilized by dihydrolipoic acid ligands, were functionalized at their surface by maltose binding proteins (MBP). The MBP were genetically modified to express a five histidine sequence at their C termini,[50] which allows strong surface interactions with the ZnS shell (Figure 2).

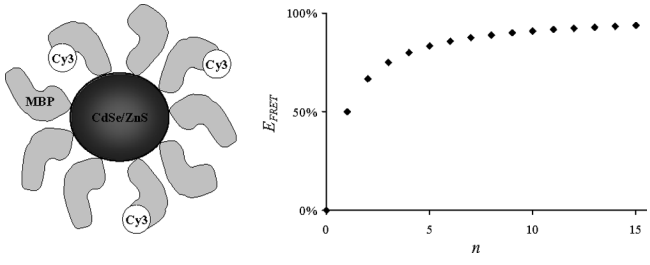


Figure 2. Schematic drawing of the interaction of CdSe/ZnS QDs and maltose binding proteins (MBP), some of which are labeled with Cy3 dyes (left). Theoretical evolution of $E_{FRET}$ as a function of $n$, the number of acceptors per QD donor for $r = R_0$ [Equation (11)].[27,48]

In their study, the authors fixed the overall ratio of $his_5$-MBP to QDs to 15 and replaced part of the precursor $his_5$-MBP by $his_5$-MBP-Cy3, the same protein labeled with a fluorescent Cy3 acceptor dye.[27,48] The number of labeled MBP ($n$) was gradually increased from 0 to 10 (out of 15 MBP) and for each value of $n$ they measured the emission spectra upon QD excitation and the changes in the QD luminescence decay times.

From the decrease in the QD emission intensity, the increase in sensitized emission of Cy3, and the decrease in the QD decay times, $E_{FRET}$ was calculated as a function of $n$ [assuming a spherically shaped system, equal distances of all Cy3 dyes from the center of the QDs, and an isotropic distribution of the D and A dipoles ($\kappa^2 = 2/3$)]. All three calculated $E_{FRET}$ functions showed excellent agreement with Equation (11).

$$E_{FRET} = \frac{nk_{FRET}}{nk_{FRET} + k_L + k_{NR}} = \frac{nR_0^6}{nR_0^6 + r^6} \quad (11)$$

With the use of $E_{FRET}$ and the corresponding $R_0$ values, the D–A distances $r$ were calculated, and the results agreed with the QD bioconjugate dimensions obtained from TEM and SAXS analysis. The experiments were verified with three different QDs as energy donors and all confirmed the agreement between experiment and theory.

As shown in the graph of Figure 2, the overall efficiency of a FRET system can be improved by the introduction of multiple acceptors in the neighborhood of a single donor [following Equation (11)]. With their large surfaces, QDs are ideally suited for this purpose with the possibility of anchoring numerous large molecules. This approach can be particularly useful within the frame of highly sensitive fluoroimmunoassays and was applied to the detection of maltose[39] or TNT[51] in nanoscale biosensors.

## QD-Based FRET Multiplexing

The ever increasing need for analytical tools applied to very large libraries of molecules (proteomics, genomics) requires the development of new technologies for high throughput screening. Spectroscopic techniques possess the possibility to divide the complete spectral information into numerous single channels. This color coding allows simultaneous measurement of multiple parameters, the so-called multiplexing. Regarding their narrow and size-tunable emission peaks, QDs are excellent candidates for multiplexing. Different approaches of color coding by using QDs have been described in the literature,[52,53] but multiplexing with FRET-based QD systems remains poorly explored. On the basis of a set of $x$ QD donor moieties combined to a set of $y$ acceptors all allowing for a substantial FRET with the $x$ QDs, up to $xy$ analytes could theoretically be detected and quantified simultaneously, assuming no cross talk of the different signals.

In a recent study,[54] Mattoussi et al. designed a series of experiments analyzing the use of QDs within the frame of multiplexed FRET systems. By using single QD donors associated to two energy acceptors (a fluorescent Cy3 and a nonfluorescent QSY-7) and a set of up to four QD donors (emitting at 510, 555, 570, and 590 nm, respectively) associated to Cy3 or QSY-7, they critically quantified and analyzed the multiplexed systems. Their experiments showed

  

that both configurations are effective for multiplexing, but that the deconvolution of the simultaneous FRET signals is a limiting factor, especially with nonsymmetric fluorescence signals of organic acceptor dyes. A promising approach should be the combination of LLCs as energy donors with QDs as acceptors. The characteristic narrow emission signals of both LLCs and QDs could significantly improve the read out of the FRET signals.

## 3. Properties of LLCs and Their Particular Advantages in FRET Experiments

The design of LLCs for the use as labels in FRET experiments is a long-term and sometimes hazardous task. As for all luminescent compounds, efficient labels first require good basic photophysical properties such as strong absorbance and high luminescence quantum yields. In the case of LLCs, this can only be achieved through judicious match of synthetic organic chemistry for the design of the ligands, coordination chemistry for incorporation of the central lanthanide ion, photochemistry for tuning the optical properties, and sometimes, a little bit of luck for obtaining a LLC with all the good features at the same time. This chapter aims at highlighting some of the important advantages of the use of LLC labels in FRET experiments in comparison to conventional fluorophores. Each point is illustrated by a typical example.

### Characteristic Emission Patterns of LLCs

The main interest in lanthanide labels resides in their electronic properties, namely, the luminescent f–f transitions. The partially filled 4f orbitals implied in these transitions have low radial expansions and are shielded from the environment by the filled 5s and 5p orbitals. This results in very weak ligand-field interactions, which can be parametrized in rare cases as for the nondegenerated $^5D_0 \rightarrow {}^7F_0$ transition of Eu,[55,56] and almost no perturbations of these transitions, which appear as narrow emission bands typical of the used emitting lanthanide.[57,58]

This point is nicely illustrated by the work of Selvin and coworkers,[61] who developed highly luminescent Eu and Tb labels on the basis of a straightforward synthesis involving the treatment of diethylenetriamine pentaacetic anhydride with a 7-amino-4-methyl-2(1*H*)-quinolinone chromophore (carbostyril 124) to form an octadentate ligand named DTPA-cs124 (Figure 3).[60] Upon complexation, DTPA-cs124 derivatives are wrapped around the lanthanide, as evidenced by the X-ray crystal structure of the Eu complex. If in the solid state the complexes appear as dimers, in solution the dimers dissociate and the vacant coordination site is occupied by a water molecule.[59] The same group also designed a series of derivatives where the labeling functionality (R in Figure 3) was modified to selectively target grafting onto the thiol residues present in, for example, cysteine-containing proteins.[59]

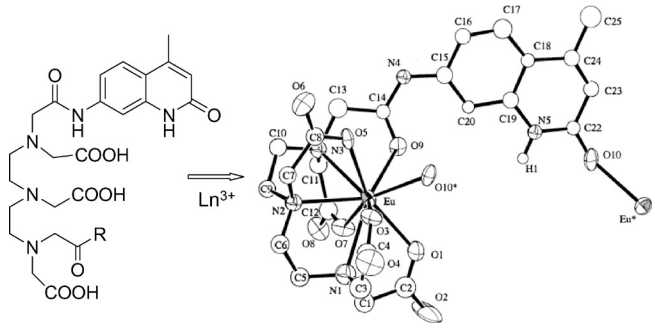


Figure 3. Ligands derived from DTPA-cs124 developed by Selvin and coworkers (left)[59] and the crystal structure of the Eu complex (R = OH, right, reproduced with permission from ref.[60]).

The Eu and Tb complexes display the characteristic emission spectra associated to the $^5D_0 \rightarrow {}^7F_J$ and $^5D_4 \rightarrow {}^7F_J$ ($J$ = 0 to 6) transitions of Eu and Tb, respectively (Figure 4), showing the typical narrow and well-separated emission bands. The use of such complexes as energy donors in FRET experiments opens spectral windows devoid of donor emissions, for example, between 505 and 525 nm or 555 and 570 nm for Tb and 655 and 680 nm for Eu. In these regions, the emission of the energy acceptors can be safely measured by using monochromators or filters (eventually in the gated mode, vide infra), without perturbations due to donor background emission. By this means, the emission signals of D and A are perfectly segregated, which results in accurate determination of the FRET efficiency.

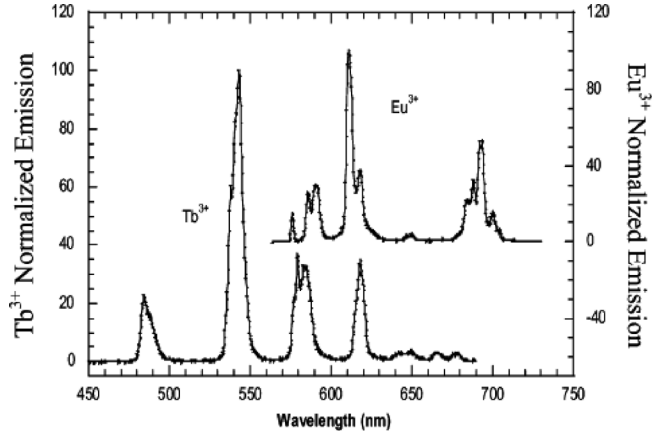


Figure 4. Characteristic emission spectra of the Tb and Eu complexes formed with DTPA-cs124 derivatives[59] (reproduced with permission from ref.[60]).

Furthermore, the emission peaks are generally spread over a broad spectral window, which allows sensitization of many possible acceptors. In one of their experiments, the group of Selvin covalently linked the Tb(DTPA-cs124) complex to the 5′ end of a DNA strand containing 10, 12, or 14 nucleotides, while labeling a complementary DNA strand with tetramethylrhodamine [absorption maximum (a.m.) at 557 nm] to be used as energy acceptor.[62] In another report, they demonstrated the use of fluorescein (a.m.

  

at 492 nm) as energy acceptor for indirect calculation of the lanthanide-centered quantum yield by FRET experiments,[63] and they also calculated the overlap integrals and $R_0$ values for a different panel of energy acceptors such as GFP (a.m. at 488 nm), Cy3 (a.m. at 552 nm), and R-phycoerythrin (a.m. at 566 nm).[61]

## The Large "Stokes Shift" of LLCs

As the f–f electronic transitions are strongly forbidden by the selection rules,[64] the corresponding molar absorption coefficients are very small. Obtaining sufficient population of the lanthanide excited states is difficult unless special excitation sources such as lasers are used. In 1942, Weissmann demonstrated that in the presence of some UV absorbing anions, excitation of Eu can be obtained indirectly through irradiation with light absorbed only by the anions.[65] Since then, this phenomenon called the antenna effect[66,67] has been largely studied and general rules[68,69] have been proposed to account for the optimization of this ligand-to-lanthanide energy-transfer process. By careful choice of absorbing ligands coordinated to the lanthanide, it is possible to obtain high populations of the lanthanide excited states and thereby strong luminescence signals. Upon excitation, the ligand reaches a first singlet excited state ($^1\pi\pi^*$, Figure 5), and it is generally assumed that the energy is first transferred to a ligand-centered triplet state ($^3\pi\pi^*$) by intersystem crossing and from there to the lanthanide-centered excited state (Ln*).[70]

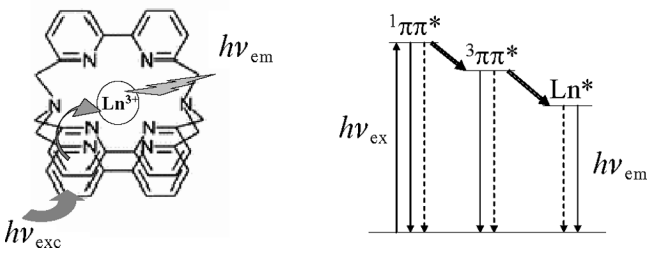


Figure 5. Antenna effect in the $[Eu(TBP)]^{3+}$ lanthanide cryptate[71,72] and the corresponding simplified energy diagram. Down arrows represent the radiative (solid line), nonradiative (dashed line), and energy transfer (thick line) deactivation pathways.

This cascade of energy transfer is schematically represented by the simplified Jablonsky diagram in Figure 5 for the europium cryptate $[Eu(TBP)]^{3+}$ (europium trisbipyridine), developed by Lehn and coworkers.[71,72] The different electronic states implied in the energy transfer have to be sufficiently well separated to prevent energy back transfer. This results in an energy gap between ligand absorption, generally in the UV domain, and lanthanide emission that often reaches few hundreds of nm {ca. 9500 $cm^{-1}$ for $[Eu(TBP)]^{3+}$}. This energy gap, often called Stokes shift (although it does not refer to the same absorbing and emitting state) is depicted by a double arrow in Figure 6.

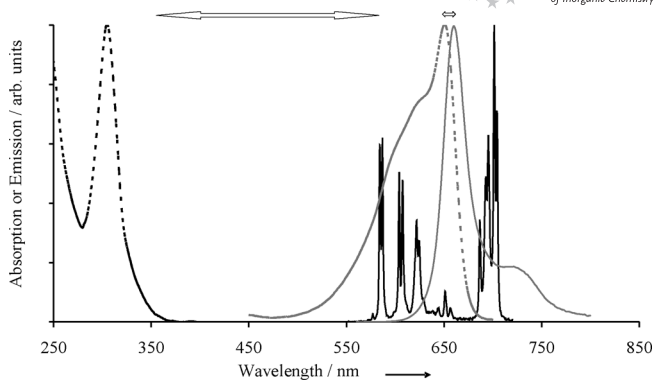


Figure 6. Absorption (dashed line) and emission (full line) spectra of $[Eu(TBP)]^{3+}$ in the presence of KF (black) and of APC (grey).

In contrast, conventional fluorescent labels or fluorescent proteins often have very small Stokes shifts (few tens of nm, ca. 500 $cm^{-1}$), as illustrated by the fluorescent protein allophycocyanin in Figure 6.[73] Thanks to the very large LLC "Stokes shift", the filtering of luminescence signals is easily obtained with simple cut-off filters, and one should fully profit from all the excitation domain. For fluorescent labels, the spectral overlap between absorption and emission renders filtering delicate, and it is often necessary to shift the excitation wavelength to remove spurious signals from the emitting channel. Within the scope of FRET experiments, the large spectral separation between donor excitation and donor as well as acceptor emission can significantly improve the measurement of the different signals.

## Long-Lived Excited-State Lifetimes of LLCs

Another result of the forbidden f–f transitions is a low probability of spontaneous emission, and the corresponding lanthanide-centered excited-state lifetimes can be very long and reach up to a few milliseconds in the cases of europium and terbium labels.[74] As already mentioned in Section 1, this allows successive FRET to short-lived acceptors and a concomitant improved brightness and sensitivity if several LLCs are placed around one acceptor. Moreover, in comparison to organic fluorophores with excited-state lifetimes of a few nanoseconds, the long-lived luminescence of LLCs is at the origin of another major advantage: the possibility to use delayed or gated acquisition of the emitted light.[75]

After pulsed excitation of a sample containing LLC labels, the insertion of a few tens of nanoseconds up to some hundreds of microseconds (depending on the labels used) delay before the acquisition of the emitted light will result in a total collapse of short-lived fluorescent signals while the label keeps on emitting. By this way, autofluorescence of the sample, fluorescence of other labels, and light scattering in the apparatus are largely suppressed.

The different emission decay profiles are illustrated in Figure 7, in which the ms time windows of the europium emission is compared to the very short prompt emission illustrated in this case by the fluorescence of rhodamine.[75]

  

Gated acquisition does not nominally increase the signal intensity, but allows a large improvement in the signal-to-background ratio.[76]

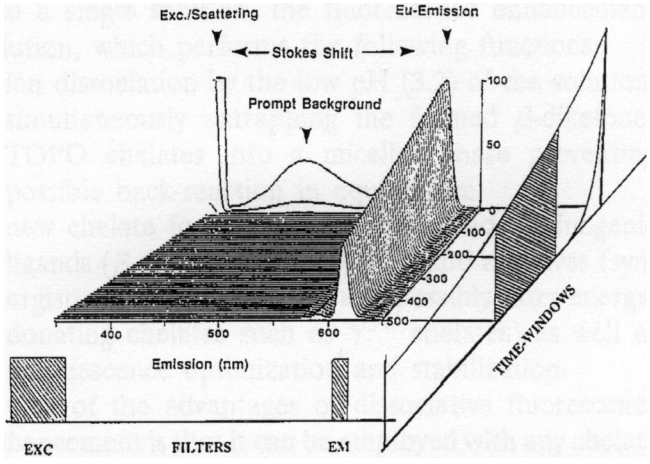


Figure 7. Emission decay profile of $Eu^{3+}$ and rhodamine (reproduced with permission from ref.[75]).

The principle of gated acquisition was studied in depth by Hemmilä and coworkers[77] within the frame of their DELFIA™ technology (DELFIA = dissociation enhanced lanthanide fluoroimmunoassay), which allows subpicomolar detection limits (50 fM in the case of Eu).[78] The DELFIA system is based on a multistep heterogeneous immunoassay in which lanthanide-labeled antibodies are fixed on a solid support in the presence of the antigen. The solid support has to be washed and the lanthanide cations are extracted by an enhancing solution (which contains antenna chromophores) prior to the reading. To allow homogeneous immunoassays in which the reading is done on the mother solution containing the antigen, it was necessary to provide lanthanide labels with included chromophoric units. Among the numerous LLC labels developed by these authors, they directed many efforts in the design of ligands of the family of (2,2′:6′,2′′-terpyridine-6,6′′-diyl) bis(methylenenitrilo)tetrakis(acetate), as exemplified by the activated dichlorotriazine phenylterpyridine (Figure 8).[79]

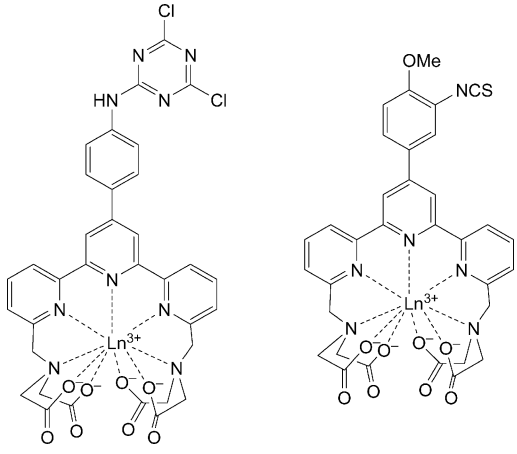


Figure 8. Examples of terpyridine-based ligands for the formation of LLC labels by using the dichlorotriazine[79,80] (left) or the isothiocyanate[81,82] (right) labeling group.

As demonstrated by the early work of Toner and coworkers,[81,82] and by numerous recent examples[83–85] the pocket provided by this kind of chelate is extremely well suited for the coordination of europium, offering good protection of the cation by its nonadentate coordination, good photophysical properties, and for numerous possible variations at the *para* position of the central pyridine ring.

To summarize, LLCs combine typical emission patterns, large "Stokes shift", and long-lived luminescence lifetimes, which makes them ideal as energy donors in time-resolved FRET experiments.[86,87] It is worth noting that this can only be obtained after tremendous synthetic effort to combine high stability, good photophysical properties, and a labeling functionality.

## 4. QDs and LLCs in FRET Experiments

In the last two sections, the specific advantages of QDs and LLCs for FRET applications were separately highlighted. This part will deal with the combined use of both and the collateral benefits. Although QDs are theoretically very well suited as FRET acceptors (e.g., extremely strong absorbance over a broad wavelength range yielding possible large overlap integrals) there are very few examples in solution.[88,89] It was shown by Mattoussi and coworkers that QDs can hardly be efficient acceptors when combined with common organic fluorophores possibly due to the short-lived excited states (ns) of these fluorophore donors.[48] Contrary to these results, in a recent publication Zhao et al. claimed FRET from FITC (FITC = fluorescein isothiocyanate) to CdSe/ZnS core/shell QDs.[89] Unfortunately, there is little spectroscopic data available (steady-state spectra only) to confirm this FRET. Other contributions deal with QDs both as donors and acceptors.[90–92] However, the drawback of short-lived donor excited states can be overcome by using LLCs as donors, which usually possess very long-lived luminescence (up to ms). Besides applications dealing with bioluminescence resonance energy transfer (BRET),[93–96] an efficient possibility of energy transfer to QDs was found to involve LLCs as donors.[97–102] We will first outline some of our results with LLC to QD FRET and then present an example of a clinically relevant immunoassay using that method.

### FRET from LLCs to QDs and the Possibility of Single-Donor Multiplexing

In order to demonstrate efficient FRET from LLCs to QDs we used the well-characterized streptavidin-biotin biological binding system as a homogeneous immunoassay model. LLCs with Eu and Tb as central lanthanide ions (Figure 9)[103] were labeled to streptavidin (LLC-Strep), whereas QDs were commercially available (Invitrogen) in biotinylated form (Biot-QD), with emission maximum at 655 nm.[98,99,101,102]

Biot-QD was added stepwise to LLC-Strep, and the formation of LLC-Strep-Biot-QD complexes was demon-

  

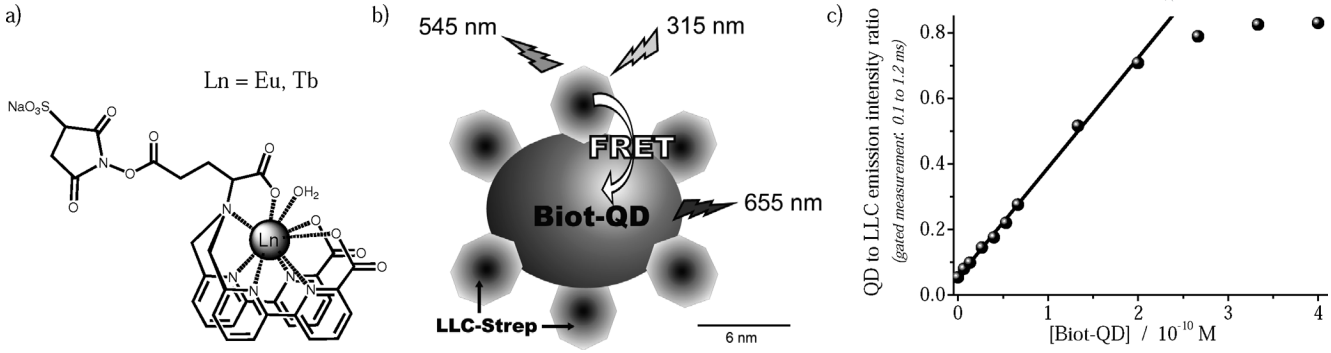


Figure 9. (a) LLC used for streptavidin labeling; (b) schematic presentation of the LLC-Strep to Biot-QD homogeneous FRET immunoassay; (c) evolution of the FRET signal (QD to LLC intensity ratio) over QD-biotin concentration with a linear fit (solid line) for determination of the subpicomolar detection limit.

strated by measuring the time-gated (e.g., time window from 0.1 to 1.2 ms) intensity ratio of QD and LLC emission at 655 and 545 nm, respectively. A schematic presentation of the complex as well as the ratio over Biot-QD concentration graph are shown in Figure 9.

The strong increase in the emission ratio caused by an increase in long-lived QD luminescence and a decrease in long-lived LLC luminescence strongly suggested FRET from LLCs to QDs. FRET from freely diffusing complexes as well as false FRET signals due to direct QD excitation were ruled out by control experiments. Moreover, in a comprehensive time-resolved study we analyzed the luminescence decay times of a FRET system with Tb-based LLC donors and Biot-QD acceptors.[101] Both donor and sensitized acceptor luminescence decay times were analyzed in detail and a more than 1000-fold increase in the QD luminescence decay time was found to result from FRET sensitization, another strong evidence for FRET. Extremely large Förster radii of ca. 100 Å and a subpicomolar detection limit for Biot-QD demonstrate the high potential of the combined FRET system for in vitro diagnostics as well as imaging applications.[103]

In further experiments,[104] we demonstrated the use of biotinylated QDs of smaller size (emitting at 605 nm) as efficient FRET acceptors within the same assay format and the same Tb-based LLC donor. Thus, homogeneous multiplexing assays with one LLC donor and multiple QD acceptors become possible.

### Clinically Relevant Assay with FRET from LLCs to QDs

In a recent publication, Härmä and coworkers demonstrated a clinically relevant application of LLC to QD FRET.[97] They used four different LLCs (three Eu and one Tb based) coupled with amino biotin and CdTe QDs directly labeled with streptavidin and estradiol-specific antibody Fab fragments. By this means, the authors could establish a closer D–A distance for their competitive biotin and estradiol assay systems relative to the use of commercial QDs with thick protective shells. In contrast, the D to A ratio was lower because each LLC was labeled to one biotin molecule and the CdTe QDs contained only few streptavidin (3-fold molar excess for QD coupling) or Fab fragments (1.5-M excess for QD coupling). Moreover, the Förster radii (not mentioned in the publication) were probably lower than in our contributions mentioned above. All these aspects resulted in relatively low FRET efficiencies and the suggestion of short spacers between the LLC donors and the CdTe QD acceptors. However, they achieved a subnanomolar detection limit within their separation-free estradiol immunoassay. Their results could lead to the conclusion of the necessity for high D to A ratios when LLCs are used with QDs as D–A pairs for sensitive in vitro diagnostics.

Both FRET partners offer the possibility to realize these conditions and further investigations in this direction are important for a better understanding. Figure 10 represents a possible FRET immunoassay arrangement with several LLCs as donors and one QD as acceptor. By using the advantages of both FRET partners extremely sensitive homogeneous immunoassays (with the potential for multiplexing) might become possible.

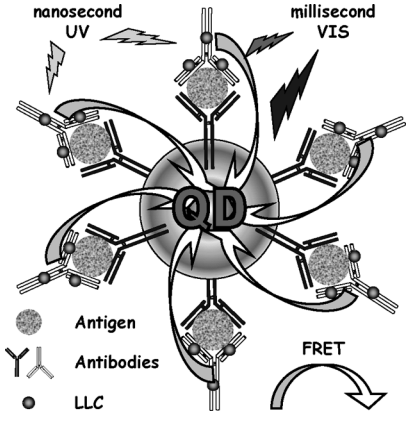


Figure 10. Homogeneous immunoassay arrangement with several LLCs labeled to primary antibodies as FRET donors and a QD conjugated with several secondary antibodies as FRET acceptor.

In summary, the combination of LLCs and QDs for FRET has a very high potential for the use in biochemical analysis with the possibility of high sensitivity and large Förster radii. For example, a sensitivity improvement of

  

more than one order of magnitude could be demonstrated for a Tb-based LLC to QD FRET bioassay, directly compared to the "gold standard" of immunoassays {[Eu(TBP)]$^{3+}$ and APC}.[102] To date, $R_0$ values for commonly used donor–acceptor pairs are rarely larger than 6 nm (only 4 out of 273 FRET pairs with $R_0$ between 6 and 7 nm[22]). Combining both LLCs (as donors) and QDs (as acceptors) in biochemical FRET applications Förster radii of more than 10 nm can be found.[98,99,102] Thus, breaking the common limit of 10 nm is possible, and investigation of QDs and LLCs for biochemical FRET applications becomes extremely valuable.

## 5. Conclusion and Perspectives

The first report on the use of luminescent lanthanide complexes for time-resolved fluoroimmunoassays is now 25 years old,[105] whereas the arrival of QDs in the field of FRET in solution is far younger[38] and their combination is only in its infancy.[97–102] Regarding the large benefits that their gathering offers, such as large Förster radii and very high sensitivity, there is no doubt that this unique combination will keep on attracting the interest of researchers.

With the ongoing improvements in water-solubilized QDs[106] and the still-running search for highly luminescent lanthanide labels,[83–85,107] there exists numerous open perspectives. As a first one, the very low detection limits reached by time-resolved acquisition techniques in FRET-based immunoassays[101] should allow improved sensitivity for the detection of analytes, an appreciated criteria for early medical diagnosis, or warfare chemical detection. Increasing the spatial amplitude of FRET with enlarged Förster radii also allows the investigation of larger systems including very large proteins and polymers. Thanks to the specific spectral signature of both partners, multiplexing may become easier with possible combinations by using two lanthanide donors, such as an europium and a terbium complex, coupled to three to four specific QD acceptors within a single fluoroimmunoassay. Assemblies of cation- or anion-responsive lanthanide complexes[108] at the surface of QDs would also be particularly well suited for sensing applications applied to microscopic techniques and would add the sensing ability to the well-established use of QDs for in vivo imaging techniques.[33,109,110]

## Acknowledgments

The authors are grateful to the French Centre National de la Recherche Scientifique and the European Commission (Specific Targeted Research Project POC4Life-LSHB-CT-2007–037933) for financial support. Professor I. Hemmilä is gratefully acknowledged for authorizing the reproduction of Figure 7.

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Received: April 1, 2008
Published Online: June 18, 2008

  