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Lanthanide Chelates as Donors in Fluorescence Resonance Energy Transfer: Exciting Prospects for Bioaffinity Assay Detection

by

Tiina Kokko

TURUN YLIOPISTO
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From
Department of Biochemistry and Food Chemistry/ Biotechnology
University of Turku
Turku, Finland

Supervised by
Professor Tero Soukka
Department of Biochemistry and Food Chemistry/ Biotechnology
University of Turku
Turku, Finland

and

Professor Timo Lövgren
Department of Biochemistry and Food Chemistry/ Biotechnology
University of Turku
Turku, Finland

Reviewed by

Docent Harri Hakala
Perkin Elmer, Wallac
Turku, Finland

and

Docent Kari Mattila
Turku University Central Hospital/ Laboratory
Turku, Finland

Opponent

Professor Michael Schäferling
Institute of Analytical Chemistry, Chemo- and Biosensors
University of Regensburg
Regensburg, Germany

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Oh, the butterflies are flying,
Now the winter days are dying,
And the primroses are trying
To be seen.
And the turtle-doves are cooing,
And the woods are up and doing,
For the violets are blue-ing
In the green.

Oh, the honey-bees are gumming,
On their little wings, and humming
That the summer, which is coming,
Will be fun.
Ant the cows are almost cooing
Ant the turtle-doves are mooing,
Which is why a Pooh is poohing
In the sun.

For the spring is really springing,
You can see a skylark singing,
And the blue-bells, which are ringing,
Can be heard.
And the cuckoo isn't cooing,
But he's cucking and he's ooing,
And a Pooh is simply poohing
Like a bird.

Winnie the Pooh

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications, referred to in the text by their Roman numerals:

I Tiina Kokko, Leena Kokko, Tero Soukka & Timo Lövgren (2007) Homogeneous non-competitive bioaffinity assay based on fluorescence resonance energy transfer. *Analytica Chimica Acta,* **585:** 120–125.

II Tiina Kokko, Leena Kokko, Timo Lövgren & Tero Soukka (2007) Homogeneous noncompetitive immunoassay for 17β-estradiol based on fluorescence resonance energy transfer. *Analytical Chemistry,* **79:** 5935–5940.

III Tiina Kokko, Leena Kokko & Tero Soukka (2009) Terbium(III) chelate as an efficient donor for multiple-wavelength fluorescent acceptors. *Journal of Fluoresence,* **19:** 159–164.

IV Tiina Kokko, Tuomo Liljenbäck, Mari T Peltola, Leena Kokko & Tero Soukka (2008) Homogeneous dual-parameter assay for prostate-specific antigen based on fluorescence resonance energy transfer. *Analytical Chemistry,* **80:** 9763–9768.

In addition, some unpublished data is presented.

ABBREVIATIONS

AF	Alexa Fluor
APC	Allophycocyanin
Bio	Biotin
B-PE	B-phycoerythrin
βhCG	β subunit of Human chorionic gonadotropin
BSA	Bovine Serum Albumin
D-A	donor-acceptor
e	Neper (~2.718)
E2	17β-estradiol
Fab	Antibody Fab fragment
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
HTS	High Throughput Screening
IC	Internal Conversion
IgG	Immunoglobulin G
ISC	Intersystem Crossing
Ln^{3+}	Lanthanide ion
LRET	Luminescence Resonance Energy Transfer
Mab	Monoclonal antibody
mRNA	messenger RiboNucleic Acid
nFRET	non-overlapping Fluorescence Resonance Energy Transfer
PSA	Prostate-Specific Antigen
PSA-F	Free, uncomplexed, Prostate-Specific Antigen
PSA-T	Total Prostate-Specific Antigen
R_0	Förster radius
RIA	Radioimmunoassay
R-PE	R-phycoerythrin
RT-PCR	Real Time-Polymerase Chain Reaction
S_o	Lowest level of singlet state
S_1,S_2	Higher, excited singlet states
SA	Streptavidin
T_1	Triplet state
TFS	TransFluorSphere
TR	Time-resolved
VR	Vibrational Relaxation
Å	Ångström (1 Å = 0.1 nm)

ABSTRACT

Fluorescence resonance energy transfer (FRET) is a non-radiative energy transfer from a fluorescent donor molecule to an appropriate acceptor molecule and a commonly used technique to develop homogeneous assays. If the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor, FRET might occur. As a consequence, the emission of the donor is decreased and the emission of the acceptor (if fluorescent) increased. Furthermore, the distance between the donor and the acceptor needs to be short enough, commonly 10-100 Å. Typically, the close proximity between the donor and the acceptor is achieved via bioaffinity interactions e.g. antibody binding antigen. Large variety of donors and acceptors exist. The selection of the donor/acceptor pair should be done not only based on the requirements of FRET but also the performance expectancies and the objectives of the application should be considered.

In this study, the exceptional fluorescence properties of the lanthanide chelates were employed to develop two novel homogeneous immunoassays: a non-competitive hapten (estradiol) assay based on a single binder and a dual-parametric total and free PSA assay. In addition, the quenching efficiencies and energy transfer properties of various donor/acceptor pairs were studied. The applied donors were either europium(III) or terbium(III) chelates; whereas several organic dyes (both fluorescent and quenchers) acted as acceptors.

First, it was shown that if the interaction between the donor/acceptor complexes is of high quality (e.g. biotin-streptavidin) the fluorescence of the europium(III) chelate could be quenched rather efficiently. Furthermore, the quenching based homogeneous non-competitive assay for estradiol had significantly better sensitivity (~67 times) than a corresponding homogeneous competitive assay using the same assay components. Second, if the acceptors were chosen to emit at the emission minima of the terbium(III) chelate, several acceptor emissions could be measured simultaneously without significant cross-talk from other acceptors. Based on these results, the appropriate acceptors were chosen for the dual-parameter assay. The developed homogeneous dual-parameter assay was able to measure both total and free PSA simultaneously using a simple mix and measure protocol. Correlation of this assay to a heterogeneous single parameter assay was excellent (above 0.99 for both) when spiked human plasma samples were used. However, due to the interference of the sample material, the obtained concentrations were slightly lower with the homogeneous than the heterogeneous assay, especially for the free PSA.

To conclude, in this work two novel immunoassay principles were developed, which both are adaptable to other analytes. However, the hapten assay requires a rather good antibody with low dissociation rate and high affinity; whereas the dual-parameter assay principle is applicable whenever two immunometric complexes can form simultaneously, provided that the requirements of FRET are fulfilled.

1 INTRODUCTION

Over the years, scientists have strived to develop technologies that could accurately and precisely measure large variety of different interesting analytes. Recently, the main topic has been how to create simple, fast and reliable assays. Therefore, the emphasis has shifted towards homogeneous assays, which are typically easier to do than traditional heterogeneous assays. Even though a lot has happened in the world of homogeneous assays, they still tend to lag behind heterogeneous assays when sensitivity issues are considered.

One, fairly popular, basis for homogeneous assays is fluorescence resonance energy transfer (FRET) from an excited donor molecule to an appropriate acceptor molecule. FRET was discovered half a century ago (Förster, 1948) and has slowly gained a high status in biotechnology. Currently, the search for "FRET" in the Pubmed database results over 2900 hits from various areas such as structural elucidation of biological molecules, *in vitro* assays, *in vivo* monitoring in cellular research, nucleic acid analysis, light harvesting and metallic nanomaterials. Most commonly, the donor/acceptor pairs consist of organic dyes. This has, however, slightly limited the attainable performance properties of the applications: The short fluorescence lifetime of organic dyes prevents the discrimination of autofluorescence during measurement and due to the small Stokes' shift, leakage of the excitation light of the donor into the measurement wavelengths is possible. Furthermore, the acceptors might also be excited directly and not via energy transfer. (Lakowicz, 1999.)

Lanthanide chelates were first introduced in 1980's but the first chelate structures were not inherently fluorescent, thus separate enhancement steps were required (Hemmilä, 1985). Later, intrinsically fluorescent lanthanide chelates were developed, which made them viable donors in FRET applications. Lanthanide chelates have unique properties that make them superior donors compared to organic dyes. Lanthanide chelates have long lifetimes that enable time-resolved measurement of acceptor emission, which significantly decreases the effect of autofluorescence and possible direct acceptor excitation. Furthermore, their emission peaks are well separated and narrow and their Stokes' shifts are long. (Hemmilä and Laitala, 2005.) Since several different lanthanides can be used (mainly europium, terbium, samarium or dysprosium) and they all have unique spectral properties, large variety of different acceptors, and hence applications, are conceivable.

In this literature survey, FRET from the viewpoint of lanthanide chelate donors is reviewed. The objective is to provide an overview of lanthanides in FRET, the appropriate acceptors for them and also what type of assay applications has been published. Even though FRET is popular among spectral imaging studies, they are not included here. Instead, the emphasis is on different bioaffinity assays applications.

2 REVIEW OF THE LITERATURE

2.1 Terminology of the bioaffinity assays

Bioaffinity assays are based on the property of biologically active substances to form stable, specific and reversible complexes. Common molecular forces such as van der Waals or hydrophobic interactions are involved in the formation of these complexes. Typically, the complexes used in bioaffinity assays are protein-protein binding (e.g. antibody-antigen (protein)), protein-ligand (e.g. streptavidin-biotin) or nucleic acid hybridisation. Some of the most commonly used bioaffinity assays are different immunoassays, which rely on the unique capabilities of antibodies to recognize and bind specific antigens. (Wild, 2005.)

Bioaffinity assays can be categorized into four different classes; hence, assays are either competitive or non-competitive and either homogeneous or heterogeneous. Based on the occupancy theory, the difference between the competitive and non-competitive assays arises from the source of the signal during measurement. If those recognizing agents that are bound to the analyte are also the source of the signal, the assay is called non-competitive assay. Thus, in non-competitive assay format the amount of signal increases with increasing analyte concentration. In competitive assays, those recognizing agents that are not bound to the real analyte create the signal. Typically, analyte analogue is labelled with some detection probe and creates the signal. Therefore, the signal of competitive assays decreases when the analyte concentration increases. In practice, competitive assays can be performed using two different principles. In back-titration method, first the recognizing agent is let to react with the analyte and, thereafter, the labelled analyte is added to react with the still available recognizing sites. The other possibility is to create an actual competition between the analyte and the labelled analyte by letting them react with the recognizing agent simultaneously. Due to the different signal generation methods, the competitive and non-competitive assay principles have inherently different sensitivity characteristics. It is generally believed that more accurate measurements can be attained when small changes are measured in low signal levels than in high signal levels. Sensitivity is typically defined as the concentration corresponding a change in the signal that can be reliably measured without it being a mere cause of the background variation. Since small changes can be measured more reliably in non-competitive assays (with low signals at zero concentration) than in competitive assays (higher signals at zero concentration), the non-competitive assays are typically considered to have better sensitivity than competitive assays. (Wild, 2005.)

In order for the bioaffinity assays to be functional, the assay principle has to be able to create a signal that is proportional to the amount of the analyte. Based on this fundamental principle, the assays can be classified to be either heterogeneous or homogeneous. In heterogeneous assays, the bound label is physically removed from

the free label and other interfering factors. This is typically done by using a solid phase to collect the sample and then, during a washing step, remove all the interfering factors away before measurement. Homogeneous assays do not have any separation steps, thus the binding reaction of the analyte or labelled analyte has to cause some measurable change in the signal level to create the correlation between analyte concentration and signal. This change may occur via various methods for example using environmentally sensitive labels, changes in fluorescence polarization or utilizing fluorescence resonance energy transfer. Since homogeneous assays do not have any separation steps that could cause variations in the signal levels, in theory, they should have better sensitivity than heterogeneous assays. However, in reality this is hardly ever the case since the presence of the sample material tends to interfere with the measurement and, thus, impair the overall sensitivity. (Wild, 2005.) Despite the sample interference issues, the homogeneous assays are typically easy to perform (ideally: mix and measure) and automate. Therefore, the homogeneous assays are gaining popularity in applications were the number of samples is large and/or the speed of the assay is important.

2.2 Introduction to fluorescence resonance energy transfer

2.2.1 Luminescence

Luminescence (emission of light that occurs from electronically excited states) is the phenomenon underlying the development of FRET based techniques. It can be divided into several categories depending on the energy source used for the excitation of the luminescent molecule. Chemiluminescence is a technique where the measured light is generated during a chemical reaction. Since no external excitation is required, the background luminescence is small. Thus, the chemiluminescence-based assays can theoretically have excellent sensitivities. (Wild, 2005.) When the light emitting reaction is catalysed by enzymes, like luciferase, bioluminescence is said to happen. (For recent review see Prinz et al., 2008, Bacart et al., 2008.) Luminescence is called radioluminescence if the exciting energy comes from radiative entities emitted by radioactive particles. (For example Erfurt and Krbetschek, 2002, Nowotny, 2007, Blair et al., 2006.) Electroluminescence occurs when the excitation energy is obtained from electric current. (For recent review see Chen et al., 2004, Godlewski, 2005, Bandaru, 2007.) Finally, luminescence can originate from a directly luminescent label molecule that can be conjugated to the assay components. There are enormous amounts of different label molecules. (Wild, 2005.) This thesis concentrates on lanthanides as directly fluorescent labels and their applications in the field of fluorescence and energy transfer.

Luminescence can also be divided into two categories based on the lifetime of the luminescence. When a photoluminescent molecule absorbs light, its electrons are raised from the lowest energy level to some of the excited levels. The dispersal of the

energy back to the ground state may occur via non-radiative processes to produce heat, or the molecule might transfer the energy to another molecule. When the absorbed energy is dissipated in the form of an emitted photon, the phenomenon is called fluorescence or phosphorescence. (Hemmilä, 1991.) The lifetime of fluorescence is typically near 10 ns; whereas the lifetime of phosphorescence is longer, from milliseconds to seconds. Lifetime of the luminescence is the time at which the intensity of the signal decreases to 1/*e* of its initial value. Lifetime is also the average amount of time a fluorophore remains in the excited state after the excitation. (Lakowicz, 1999.) The events during the excitation and the following emission can be presented using a Jablonski diagram. (Figure 1)

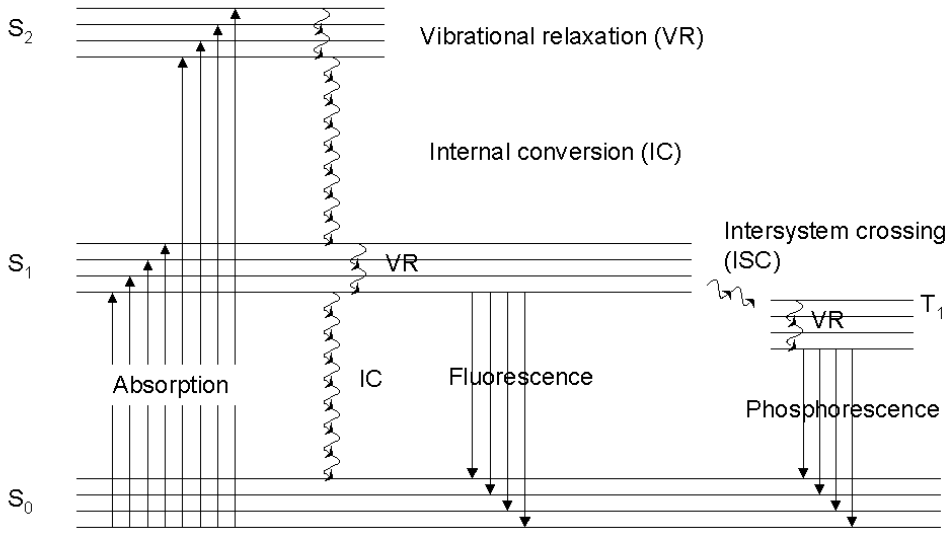


Figure 1 Energy flow diagram (Jablonski diagram) for a fluorescent and phosphorescent component (Hemmilä, 1991).

When a molecule absorbs light, one of its electrons from the occupied orbital is promoted to an unoccupied orbital. The absorbed energy is discharged rapidly to the lowest level of singlet by vibrational relaxation (VR) and in the case of higher excited singlets (S_2 =>), to the lowest excited singlet (S_1) by internal conversion (IC). The actual fluorescence occurs from the lowest vibrational sublevel of the S_1 level and the electron can return to any of the vibrational levels of ground state (Figure 1). Because the emission always happens from the lowest excited level, the used excitation wavelength does not affect the shape of the emission spectrum. In some cases, a singlet-triplet (T_1) intersystem crossing (ISC) might occur in which case the excited emission is called phosphorescence. This transfer is an unlikely process but some phosphorescent compounds exist. (Hemmilä, 1991.) As can be seen in the figure 1, the energy of the emission is typically lower than the energy of the absorption. Therefore, fluorescence usually occurs at lower energies or longer wavelengths than absorption.

2.2.2 Principles and requirements of FRET

Fluorescence resonance energy transfer (sometimes called Förster resonance energy transfer, FRET) can be defined as a non-radiative energy transfer from an excited donor molecule to an acceptor molecule (Selvin et al., 1994). Due to FRET, the intensity of the donor fluorescence decreases. The acceptor can be fluorescent or non-fluorescent molecule. Based on the donor and the acceptor molecules, two categories of FRET can be identified: Heterotransfer occurs between two chemically different molecules, thus donor and acceptor are distinctively different molecules. If the Stokes' shift of the molecule is small, FRET can also occur between two chemically identical molecules, which is called homotransfer. (Lackowicz, 1999.) In order FRET to occur, spectral overlapping of the donor and the acceptor, close enough proximity and dipole-dipole angels other than 90 degrees are required. The requirement of the overlapping of the donor emission spectrum with the excitation spectrum of the acceptor is commonly called the Förster principle (Förster, 1948, Figure 2).

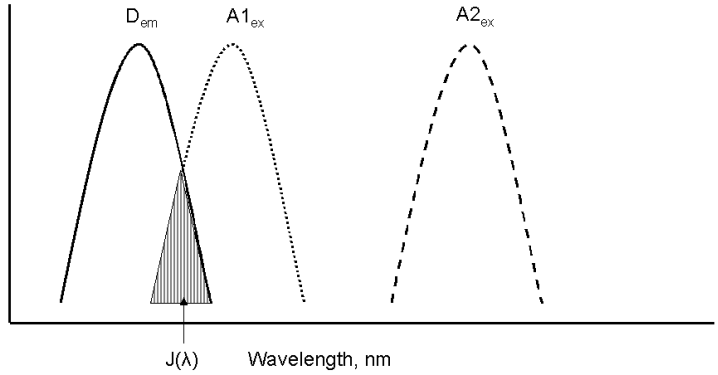


Figure 2 Schematic picture of the Förster principle. The emission spectrum of the donor (straight line) overlaps well with the excitation spectrum of the acceptor 1 (dotted line), thus FRET could occur; whereas, there is no overlapping with the excitation spectrum of the acceptor 2 (dashed line). In this case, no FRET could occur.

From the spectral overlapping, the theoretical intensity of FRET between a specific donor-acceptor pair can be estimated with Förster radius or distance (R_0) (Förster, 1948). Förster radius is the distance of the donor and the acceptor when the efficiency of the energy transfer is 50% from the maximum value and can be calculated using equation:

$$R_0 = 0.211\left[\kappa^2 n^{-4} Q_D J(\lambda)\right]^{1/6} \quad (1)$$

Where *n* is the refractive index of the medium (typically ~1.4 for aqueous solutions), Q_D is the quantum yield of the donor in the absence of the acceptor and J(λ) is the spectral overlapping of the donor-acceptor pair (figure 2). The κ^2 is a factor describing the relative orientation in space of the transition dipoles and the acceptor (usually

assumed to be equal to 2/3). (Lakowicz, 1999.) The quantum yield can be environmentally sensitive, thus the R_0 values for a D-A pair can vary if the solution conditions change. Also the spectral properties of the donor or acceptor may change due to environmental factors. (Wu and Brand, 1994.) For example, presence of certain metal ions can shift the absorption maximum of the acceptor and thus either allow or prohibit FRET. These properties have been used to create FRET based metal ion assays, so called FRET on-off or off-on assays. (Lee et al., 2007, Lee et al., 2008.) Typically, donor-acceptor pairs that have R_0 between 20-90 Å are used to study biological macromolecules (Lakowicz, 1999). However, if the donor and the acceptor are chosen appropriately, pairs with R_0 under 10 Å can be used to study smaller molecules (Sahoo et al., 2006, Sahoo et al., 2007).

The electron cloud of a molecule has a limited volume. Typically, the interactions of the molecule are limited to this volume. However, some molecules can interact with other molecules at much greater distances by projecting an electromagnetic field, which exists in a molecule if the molecule has one region with net positive charge and other with net negative charge. This charge difference creates a dipole. If the molecule is asymmetric, these fields typically have a specific orientation. In FRET, the excitation light induces an oscillating field in the donor. Thereafter, this oscillating emission dipole can influence the absorption dipole of the acceptor to oscillate in synchrony. If the emission dipole of the donor is in 90 degrees angle to the absorption dipole of the acceptor, the oscillations will cancel each other out, thus no FRET can occur. Therefore, FRET requires that the dipole-dipole angles are not perpendicular to each other. (Vogel et al., 2006, Lakowicz, 1999.) It has also been shown that the coupling responsible for FRET generates a mechanical force between the D-A-pair that is distinct from the typical van der Waals force between ground state molecules. This underlying potential is also depended of the distance of the donor and the acceptor. (Cohen and Mukamel, 2003.)

Finally, FRET can occur only if the donor and the acceptor are close enough proximity to each other. Typically, the distance should be 10-100 Å (Lakowicz, 1999). The rate of the energy transfer can be calculated as:

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \tag{2}$$

Where the τ_D is the decay time of the donor in the absence of the acceptor and r is the distance between the donor and the acceptor. (Lakowicz, 1999.) The strong dependence between the rate of FRET and the distance of the D-A-pair makes FRET a feasible technique to study average distances between donor and acceptor. However, there are some shortcomings with using FRET to measure average distances. First, all

the distances are determined in reference to the Förster distance, which is a theoretical value calculated from the spectral properties of the D-A pair (Wu and Brand, 1994). Second, the orientation factor (κ^2) needed for the R_0 calculations embodies some uncertainties. The orientation factor can differ from 0 to 4 (value of 2/3 is typically used) and at the moment, there are no means to measure the value exactly, thus the results from the distance measurements may deviate from the true distance. (Lakowicz, 1999.) These uncertainties can be reduced by, for example, utilizing fluorescence polarization anisotropy measurements to study the feasible upper and lower limits of the orientation factor (Dale et al., 1979) or by using time resolved decay studies to measure distributions of the defined distance (Wu and Brand, 1992). However, it has been shown that when the κ^2 value of 2/3 is used, the maximum error in the calculated distance is 35%, thus reasonably accurate results can be obtained (Lakowicz, 1999). Furthermore, these calculations usually assume that κ^2 and r are independent of each other. However, in some cases, for example when the other probe is free and the other is fixed, these values might be dependent (VanBeek et al., 2007). Thus, assumptions of independence should be considered before calculations.

To conclude, the range of distances available between donor and acceptor and the efficiency of FRET are regulated by the spectral properties of the D-A pair. The molecular processes occurring during FRET are presented in Jablonski diagram in figure 3.

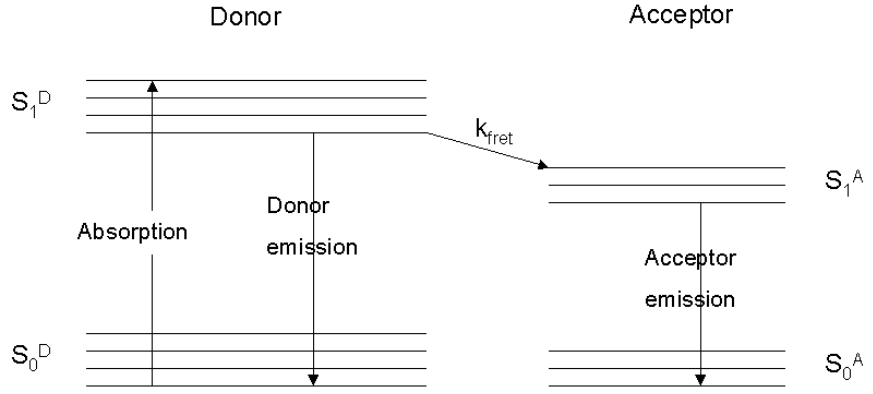


Figure 3 Simplified Jablonski diagram of the FRET process (Lakowicz, 1999).

First, the excitation of the donor occurs as described earlier. If an appropriate acceptor is nearby and the other requirements of FRET are realized, a non-radiative energy transfer from donor to acceptor may occur (k_{fret} = the efficiency of the energy transfer). If the acceptor is a fluorescent molecule, acceptor emission may result. As can be seen from figure 3, the process of FRET does not involve any intermediate photons. Instead, the energy transfer is based on a theory of fluorophore as an oscillating dipole, which can exchange energy with another dipole with a similar resonance frequency. (Lakowicz, 1999.)

2.2.3 FRET measurements

When the emission of a fluorophore (donor or acceptor) is measured, two fundamental methods can be distinguished: steady-state and time-resolved (TR) measurements. In steady-state measurements the fluorophore is excited with a continuous beam of light and the intensity of the emission is measured. This is the most common type of fluorescence measurement. In TR measurements, the fluorophore is exposed to pulsed excitation light, where the pulse width is shorter than the decay time of the fluorophore emission. Between excitation and measurement of the emission, a short time is waited, during which the possible background fluorescence of the sample components and autofluorescence of other interfering materials is decreased. Thus, the measurement is more specific to fluorophore emission than the steady-state measurements. However, TR measurements require that the lifetime of the fluorophore emission is long enough and also somewhat sophisticated instrumentation, thus these measurements are not as common as the steady-state measurements. (Lakowicz, 1999.) The occurring FRET can be measured using number of different methods. Essentially, the methods can be divided into four basic principles: measurements of changes in donor emission, acceptor emission, both donor and acceptor emission simultaneously (Figure 4) or orientation of the fluorophores.

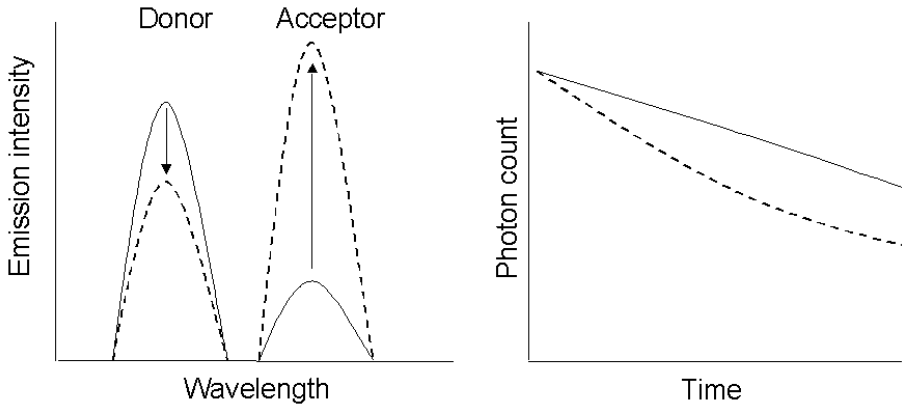


Figure 4 Schematic figures of the emission changes underlying the FRET measurements. Solid line: no FRET and dashed line: FRET situation. **Left:** If FRET occurs, the emission of the donor decreases and the emission of the acceptor increases (if fluorescent). **Right:** As a result of FRET the lifetime of the donor emission is decreased. (Modified from Vogel et al., 2006.)

The measurements of the changes in the donor emission are typically done by either lifetime or fluorescence intensity (quenching) measurements. In the quenching measurements, the intensity of the donor emission is measured in the presence and absence of the acceptor. If FRET occurs the emission of the donor is decreased, which can be quantified. The measurement wavelength is selected in a way that the possible acceptor emission does not contribute to the measurement. (Wu and Brand, 1994.) As a

result of FRET, also the fluorescence lifetime of the donor shortens, which can be measured (Ozinskas et al., 1993). The efficiency of energy transfer (E) in quenching and lifetime assays can be calculated from:

$$E = 1 - \frac{F_{DA}}{F_D} \qquad \text{or} \qquad E = 1 - \frac{\tau_{DA}}{\tau_D} \tag{3, 4}$$

Where the F_{DA} and F_D are the fluorescence of the donor and τ_{DA} and τ_D the fluorescence lifetimes of the donor in the presence (DA) and absence (D) of the acceptor. (Lakowicz, 1999.) However, it has been shown that the efficiency cannot be determined accurately, unless the sample contains only D-A complexes (no free donors or acceptors). Furthermore, all the complexes need to be perfectly labelled. (Wlodarczyk et al., 2008.) In addition, three things need to be considered when donor emission changes are measured. A) If the donor is conjugated to a large molecule and the acceptor functions as a ligand, the binding of the ligand/acceptor might cause changes in the fluorescence properties of the donor through conformational changes of the large molecule. If this happens, it should be noticed in the efficiency calculations. B) If the acceptor absorbs at the wavelength of the donor excitation, it might cause some inner filtering, thus decreasing the observed donor emission. It can be corrected using absorbance measurements. C) Intermolecular energy transfer may occur if the acceptor concentration is very high. The critical concentration of the acceptor, in which the energy transfer is 76%, can be calculated (as moles per liter) from: $447/R_0^3$, where R_0 is the Förster distance in Ångströms. However, the acceptor concentration should be much lower than that because when the acceptor concentration exceeds on hundredth of the critical concentration, possibility of intermolecular energy transfer exists. (Wu and Brand, 1994, Lakowicz, 1999.)

If the acceptor is a fluorescent molecule then one possibility to measure FRET is to monitor the changes in the acceptor emission (also called sensitized emission) as a result of donor excitation. The sensitized emission can be measured both with steady-state and time-resolved methods. For both of the methods, it is important to ensure that no donor emission leaks through the acceptor emission filter and alters the results. (Gordon et al., 1998.) Furthermore, if the absorption spectra of the acceptors overlap with the excitation wavelength, the acceptor might be directly excited without any FRET (Vogel et al., 2006). Thus the measurement conditions need to be carefully considered. In addition, if a large molecule (e.g. antibody) is labelled with multiple acceptors, the proximity of the acceptors might cause energy transfer between acceptors (if the absorption and emission spectra of the acceptor overlap substantially) and the observed sensitized emission decreases from the true levels (Miller, 2005). Sometimes it is convenient to measure both donor and acceptor emissions simultaneously. In immunoassays, this double-wavelength detection enables real-time

correction for optical transmission making the assay free from media interactions (Mathis, 1995). Also, some applications of the spectral imaging microscopy utilize the simultaneous measurement of both donor and acceptor emissions. These techniques can be used to measure the efficiency of FRET as well as the abundance of the donors and acceptors. For a recent review see for example Sekar and Periasamy, 2003, Jares-Erijman and Jovin, 2003.

Also fluorescence polarization anisotropy can be used to measure FRET. These methods compare the orientation of the molecules excited with the orientation of the molecules that emit photons using linearly polarized light. If no FRET occurs then the same molecule is excited and emits the light, thus its orientation will be highly correlated. If FRET, however, occurs then the emitting molecule might be different than the excited molecule and, thus, correlation between their orientations will decrease. (Vogel et al., 2006.)

To conclude, the efficiency of FRET can be measured using several different approaches. However, the evaluation of the accuracy of FRET measurements is challenging. This is mainly due to the fact that there are no widely accepted FRET standards (molecules with known FRET efficiencies), against which different methods could be compared. Furthermore, the FRET results are, in many cases, presented using indices (relative measures) instead of the actual efficiency values. These indices methods typically vary between different applications. (Berney and Danuser, 2003.) Therefore, Vogel et al. suggest that, if possible, indices should be avoided and FRET efficiencies should be used (Vogel et al., 2006).

2.3 Lanthanide chelates as donors in FRET

The first successful lanthanide chelate technology was published during the 1980's. These chelates required dissociative fluorescent enhancement step to convert a non-fluorescent chelate into a highly fluorescent one. This was also commercialised as a DELFIA (Dissociation Enhanced Lanthanide FluoroImmunoAssay) system by Wallac, currently part of Perkin Elmer Life and Analytical Science. (Soini and Kojola, 1983, Mukkala et al., 1989.) From those days, also directly (intrinsically) fluorescent lanthanide chelates that do not require enhancement steps have been developed. These lanthanide chelates (mainly europium, terbium, samarium and dysprosium) have many beneficial fluorescence characters that make them ideal candidates for donors in FRET. Only the intrinsically fluorescent chelates can act as donors, thus only they are reviewed in this thesis. For a recent review of the other chelate technology see for example Hemmilä and Mukkala, 2001. It should be noted that the precise term for the energy transfer, when lanthanides are used as donors, is luminescence resonance energy transfer (LRET), since the lanthanide emission is not, technically, considered to be fluorescence. However, LRET obeys the same previously described principles as

FRET. Nonetheless, term FRET is very commonly used in the literature with lanthanides and, thus, is also used throughout this thesis.

2.3.1 Structure of lanthanide chelates

In order to be a functional label, the candidate molecule has to fulfil certain requirements. It has to be stable and soluble. For the labelling reactions to other molecules, such as antibodies, the molecule has to be small and hydrophilic, preferably with a negative net charge to avoid decreased solubility and increased non-specific interactions of the conjugate (Hemmilä et al., 1997). Furthermore, the binding of the label to the antibody or antigen should not have negative influence to their properties. In addition, a high luminescent intensity is required for a sensitive detection (Hemmilä, 1985). Lanthanide ions cannot fulfil these requirements alone. However, when combined with an organic chromophore (called antenna or sensitizer), which absorbs the excitation light and transfers it to the lanthanide ion, powerful fluorescent probes can be developed (Selvin, 2002). For efficient energy transfer, the organic choromophore and the lanthanide ion need to be in close proximity to each other (Hemmilä et al., 1997). The most commonly used antennas are based on pyridine, bipyridine, terpyridine, salicylate, coumarin derivatives, phenanthroline and pyrazole derivatives (Hemmilä and Laitala, 2005). The ligand binds to the lanthanide ion tightly and shields the lanthanide ion from the quenching effects of water. The fluorophore also includes some reactive group that can be used to conjugate the complex to biomolecules (Selvin, 2002). Some of the typical intrinsically fluorescent lanthanide chelate structures are presented in figure 5.

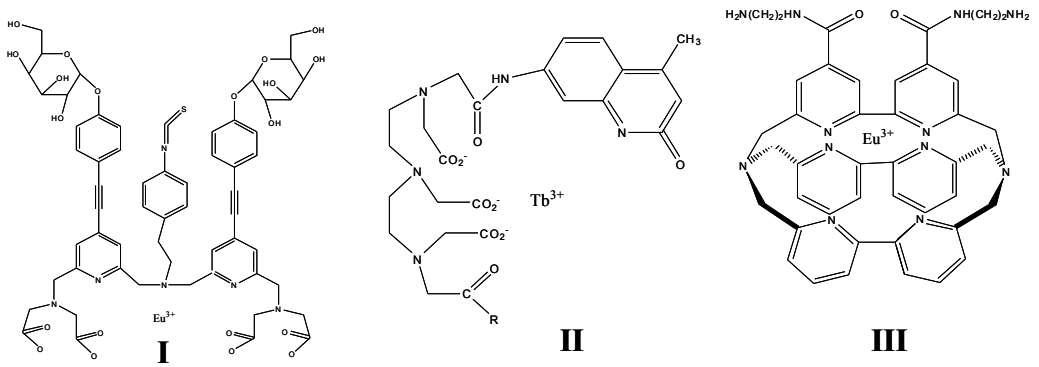


Figure 5 Some representatives of different chelate structures **I:** nona-dentate pyridine based europium chelate (von Lode et al., 2003) **II:** polyaminocarboxylate terbium chelate (Selvin and Hearst, 1994) **III:** europium tris-bipyridine cryptate (Bazin et al., 2001).

As can be seen from the figure 5, in the pyridine and cryptate based probes (I, III) the antenna participates to the binding of the lanthanide ion; whereas, in the polyaminocarboxylate chelates (II) the antenna and the chelating part are separate

entities (Selvin, 2002). Pyridines are the most commonly used light-absorbing aromatic groups in lanthanide chelates. However, the pyridine based lanthanide probes are susceptible to the quenching of water molecules because they have a strong tendency to fill their first coordination sphere with water molecules up to the coordination number nine. Therefore, increasing the number of coordinating groups with the metal ion can enhance the stability of the chelates. Furthermore, the water solubility can be attained with sugar moieties in the chelate structure. (von Lode et al., 2003.) Terbium does not necessarily form fluorescent chelates with same ligands as used for europium. Therefore, for terbium, one or two of the pyridines are often substituted with five-member rings, such as pyrazole, imidazole or thiazole. (Hemmilä and Mukkala, 2001.) The cryptates are a cage like chelates where the ions are encapsulated inside the cavity and are, therefore, highly inert and can be used under drastic chemical conditions, for example in acidic media (Bazin et al., 2001). The polyaminocarboxylate chelates have a tendency to bind the lanthanide ion extremely tightly (Selvin and Hearst, 1994). In conclusion, the fluorescent lanthanide chelates can be constructed using numerous different building blocks. In the end, stable and highly fluorescent probes should be attained.

2.3.2 Fluorescence properties of lanthanide chelates

The lanthanides are fluorescent metals, which display emission in aqueous solutions and decay times of 0.5-3 ms. The absorption coefficients of lanthanides are very low and the emission rates are slow, which results in long lifetimes. Due to the weak absorption, this luminescence is observed only at high lanthanide concentrations or when excited directly by lasers. (Leonard and Gunnlaugsson, 2005.) However, these shortcomings can be avoided by using an organic antenna as an energy transfer moiety (Lakowicz, 1999). The most used lanthanides are terbium and europium, which emit at the visible wavelengths. Also samarium and dysprosium emit at visible but their fluorescence intensity is weaker; thus, they are less common than europium and terbium. (Xu et al., 1992.) The Jablonski diagram for lanthanide chelate (using terbium chelate as an example) is presented in figure 6.

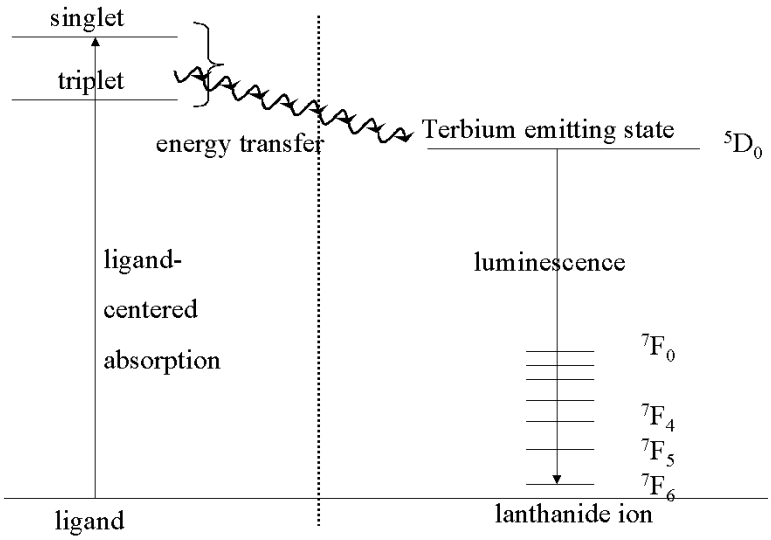
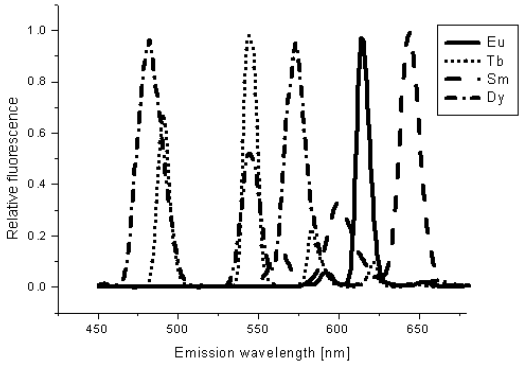


Figure 6 Simplified Jablonski diagram for the fluorescence process in a terbium chelate (Lakowicz, 1999).

The lanthanide excitation is a two-step process. First, the antenna is excited in the ultraviolet wavelengths. Second, the energy is transferred through an intramolecular energy-transfer process to the lanthanide ion. Thereafter, the excited lanthanide may express luminescence. The lanthanide ions have several ground states and the transition distribution to these ground states (^7F_x in figure 6) creates the ion- and chelate specific emission profiles. (Hemmilä and Laitala, 2005.) (Figure 7) Due to the chelate structure that shields the lanthanides from the broadening effects of the solvent, the emission is typically narrow-band line-type emission (the emission peaks are narrow and well defined), whereas the excitation region is typically large (Selvin, 2002). The multitude of well separated emission peaks enables the viability of lanthanide chelates in heterogeneous multi-parameter assays (Hemmilä and Mukkala, 2001) and recently also in homogeneous assays (Karvinen et al., 2004a, Kupcho et al., 2007, Jeyakumar and Katzenellenbogen, 2009). Since the excitation wavelengths are ultraviolet and the emissions are visible, the Stokes' shift of the lanthanide chelates are large (> 250 nm), which facilitates the emission measurement without interference from the excitation. In addition, the lifetime of the luminescence of the lanthanide chelates is exceptionally long; thus enabling time-resolved measurements (Hemmilä, 1985). The fluorescence is also unpolarized, which reduces the previously described orientation dependency of FRET and makes the distance determinations more accurate (Selvin, 2000). The lanthanide chelates do not suffer from self-quenching, thus they can be packed inside a nanoparticle without degrading the fluorescence properties of the probe. These particulate labels are typically latex or silica-based. (Hemmilä and Laitala, 2005.) Furthermore, the shell protects the lanthanide chelates inside the particle from interferences, such as water molecules or metal ions (Ye et al., 2004, Kokko et al., 2007).



Cation	Emissions	Cation	Emissions
Eu^{3+}	580 nm	Tb^{3+}	490 nm
	590 nm		**545 nm**
	613 nm		590 nm
	650 nm		620 nm
	690 nm		650 nm
	710 nm	Sm^{3+}	560 nm
Dy^{3+}	483 nm		598 nm
	575 nm		**643 nm**
	660 nm		710 nm

Figure 7 Left: The normalized emission spectra of Eu(III), Tb(III), Sm(III) and Dy(III) chelates. (Modified from Huhtinen et al., 2005.) **Right:** The major emission lines for the lanthanide chelates. The dominant peak in bold. (Hemmilä, 1991.)

The previously described europium, terbium, samarium and dysprosium chelates are so-called down-converting probes; the emission occurs at longer wavelengths than the excitation. Some lanthanides, mainly erbium, thulium, praseodymium, holmium and ytterbium, can also be used to create up-converting probes, where the excitation occurs at the infrared area and emission at visible wavelengths. These systems are free of background interferences because up-conversion does not occur in the nature. However, these probes are not based on chelate structures; instead they are inorganic particles that consist a crystalline host lattice doped with rare earth lanthanide ions. For a recent review see Soukka et al., 2005 and Soukka et al., 2008.

2.3.3 Other long lifetime fluorescent probes

Lanthanides are not the only metals that can be used to create a long lifetime fluorescent probes. Also transition-metal-ligand complexes and metallo-porphyrins can be utilized. The transition-metal-ligand complexes are typically based on ruthenium, rhenium or osmium with one or more diimine ligands. These complexes are highly stable, so the metals do not dissociate from the ligands significantly. (Lakowicz, 1999.) The metallo-porphyrins are typically complexes of platinum or palladium. Particularly platinum is used due to a higher quantum yield in aqueous solutions and room temperature (de Haas et al., 1997). Examples of these structures are presented in figure 8.

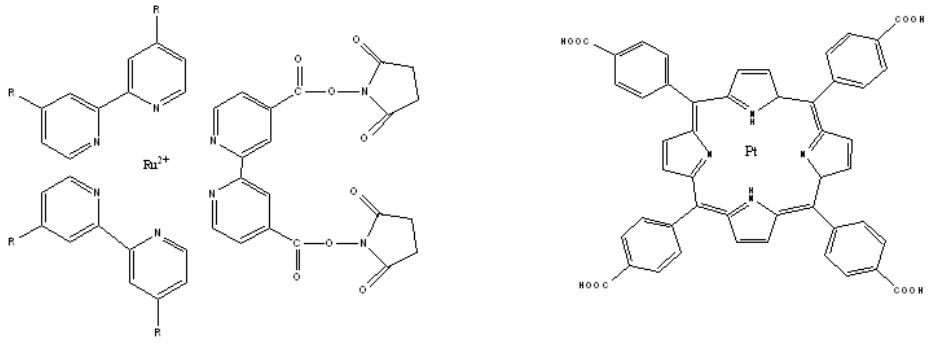


Figure 8 Left: Ruthenium-ligand complex (Terpetschnig et al., 1995) and **Right**: platinum porphyrin (de Haas et al., 1997).

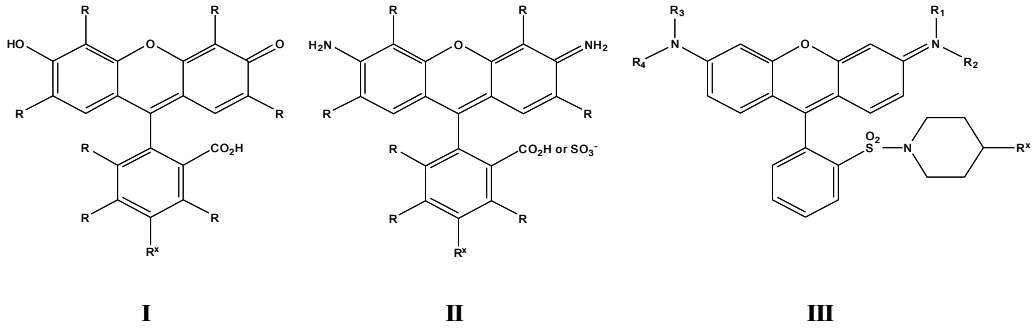
The luminescence process of the transition-metal-ligand complexes is reversed compared to the lanthanide chelates; the energy transfers from the metal ion to the ligand. This is partially forbidden, thus the lifetimes of the complexes are long (10 ns to 10 μs). (Lakowicz, 1999.) Both the transition-metal and porphyrin complexes display large Stokes' shift (Lakowicz, 1999, Eastwood and Gouterman, 1970). The emission spectra of the transition-metal-ligand complexes consist of a one large peak where the wavelength of the emission maxima can be changed by using different metal ions or ligands (Kober et al., 1985, Terpetschnig et al., 1995). The emission peaks of porphyrin complexes are narrower but contain typically only one peak (Eastwood and Gouterman, 1970, de Haas et al., 1999).

2.4 Acceptors in FRET

Numerous different acceptors are available. The decision which acceptors are to be used is made based on the properties of the donor and the performance expectations of the application. In this thesis, only acceptors that absorb and emit (if fluorescent) at visible or near IR area, and are thus suitable for lanthanide chelate donors, are reviewed. For a more complete review of the various acceptors see for example Sapsford et al., 2006.

2.4.1 Organic dyes

Organic dyes (either fluorescent or non-fluorescent) are the most commonly used acceptors in FRET. In fact, they are usually utilized both as donor and acceptor; typically as pairs of coumarin/fluorescein, fluorescein/rhodamine and Cy3.5/Cy5 or their derivatives. (Massey et al., 2006.) Structures of some of the organic dyes are presented in figure 9. Due to the high number of cyclic structures, the dyes tend to be hydrophobic. This can be problematic when conjugating these dyes to other molecules, thus also more hydrophilic dyes have been developed (Zheng et al., 2007).



I **II** **III**

Figure 9 Structures of common acceptors: fluorescent dyes fluorescein (**I**) and rhodamine (**II**) and a non-fluorescent dye family of QSY (**III**) (Sapsford et al., 2006).

A wide variety of different emission maxima can be obtained by using different dyes (figure 10); thus these dyes can be used as acceptors with numerous donors and applications. Organic dyes typically have a short fluorescence lifetime together with broad absorption and emission spectra and, thus, small Stokes' shifts. (Lakowicz, 1999.) This could cause excitation light to leak to the emission measurement results and self-quenching (Sapsford et al., 2006). However, the lanthanide donors extend the lifetime of the fluorescence of the organic dyes; hence a time resolved measurement of the sensitized emission could be used. This prevents the leakage of the excitation light and possible background fluorescence not originating from FRET (Selvin, 2002). To avoid self-quenching, appropriate distance between organic dyes (for example when proteins are labelled) is required (Lakowicz, 1999). The organic dyes can also be environmentally sensitive. When near IR dyes were studied, it was noticed that some of the emission maxima shifted and lifetimes changed due to ethanol or detergent additions. Furthermore, when the dyes were conjugated to biotin and streptavidin was added, a clear change in the spectral properties of both the dye-biotin and dye-biotin-streptavidin complexes were observed compared to free dyes. (Buschmann et al., 2003.) Thus, knowledge of the behaviour of the dyes is important when new assays are designed.

Considerable numbers of different acceptors have been developed and are commercially available. These commercial dyes are easy to use since they typically are in a reactive form and can be conjugated directly to various molecules through different reactive moieties. Some of the most common acceptors and their manufacturers are presented in figure 10 together with their feasible emission wavelengths for fluorescent acceptors and absorption wavelengths for the non-fluorescent acceptors (quenchers).

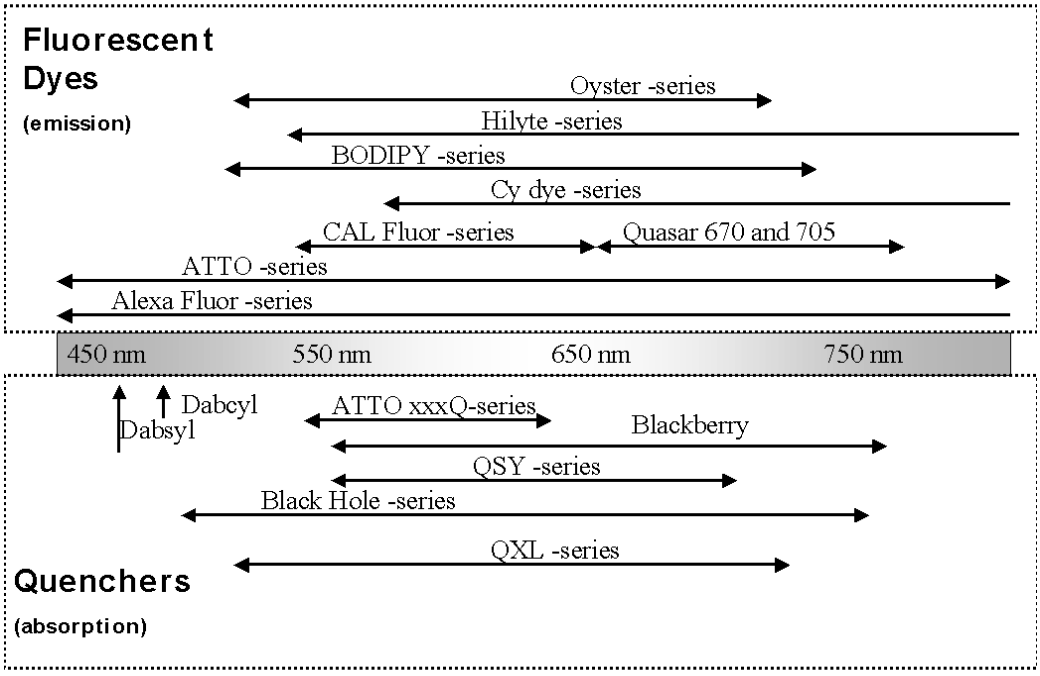


Figure 10 Some of the most common commercially available organic dyes and their emission wavelengths (for fluorescent dyes) and absorption wavelengths (for quenchers). The Alexa Fluor, BODIPY, Dabcyl and QSY –series are sold by Molecular Probes, Invitrogen. Cy –series is a product of GE Healthcare. Hilyte Fluors and QXL quenchers are manufactured by AnaSpec. Oyster dyes are products of Denovo biolabels. Bioresearch manufactures Black hole quencher and the CAL Fluors and Quasar dyes. Atto dyes and quenchers are products of Atto-Tec and Blackberry comes from Berry and Associates.

In principle, the signal levels of an assay could be increased by using multiple fluorophores as donors and/or acceptors. However, excess labelling might change the function of the molecule. Furthermore, with organic dyes the self-quenching inhibits high labelling degrees. Therefore, fluorescent nano- and microparticles soaked with fluorophores have been developed. (Sapsford et al., 2006.) In addition, these particles can be doped with varying amounts of different organic dyes simultaneously and, thus, different colours can be exhibited using only one excitation wavelength, technique

called barcoding (Wang and Tan, 2006). Commercially available TransFluospheres (TFS) are particles that contain optimal combination of dyes in order to optimise the internal FRET and also to yield large Stokes' shift (Molecular Probes, Invitrogen).

2.4.2 Proteins as acceptors

Fluorescent proteins, mainly phycobiliprotein family or green fluorescent protein and its derivatives, are used intensively as donors and acceptors in various FRET applications, mainly in diverse imaging techniques. However, they also have spectral properties that enable them to be used as acceptors for lanthanide chelates as well.

The most commonly used phycobiliproteins are B- and R-phycoerythrins (B- and R-PE) together with allophycocyanin (APC). These proteins are found in cyanobacteria and red algae. The subunit structure of B- and R-phycoerythrins [$(\alpha\beta)_6\gamma$] is extremely stable (Oi et al., 1982), whereas the trimer structure [$(\alpha\beta)_3$] of APC tends to dissociate at low concentrations. Therefore, more stable, cross-linked APC, has been developed (Yeh et al., 1987). The spectral properties of R-PE and APC are presented in figure 11.

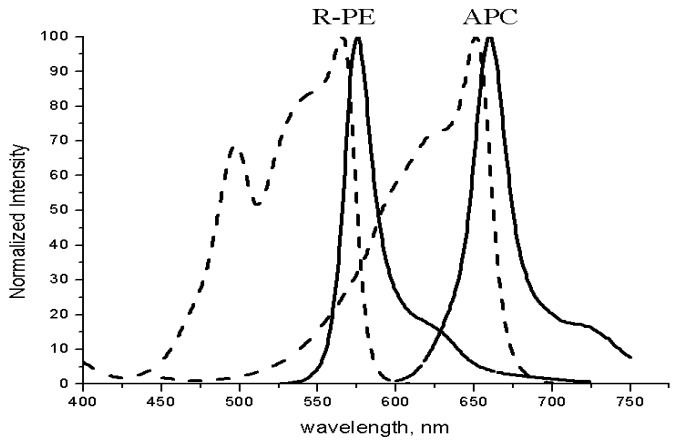


Figure 11 Absorption (dashed line) and emission (solid line) spectra of R-PE and APC (Obtained from Molecular Probes, Invitrogen).

It has been shown that the quantum yields of the emission of the phycobiliproteins are wavelength-independent, thus they can be excited using large variety of excitation wavelengths (Yeh et al., 1987). Therefore, they can have somewhat large Stokes' shifts, even though their absorption and emission maxima are in close proximity. They also have high quantum yields. Furthermore, phycobiliproteins are water-soluble and stable, thus they endure long storage time. (Lakowicz, 1999.) In addition, their spectral properties make them good candidates to act as acceptors for lanthanide chelates.

The green fluorescent protein (GFP) was discovered by Osamu Shimomura in 1960's from jellyfish *Aequorea Victoria* and has since, together with its derivatives (blue, cyan and yellow fluorescent proteins), became one of the most powerful imaging techniques used in fluorescence based optical microscopy (Nienhaus, 2008). Furthermore, in the year 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien won the Nobel price for the discovery and development of the GFP. The GFP has 238 amino acids that fold into a rigid 11 stranded β barrels with a central helix running along its axis (Tsien, 1998). The fluorescent group is situated within a highly constrained and protected region of the protein (Lakowicz, 1999). GFP can be co-expressed with other proteins intracellularly and when visualized, reveal the location and relative expression level (Sapsford et al., 2006). GFP has an absorption maximum at 395 nm and a shoulder at 470 nm. Maximum emission occurs at 509 nm. (Chalfie et al., 1994.) Thus, GFP could be a suitable acceptor for at least terbium chelates (Riddle et. al., 2006). For a review of the spectral properties of other fluorescent proteins, see for example Shaner et al., 2007.

2.4.3 Quantum dots as acceptors for lanthanide chelates

The previously presented organic dyes and fluorescent proteins are the most commonly used acceptors for lanthanide chelates. However, some other probes have also spectral properties that enable them to be used as acceptors.

Quantum dots are nanocrystal fluorophores that consist of a semiconductor core followed by a semiconductor layer and have variable surface layers depending on needed surface functionalization. The core is typically composed of elements from the groups II and IV (cadmium/selenium being the most common combination) or groups III and V (for example indium/phosphorus). The second semiconducting layer is made of material that has a larger band gap than the core semiconductor, such as zinc/sulphur. Finally the surface layer is structured to make the dots water-soluble and also compatible for different bio-conjugations. (Alivisatos et al., 2005.) Quantum dots have very wide absorption spectra, thus several excitation wavelengths can be used. Whereas, the emission spectra is narrow and the emission wavelength can be controlled by changing the core size or composition of the dot. (Jamieson et al., 2007.) Therefore, quantum dots can be engineered to emit light at a variety of precise wavelengths typically ranging from 450 nm to 850 nm (Yezhelyev et al., 2006). Thus, quantum dots could potentially be used to create multi-parameter assays where with one excitation wavelength several different emission wavelengths are achieved.

2.5 Applications of FRET using lanthanides as donors

The number of published FRET applications is enormous. Most commonly the D-A pairs are consisted of organic dyes. However, lanthanide chelates have been used in various applications. The emphasis of this chapter is to present different assay

techniques together with diverse lanthanide-acceptor pairs, in order to show the versatility and possibilities of the lanthanide chelates as donors in FRET based assays. All of the presented techniques are based on exploiting the beneficial characters of the lanthanide chelates (more detailed descriptions previously): The long lifetime of the lanthanides extends the lifetime of the sensitized emission of the acceptors, thus enabling time-resolved measurement of the emission. In addition, the long Stokes' shift diminishes leak of the excitation light to the measurement and the sharp emission peaks alongside with the emission valleys provide several appropriate wavelength areas for designing FRET assays with small contribution of the donor emission to the background signal. The basic principles of some of the FRET based assays are presented in figure 12.

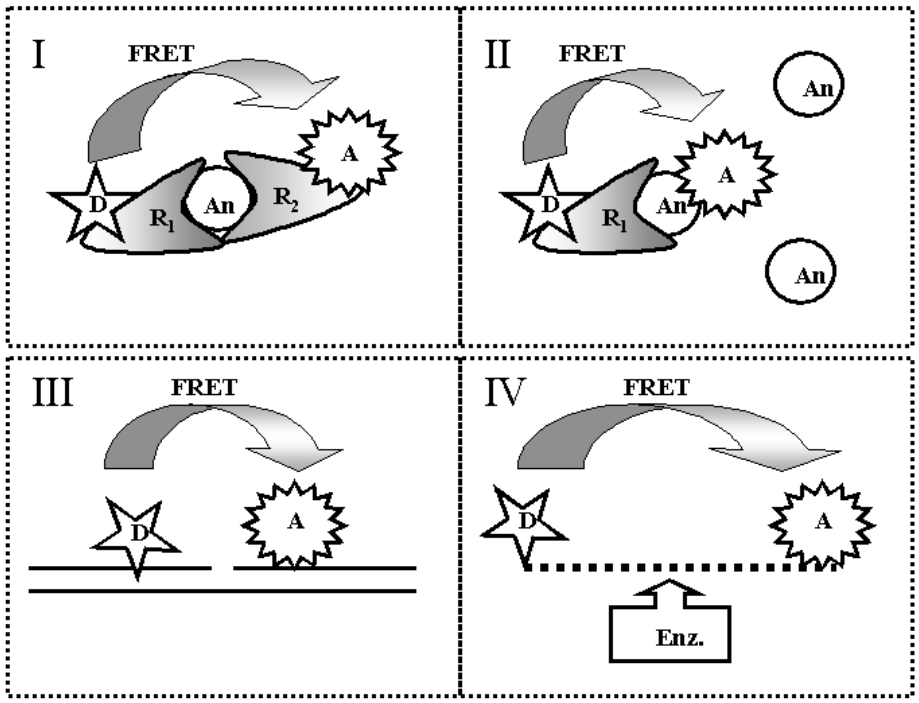


Figure 12 The underlying main principles of the published FRET based assays utilizing lanthanides as donors. **I:** Homogeneous sandwich assay, which uses a donor (D) and acceptor (A) labelled moieties ($R1_1$ and R_2) that can recognize the analyte. FRET can occur if both of the recognizing agents are bound to the analyte (An). **II:** In competitive assay format, the analyte competes of the recognizing moiety with the acceptor labelled analyte analogue. Thus increasing analyte concentration decreases the obtained signal. **III:** In DNA-hybridisation assays, FRET can occur if both the donor and the acceptor oligonucleotides are hybridised to the target DNA. **IV:** In the protease/nuclease activity assays, FRET can typically occur in the absence of the active enzyme. In the presence of the enzyme (Enz.) the substrate is cleaved between the donor-acceptor pair and, thus, the distance is too long for efficient FRET to occur.

Perhaps the simplest homogeneous assay principle is based on the traditional heterogeneous sandwich immunoassay. In the homogeneous version, one of the antibodies is labelled with the donor and the other with the acceptor. FRET can occur when both of the antibodies are bound to the antigen (Figure 12, I). This technique has been used to create an immunoassay for the β subunit of human chorionic gonadotropin (βhCG) utilizing terbium chelate as a donor and rhodamine as an acceptor. The obtained performance of the assay correlated well with a conventional heterogeneous immunoassay. In that study, it was also shown that homogeneous assays do not require shaking of the plates, which simplifies the assay protocols even further. (Blomberg et al., 1999.) One of the main disadvantages in the homogeneous assays is the possible sample interference during measurement. It was shown; with a sandwich assay using europium cryptate as a donor and APC as an acceptor that by using the proportion of two measurement wavelengths, one at the donor emission and one at the acceptor emission, the interference of the sample could be reduced and the performance of the assay was significantly improved. Even with human serum samples, the results of the homogeneous assay correlated well with a heterogeneous radioimmunoassay (RIA). (Mathis, 1993.) Recently, also terbium cryptates have been used in homogeneous assays. For example, Leifert et. al have used terbium cryptate as a donor and Alexa Fluor 546 as an acceptor while studying the G-protein subunit interactions (Leifert et al., 2006). Particulate labels can also be used to design homogeneous sandwich assays. Valanne et. al used a europium particle as donor and TFS particle as an acceptor to design a homogeneous PSA assay. With this protocol, a good sensitivity of ~0.1 ng/mL was obtained. However, the dynamic range was somewhat low since the signal levels started to decrease after 25 ng/mL. (Valanne et al., 2005.) Particulate labels have high donor density, thus several chelates can participate to the energy transfer. Therefore, in these cases, the sensitized emission of the acceptor can be very high, sometimes even high enough for the detection of FRET at the single-nanoparticle level (Casanova et al., 2006). As described previously, the emission spectrum of terbium chelate has several sharp and well-separated peaks and valleys. This quality was exploited when terbium chelate (conjugated to an antibody that could recognize the receptor) was used as a single donor for two different acceptors (fluorescein and Alexa Fluor 633 conjugated to two different peptides) simultaneously. Based on whether the receptor had bound to the target ligand or not, one of the two peptides could bind to the receptor surface and, thus, close enough for FRET to occur. During measurement, the wavelength of the sensitized emission of the acceptor could indicate the presence or absence of the ligand-protein-interaction. (Kupcho et al., 2007.)

The sandwich assay technique is usually impropriate when small analytes (haptens) are to be measured, due to the fact that two antibodies cannot bind to haptens simultaneously. Therefore, haptens are typically measured using competitive assays where the analyte competes of the binding sites of the recognizing agent with a labelled analyte analogue (Figure 12, II). The conjugation of the label to the analyte can be achieved using different methods. For example, the analyte could be conjugated to

bovine serum albumin (BSA) molecule together with europium chelates to form a large donor molecule. Together with a Cy5 dye labelled T3 thyroid hormone specific antibody, a homogeneous T3 assay was designed that had excellent correlation with a heterogeneous RIA. (Wang et al., 2006.) More universal donor complexes can be created with terbium chelate labelled streptavidin. The antigen analogue was biotinylated and, thus, capable of binding to the terbium-streptavidin complex. This time Cy-labelled herbicide specific antibody was used as an acceptor complex. With this protocol the number of donors participating to the energy transfer was increased compared to a conventional antigen-label complex and the assay was able to reach the performance of generally used HPLC method. (Wang et al., 2001.) Hapten assays can also be non-competitive. For example, Cy5 labelled antibody recognizing immuno-complex of another antibody (labelled with europium chelate) and morphine was used to design a non-competitive homogeneous assay. To further improve the assay, antibody Fab fragments were used to reduce the amount of futile donor and acceptor molecules too far to participate in FRET and, thus, only contributing to the level of background. With this protocol a detection limit of 5 ng/mL was achieved while the cut-off detection level of opiates in oral fluids is typically 40 ng/mL. (Pulli et al., 2005.) However, the development of these anti-IC antibodies can be somewhat challenging. Also other than the conventional acceptors can be used. Hildebrandt et. al have shown that a terbium chelate could be used as a donor for CdSe/ZnS core shell quantum dots. With that system detection limit of 3.3 pM was achieved. (Hildebrandt et al., 2005.) However, the method was tested using biotin-streptavidin complex, which is based on very strong and highly stable interactions, thus the performance of an actual assay system should be evaluated before final conclusions.

In addition to immunoassay detection, FRET can also be used in distance measurements. Lanthanide chelates can be synthesized to contain various reactive groups (such as thiols) and can, therefore, be labelled into specific sites of different biomolecules. Thus, the distance of the D-A pair can be controlled. These properties have been utilized in distance measurements, for example, when conformational changes in myosin (Xiao et al., 1998) and in AE1 protein from red blood cells (Knauf and Pal, 2004) were studied. Both of these applications used terbium chelate as a donor together with appropriate acceptors.

Since homogeneous assays are typically very easy to perform, in ideal cases mix and measure assays, they are very appealing techniques to be used in high throughput screening (HTS) applications in pharmaceutical industry when new, potential drug compounds are searched. Commonly, these applications study whether the target molecule has some effect to a specific biological incident, for example protease/nuclease activity. In these enzyme activity assays, the substrate is typically labelled with both donor and acceptor. If the enzyme cleaves the substrate, the donor and acceptor end up too far from each other for FRET to occur. (Figure 12, IV) Karvinen et. al has used this principle to create different enzyme activity assays for caspase, helicase and phosphatase. All of these assays were based on removing the

quencher molecule from the proximity of the lanthanide chelate donor via enzyme activity. When europium chelate was used as a donor, some remarkably high quenching efficiencies were obtained (99.97% with QSY7 as an acceptor/quencher). It was also shown that the D-A pair optimisation is essential since the different assays showed best energy transfer properties with different quenchers, even though donor was europium chelate in every assay. Thus the energy transfer distance and the ionic environment around the donor and acceptor had an effect to the performance of different D-A-pairs. (Karvinen et al., 2004b.) Similar principle was also used to multiplex caspases 1, 3 and 6. In that research, the substrates were labelled with samarium, terbium or europium chelates together with appropriate quenchers. Since the emission peaks of the different lanthanide chelates are sited at different wavelengths (see page 21, figure 7), the emissions could be measured simultaneously. Thus, the assay was able to detect the activity of a mixture of the three enzymes in the same well. (Karvinen et al., 2004a.) Another caspase assay utilized a universal donor (streptavidin coated europium nanoparticles) together with Alexa Fluor 680 and BlackBerry quencher conjugated to the substrate. When the substrate was intact, the energy could first transfer from europium particle to Alexa Fluor 680 and from there to the BlackBerry. Thus, double FRET occurred. If the substrate was cleaved, there was no energy transfer from Alexa to BlackBerry, thus no quenching happened, and the emission of the Alexa Fluor could be measured. (Valanne et al., 2008.) Enzyme activity assay can also be based on acceptor emission measurement. Kinase assay was developed where energy transfer from terbium chelate (conjugated to a phosphospecific antibody) to either fluorescein or green fluorescent protein could only happen if the substrate was phosphorylated by the kinase. Since GFP can be used as a fusion protein, this assay principle enables the screening of GFP fusions of native, physiologically relevant substrates. (Riddle et al., 2006.) Important issue to consider, in these activity assays, is the distance between the D-A pair. The flexibility of the different substrates vary, thus in truth the donor and the acceptor could be much closer to each other than theoretically seems. If the true distance of D-A pair is small relative to R_0, the energy transfer might be too rapid to be efficiently measured using the typical parameters of lanthanide chelates for time-resolved measurements. Thus, the performance of the assay might be improved if the D-A distance is increased. (Vogel and Vedvik, 2006.) On the other hand, it has been shown that with extremely short D-A pair distance together with high efficiency FRET and a very short delay time before the measurement the sensitivity of the assay can be improved (Laitala, 2005). However, these systems require very sophisticated instrumentation.

Also HTS assays that measure small analytes have been developed, for example, competitive estradiol assay that utilized FRET between antibody-coated europium particle and estradiol-Alexa Fluor 680. Compared to conventionally labelled antibody as a donor, the particle-based assay achieved 20 times better sensitivity. (Kokko et al., 2004, Kokko et al., 2008.) Protein-protein interactions might provide a feasible base for HTS assays. Glaser et. al have published an assay that could screen molecules that might affect the growth of different bacteria. In that research, two proteins, crucial for

the survival of the bacteria (*Escherichia coli*), were labelled with terbium chelate or Cy3 dye. If any of the screened substances prevented the complex formation of these two proteins, no FRET could occur. (Glaser et al., 2007.) The combination of europium chelate as a donor and APC as an acceptor has inspired couple of research groups to develop different HTS applications. They have been used to create a screening assay for small-molecule inhibitors of HIV-1 fusion and possibly the viral entry (Dams et al., 2007). Also, a small-molecule inhibitors of ubiquitination, and thus possibly modulators of the turnover of growth-regulatory proteins, have been screened with Eu/APC pair. When this assay was compared to a conventional heterogeneous assay, the performance of the homogeneous assay was slightly inferior but the homogeneous assay had better precision. (Boisclair et al., 2000.) In addition, this D-A pair was used to screen molecules that affect ligand interactions to herpes virus entry mediator receptor. This assay principle was functional in 384-well format, which makes it particularly useful in HTS studies. (Moore et al., 1999.)

FRET can also be utilized to create homogeneous DNA hybridisation assays where the objective is to detect the target DNA in solution without immobilization of the DNAs or separation of the reaction solution. The assays are typically based on two DNA probes, labelled with donor or acceptor, and each being complementary to two different regions of a target DNA. The chosen probes are in close proximity and FRET can happen between the D-A pair if the target DNA is present. (Figure 12, III) Hybridisation assays can be used, for example, in gene expression analysis or in diagnosis of infectious and genetic diseases. Mathis has used the previously described europium cryptate/APC pair to create a DNA hybridisation assay that had equivalent analytical sensitivity but better capturing efficiency and speed than a corresponding heterogeneous assay (Mathis, 1995). Sueda et. al utilized the benefits of using multiple labels as donor complex to create two DNA hybridisation assays using either europium or terbium labelled streptavidin as the universal donor. The probes where conjugated to either biotin or appropriate Cy-dye. With these assays, detection limits for the target DNA were 200 pM and 30 pM using europium or terbium, respectively. (Sueda et al., 2000, Sueda et al., 2002.) It is also possible to create a DNA hybridisation assay using only one probe labelled with both donor and acceptor (europium chelate and Alexa fluor 647 or 700). In this assay the coiling of the unhybridised probe brought the donor and acceptor in very close proximity resulting very high intensity acceptor signal with a short lifetime. When the probe was hybridised to the target DNA, the distance of the D-A pair increased and the lifetime and the intensity of the acceptor emission were changed. (Laitala et al., 2007.) All of the previously presented applications were based on the Förster principle of the spectral overlapping of the D-A pairs. However, it has been shown that lanthanide chelates can be used to create systems that do not fully comply with the Förster theory. Laitala and Hemmilä have shown that, sometimes, sensitized emission of the acceptor can be measured even if there is virtually no overlapping of the spectra, thus, this phenomenon is called non-overlapping i.e. nFRET. Using this technique with DNA hybridisation assay, quenching efficiencies of 80-90% of the fluorescence of europium chelate were achieved even though the Förster

radii were only 3-5.5 Å. (Laitala and Hemmilä, 2005.) In addition to DNA hybridisation assays, FRET has also been used to improve lanthanide based RT-PCR application. In RT-PCR, the accumulation of the PCR product is read in real time; whereas in end point applications the results are measured only once after the PCR reaction has ended. Nurmi et. al have used an environmentally sensitive terbium chelate to design RT-PCR assays. The chelate was conjugated to the probe, which was cleaved by the nucleic acid polymerase during the amplification of the target DNA. Since the chelate was highly fluorescent only in solution and only mildly fluorescent when connected to the probe, the amplification of the DNA resulted increasing fluorescence of the terbium chelate. (Nurmi et al., 2000.) This technique was further improved by adding another oligonucleotide conjugated to a quencher (QSY7) complementary to the lanthanide-probe. Thus the fluorescence of the intact probe in the solution was quenched using the QSY-7-oligonucleotide. This enhanced the signal to background ratios and enabled detection of 100 copies of the mRNA, whereas with commonly used Taqman system (Applied Biosystems) 1000 copies were needed for similar detection. (Nurmi et al., 2002.)

Some of the previously described D-A pairs and technologies have also been commercialised. LanthaScreen by Invitrogen utilizes a terbium chelate as a donor and fluorescein as an acceptor. The europium cryptate and APC applications using two different wavelengths during measurement are manufactured as Kryptor systems by Brahms. Europium and recently also terbium (Lumi4-Tb) cryptates are used in HTRF/TRACE assays together with appropriate acceptors by CisBio International (see also Mathis, 1999). In addition, Wallac, Perkin Elmer Life and Analytical Sciences has been active in creating homogeneous assay formats: The previously described quenching based enzyme activity assays are manufactured as TruePoint assays. Whereas the LANCE technology utilizes two different europium chelates together with appropriate acceptors (Hemmilä, 1999).

In conclusion, it can be said that fairly many different types of FRET applications utilizing lanthanides as donors has been published. It appears that most of the applications are based on FRET between lanthanide chelates and organic dyes. However, it seems that excellent results are obtained with these pairs, thus their prevalence is somewhat justified. Based on the increasing number of publications in this area, the future prospects of the FRET applications, using lanthanides as donors, appear to be bright and exciting.

3 AIMS OF THE STUDY

The aim of the present study was to research the donor properties of lanthanide chelates (europium and terbium) in FRET. By employing these properties, the possibilities of novel homogeneous assay techniques were explored.

More specifically the aims were:

I To study the appropriate acceptors for efficient quenching of fluorescence of europium(III) chelate. By using these quenchers, create a non-competitive homogeneous assay design for haptens employing biotin-streptavidin interaction as a model system.

II To prove that the previously presented assay technique (**I**) can be used in an actual non-competitive homogeneous assay for hapten (E2). Also the interference of the biological matrix (human serum) to FRET was studied.

III To study the possibility of using terbium(III) chelate as a single donor for multiple wavelength acceptors. For this, the performance parameters of the competitive E2 assay using various acceptors were determined together with interference measurements between different acceptors.

IV To create an actual homogeneous dual-parameter assay for free and total PSA utilizing terbium(III) chelate as a single donor together with two acceptors and to study the possibility of using the assay technique with actual human samples (lithium-heparin plasma).

4 SUMMARY OF MATERIALS AND METHODS

In this chapter, an overview of materials and methods is given. More detailed information about materials and methods can be found in the original publications (**I**–**IV**).

4.1 Measurement of fluorescence and sensitized emissions

Both the fluorescence of the lanthanide chelates and the sensitized emission of the fluorescent dyes were measured using time-resolved fluorometry. The fluorescence of the europium(III) and terbium(III) chelates was measured with 1420 Victor Multilabel Counter (Perkin Elmer Life and Analytical Sciences, Boston MA) using protocols installed by the manufacturer. The excitation wavelength for both chelates was 340 nm and the emission filters were 615 nm and 545 nm for europium(III) and terbium(III) chelates, respectively. Both of the lanthanide fluorescence measurements had delay time of 400 μs and measurement time of 400 μs.

The sensitized emissions of Alexa Fluor (AF) 488, 555, 595 and 680 were measured using 1420 Victor Multilabel Counter with appropriate emission filters (Table 1). For the measurement of the emission of AF 680 a red-sensitive R4632 photomultiplier tube was used (Hamamatsu Photonics, Shimokanzo, Japan). (**IV**)

Table 1 The emission filters for the fluorescent dyes

Fluorescent dye	Emission, nm	Manufacturer
AF 488	520	Chroma Technology Corp. (Rockingham, VT)
AF 555	572	installed by instrument manufacturer
AF 594	615	installed by instrument manufacturer
AF 680	720	Coherent Inc. (Santa-Clara, CA)

In addition, for the measurement of the sensitized emission of AF 680 a modified 1234 Delfia Research Fluorometer (Wallac, Perkin Elmer Life and Analytical Sciences) with a red-sensitive R2949 photomultiplier tube (Hamamatsu Photonics) was used. (**III**) For all the measurements, the delay time was 75 μs and the measurement time was either 400 μs (**III**) or 50 μs (**IV**).

4.2 Conjugation of proteins to lanthanide chelates

For the study, various protein molecules were labelled with lanthanide chelates. Streptavidin (**I,** Societa Prodotti Antibiociti, Milan, Italy) and E2 specific antibody Fab fragment (**II**, produced and purified as described by Kokko et al., 2004) were labelled with intrinsically fluorescent europium(III) chelate (2,2',2'',2'''-{[2-(4-isothiocyanatophenyl) ethylimino]-bis(methylene)bis{4-{[4-(α- galactopyranoxy)

phenyl]ethynyl}-pyridine-6,2-diyl}-bis(methylenenitrilo)}tetrakis(acetato) europium(III)) (von Lode et al., 2003). E2 specific antibody Fab fragment (**III**) and PSA specific Mab 5E4 (**IV**) were labelled with intrinsically fluorescent terbium(III) chelate (2,2'2'',2'''-{{6,6'-{4''-[2-(4-isothiocyanatophenyl)ethyl]-1H-pyrazole-1'',3''-diyl} bis(pyridine)-2,2'-diyl}bis(methylenenitrilo)]tetrakis(acetato) terbium(III)) (Rodrigues-Ubis et al., 1996).

For the labelling reactions 160 to 480 µg of protein and 5 to 100 molar excess of lanthanide chelate dissolved in water was used. The pH was adjusted to 9.8 by adding carbonate buffer (final concentration of 50 mM). Reactions were incubated over night. The next day, reactions were purified using size separation with gel filtration. The amount of protein in the purified products was determined by absorbance measurement at 280 nm, and the amount of the lanthanide chelate was defined by comparing the fluorescence of the product to the fluorescence of a known standard.

4.3 Conjugation of acceptors

During this research, a selection of various fluorescent and non-fluorescent organic dyes was used as acceptors (Table 2).

The appropriate dyes were selected based on the overlapping of their absorption spectra with the emission spectra of the donors together with either their quenching properties (**I** and **II**) or their fluorescence properties (**III** and **IV**). Acceptors were conjugated to amino-modified biotin (**I**, N-(2-Aminoethyl)biotinamide, Molecular Probes Inc, Eugene, OR), amino-modified estradiol (**II** and **III** (Mikola and Hänninen, 1992)) or PSA specific antibodies H50 and 5A10 (**IV** (Piironen et al., 1998)). For the conjugation, 10 to 100-fold molar excess of the organic dye was used. The pH of the reactions was adjusted to 9.0-9.3 by adding carbonate buffer. The reactions were incubated for 4 hours in +37 ˚C and either frozen until purification (**I-III**) or purified immediately (**IV**). The small molecule conjugates (**I-III**) were purified using reversed-phase HPLC technology (Instrumentation from Thermo Electron Corp. Waltham, MA) with Genesis C18 column (Jones Chromatography, Grace Vydac, Hesperia, CA). Gradient purification was done using 50 mM triethylammonium acetate (TEAA, Fluka Biochemica, Steinheim, Switzerland) in water or in acetonitrile. The antibody-acceptor conjugates (**IV**) were purified with gel filtration using NAP5 and NAP10 columns (GE Healthcare, Chalfont St Giles, UK). The amounts of the acceptor dye in the purified products were defined by absorbance measurements (appropriate wavelengths provided by manufacturer).

Table 2 Physical characteristics of the acceptor dyes provided by the manufacturers.

Name	Molecular weight (Da)	Absorption maximum (nm)	Emission maximum (nm)	Extinction coefficient 1/(cmM)	Manufacturer
Dabcyl	366	454	--a	32700	Molecular Probes, Invitrogen (Carlsbad, CA)
Atto612Q	888	615	--	114000	ATTO-TEC (Siegen, Germany)
QSY7	791	560	--	84300	Molecular Probes, Invitrogen (Carlsbad, CA)
QSY9	951	562	--	88200	Molecular Probes, Invitrogen (Carlsbad, CA)
QSY21	815	661	--	89600	Molecular Probes, Invitrogen (Carlsbad, CA)
Cy5	792	649	670	250000	GE Healthcare Chalfont St Giles, UK
Cy7	818	747	776	200000	GE Healthcare Chalfont St Giles, UK
Alexa Fluor 488	643	495	519	71000	Molecular Probes, Invitrogen (Carlsbad, CA)
Alexa Fluor 555	~1250	555	565	150000	Molecular Probes, Invitrogen (Carlsbad, CA)
Alexa Fluor 594	820	590	617	90000	Molecular Probes, Invitrogen (Carlsbad, CA)
Alexa Fluor 680	~1150	679	702	184000	Molecular Probes, Invitrogen (Carlsbad, CA)

a) non-fluorescent dye

4.4 Efficiency of FRET

4.4.1 Quenching efficiency

The efficiency of the energy transfer was estimated by comparing the fluorescence of the donor in the absence and in the presence of the acceptor (quenching efficiency).

$$Q\% = \left(1 - \frac{F_{DA}}{F_D}\right) \times 100\% \tag{3}$$

Where F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor and F_D is the fluorescence intensity in the absence of the acceptor. (Lakowicz, 1999)

4.4.2 The Förster radii

The theoretical efficiency of the energy transfer was estimated by calculating the Förster radii for the different donor-acceptor pairs.

$$R_0 = 0.211\left[\kappa^2 n^{-4} Q_D J(\lambda)\right]^{1/6} \tag{1}$$

where

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

Where J is the spectral overlapping of the D-A pair (see figure 2, page 13); κ is a factor describing the relative orientation in space of the transition dipoles of the donor and the acceptor; *n* is the refractive index of the medium; Q_D is the quantum yield of the donor; $F_D(\lambda)$ is the corrected fluorescence intensity of the donor and $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor. (Lakowicz, 1999.)

4.5 Model analytes

In this research, two different homogeneous assay principles were developed. Both of these principles needed an appropriate model analyte, in order to test the performance of actual assays. In this chapter, the analytes are introduced briefly.

For the non-competitive hapten assay, 17β-estradiol (E2) was selected as the model analyte. E2 is a small molecule (~270 MW) and the most important female sex

hormone. It regulates the female menstrual cycle as well as maintenance of the bone mass and cardiovascular system for both genders. Normal E2 levels vary according to age and menstrual cycle. In diagnostics, E2 levels are used, for example, when monitoring hormonal infertility treatment, assessing ovarian function or diagnosing tumors. It has also been shown that increased amounts of E2 and its metabolites stimulate the proliferation of cancer cells. Therefore, the pharmaceutical industry uses E2 assays in high throughput screening while searching for a proper inhibitor for E2 synthesis in cancer cells (Kokko et al., 2005). Due to human activity, E2, and other estrogens, are also found in many aquatic environments from where they can be carried into aquatic animals. This external E2 can cause several serious problems to water animals, such as abnormal sexual development (Guillette et al., 1994) or induced vitallogenin production in male trout (Tanaka et al., 2004). Thus, analyzing E2 from environmental samples is of growing importance.

Prostate-specific antigen (PSA) is a member of the human kallikrein protease family (Yousef and Diamandis, 2002). PSA is produced by prostatic epithelial cells (Henttu et al., 1992) and occur mainly complexed to α1-antichymotrypsin (65-95% of the total PSA in blood).) Some of the PSA (5-35%) is present in free, uncomplexed, form. (Lilja et al., 1991, Stenman et al., 1991.) It has been shown that the ratio between total PSA and free PSA is less in cancer cases than in benign prostatic hyperplasia (Christensson et al., 1993). Thus, there is a diagnostic demand to measure both free and total PSA from a single sample. Furthermore, PSA has multiple epitopes and a large variety of different monoclonal antibodies have been developed for these epitopes. This makes PSA a good candidate as a model analyte in the homogeneous dual-parameter assay. Typically, healthy young men (<45 years) have PSA levels of less than 1 ng/mL. PSA values correlate strongly with age because benign prostate enlargement with increasing age will increase PSA concentration in blood (Preston et al., 2000). Therefore, the upper limits for suggested reference ranges in men are 2.5, 3.5, 4.5 and 6.5 ng/mL for ages 40-59, 50-59, 60-69 and 70-79 years, respectively (Oesterling et al., 1993).

4.6 Homogeneous assay protocols

All the developed assays in this research were homogeneous assays based on FRET. A summary of the assay specifications is presented in table 3 (page 41). More detailed instructions can be found in the original publications.

The developed assays had either competitive or non-competitive nature. In this thesis, non-competitive assay is defined as an assay that measures directly the amount of analyte. Thus, the signal is created by those complexes containing the analyte and, therefore, the amount of signal increases when the concentration of the analyte increases. Whereas in competitive assays the amount of analyte is measured indirectly and the measured signal is created by complexes that do not contain any analyte. (Instead, the analyte is replaced by analyte-analogue conjugated to an acceptor.) Thus,

the amount of signal decreases when the concentration of the analyte increases. To conclude, the key difference between the basic assay techniques is not the presence or absence of competition but the origin of the measured signal.

A schematic picture of the fundamental principles underlying the developed assays is presented in Figure 13. If the assay principle was based on quenching (**I**, **II**), the FRET between donor and acceptor decreased the donor emission in proportion to the analyte amount. When fluorescent dye was used (**III**, **IV**), the amount of sensitized acceptor emission could be measured if the recognizing agent had bound the acceptor-conjugate. Thus, the amount of sensitized emission was either decreasing (**III**) or increasing (**IV**) with the analyte concentration.

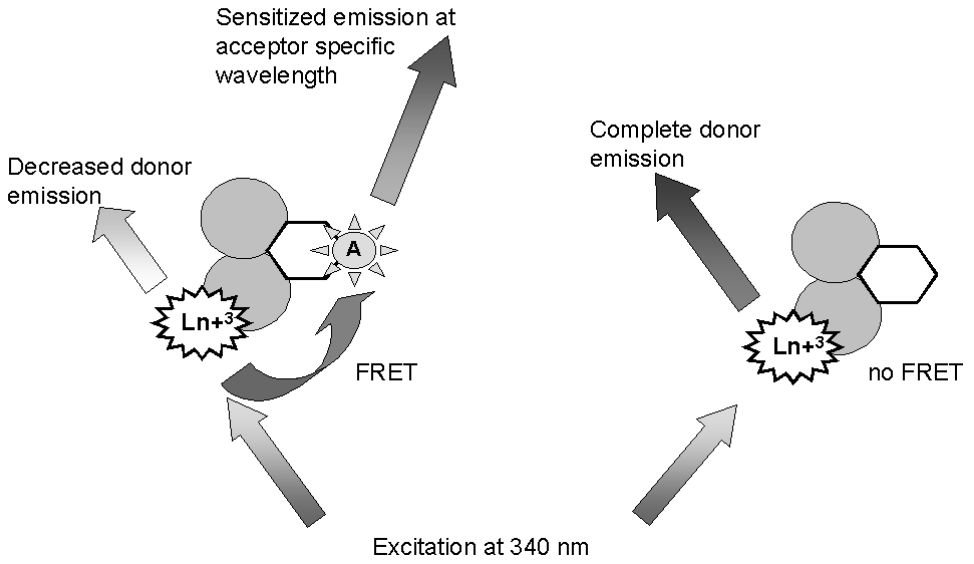


Figure 13 Basic principles underlying all the developed assays. **Left:** If the donor (Ln^{3+}) and the acceptor (A) complexes are bound together either directly (**I-III**) or via analyte as a sandwich complex (**IV**), FRET can occur. Thus, the emission of the lanthanide chelate is decreased (**I**, **II**) or in the case of fluorescent dyes, sensitized emission of the acceptor can be measured (**III**, **IV**). **Right:** If the donor-binder complex is not bound to the acceptor–conjugate, no FRET occurs. Thus, there is no sensitized emission and the emission of the lanthanide chelate is not inflected.

Table 3 Summary of the assay specifications used in the original publications.

Original publication	**I**	**II**	**III**	**IV**
Analyte (volume)	Biotin (50 µL)	E2 (50 µL)	E2 (10 µL)	F- and T-PSA (25 µL)
Assay principle	Non-competitive	Non-competitive	Competitive	Non-competitive
Donor complex	Eu(III)-SA[a] (3 nM, 50 µL)	Eu(III)-FabS16[b] (10 ng/ 25 µL)	Tb(III)-FabS16[b] (10 ng/ 40 µL)	Tb(III)-5E4 (10 ng/ 75 µL)[c]
Acceptor complex	Bio-QSY9, Bio-Cy7 & Bio-Atto612Q (20-25 nM, 50 µL)	E2-QSY21 (125 nM/ 25 µL)	E2-AF488, E2-AF555, E2-AF594 & E2-AF680 (50 nM /50 µL)	H50-AF680 & 5A10-AF488 (20 ng/ 75 µl)[c]
Matrix	--	Serum (10%)[d]	--	Plasma (10%)
Incubation	30 min + 5 min, RT[e]	30 min + 15 min, RT	30 min + 15 min, RT	60 min, RT
Measurement wavelength	615 nm	615 nm	520 nm, 572 nm, 615 nm & 720 nm	520 nm & 720 nm
What was measured?	Donor emission	Donor emission	Acceptor emission	Acceptor emission

[a] Europium(III) chelate labelled streptavidin, the concentration of the stock solution presented in parenthesis, [b] Europium(III) chelate or terbium(III) chelate labelled E2 specific antibody Fab fragment, [c] the donor and acceptors were mixed and 75 µL of mixture was added to wells, [d] the volume percentage of the matrix in the total well volume, [e] RT= in Room Temperature

5 SUMMARY OF RESULTS AND DISCUSSIONS

Summary of the results and discussions of the original publications and some unpublished data are presented in the following section.

5.1 Components of the homogeneous assays

5.1.1 Protein-lanthanide chelate donors

In all of the developed assays, proteins labelled with intrinsically fluorescent lanthanide chelates acted as donors. The emission spectra of the used lanthanide chelates (europium(III) and terbium(III)) are presented in figure 14.

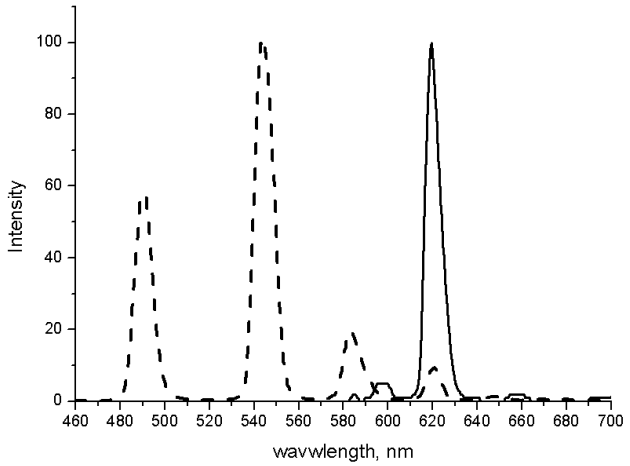


Figure 14 Time-resolved **e**mission spectra of the intrinsically fluorescent terbium(III) chelate (dashed line) and europium(III) chelate (solid line).

For both chelate donors, the labelling degrees (lanthanide chelates per protein molecule) were determined by measuring the protein amount with absorbance measurements. The amount of lanthanide chelate was measured by comparing the fluorescence of the labelled product to the fluorescence of a known lanthanide standard. Usually several labelling reactions were done and the product with the most suitable labelling degree was chosen for further studies. The obtained labelling degrees of the used donors are presented in table 4.

Table 4 The labelling degrees of the donors used in the research.

Donor	Labelling degree	Original publication
SA-Eu	3.2, 4.2 and 6.3	**I**
FabS16-Eu	1.7[a]	**II**
FabS16-Tb	4.0	**III**
Mab 5E4-Tb	12.4	**IV**

[a]After the gel purification some free europium(III) chelate was detected in the purified product. Therefore, fractions were dialyzed in order to remove the remaining free label.

5.1.2 Selection of the acceptors

In order to FRET to occur, short distance between the donor and the acceptor as well as spectral overlapping between the emission of the donor and the excitation of the acceptor are required (Förster, 1948). There are wide selection of different fluorescent and non-fluorescent dyes that can act as acceptors. Thus, the appropriate acceptors for each of the studies were selected based on the features required by the assay principle.

For the development of the non-competitive homogeneous assay for hapten (**I** and **II**), the key factor was the acceptors ability to quench the fluorescence of the europium(III) chelate. Thus, the absorbance spectrum of the acceptor needed to overlap well with the emission spectrum of europium(III) chelate (Figure 15).

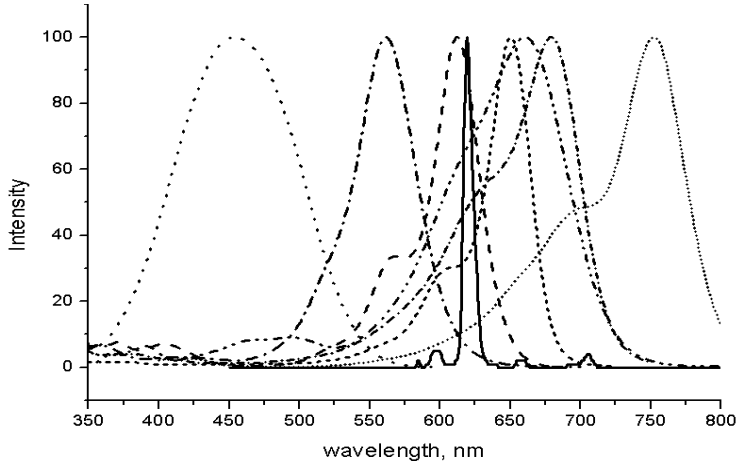


Figure 15 The absorbance spectra of the appropriate quencher dyes for europium(III) chelate (solid line) from left to right: Dabcyl (dotted line), QSY9 (dash dotted line), Atto612Q (dashed line), QSY21 (dash dot dot line), Cy5 (short dashed line), Alexa Fluor 680 (short dash dot line), Cy7 (short dotted line).

When the possibility to use terbium(III) chelate as a single donor in a homogeneous multiplex assay was studied (**III** and **IV**), the fluorescence properties of the acceptors were dominating factors. The chosen acceptors had emission maxima at the emission valleys of the terbium(III) chelate. Minimum spectral overlapping between the acceptors was also required. (Figure 16)

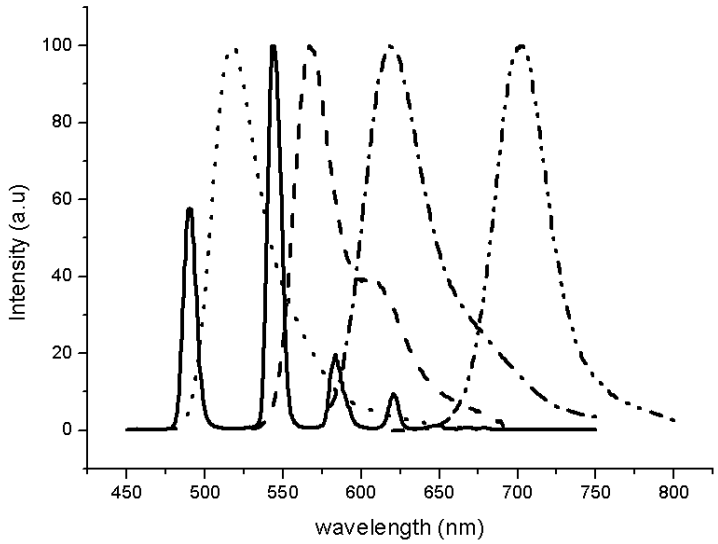


Figure 16 Emission spectra of the fluorescent dyes suitable as acceptors for terbium(III) chelate (solid line), from left to right: AF 488 (dotted line), AF 555 (dashed line), AF 594 (dash dot line), AF 680 (dash dot dot line).

In most of the assay applications, it was possible to conjugate only one organic dye per acceptor component (**I-III**). In these cases, the fractions from the purifications were characterized and fractions containing both the organic dye and the antigen were selected. For the homogeneous PSA assay (**IV**) monoclonal antibodies were conjugated with organic dyes. The labelling degrees for the free PSA (F-PSA) specific Mab5A10-AF488 and total PSA (T-PSA) specific MabH50-AF680 were 13.9 and 5.4, respectively.

5.2 Efficiency of FRET (I, III)

The theoretical efficiency of energy transfer between donor and acceptor can be estimated by calculating the Förster distance for the pair. Föster radius is the distance between donor and acceptor at which FRET is 50% efficient. Typically, the Förster radii are 20 to 90 Å. The longer the Förster distance of the donor-acceptor pair the more efficient is the energy transfer between them. (Lakowicz, 1999.)

The Förster radii were calculated for both of the donors (europium(III) chelate and terbium(III) chelate) with various acceptors (Table 5) using equations presented in 4.4.2. Since all the measurements were performed in aqueous solution, the refraction index (n) was 1.33. The κ^2 is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor. It is usually assumed to be equal to 2/3, which was used in these calculations as well. It has been previously reported that this assumption can produce only ±35% error in the calculated distances. (Lakowicz, 1999.) The quantum yields of the donors were 0.127 for europium(III) chelate (von Lode et al., 2003) and 0.42 for terbium(III) chelate (Rodrigues-Ubis et al., 1996). The observed efficiency of energy transfer can be estimated by calculating the amount of donor emission quenched by FRET. The higher the quenching efficiency the more efficient the energy transfer is.

Table 5 Calculated Förster radii and the observed quenching efficiencies for the donor-acceptor pairs (contains unpublished data).

Acceptors for Eu(III) chelate	**Förster radii (Å)**	**Quenching efficiency %**	**Acceptors for Tb(III) chelate**	**Förster radii (Å)**	**Quenching efficiency %[a]**
Dabcyl	9.3	90	**AF 488**	46	29
Atto612Q	56	98	**AF 555**	61	52
QSY9	36	97	**AF 594**	53	46
QSY21	53	95	**AF 680**	52	42
Cy5	56	67			
Cy7	47	96			
AF 680	57	78			

[a] Approximately half of the antibody Fab fragment used in the tests was found to be inactive and thus not capable of binding the antigen. Therefore the maximum attainable quenching efficiency was ~60%.

Since the selection of the suitable acceptors was based on spectral overlapping of the emission spectra of donors and the absorption spectra of acceptors, the theoretical Förster radii were very similar with all the pairs. However, the Förster distance of the europium(III)-Dabcyl pair was very short. Nevertheless, Dabcyl was able to quench nearly 90 % of the emission of the Eu(III) chelate. Similar results have been obtained also previously and Dabcyl has been successfully used as an acceptor with europium chelate by others as well (Karvinen et al., 2002, Gopalakrishnan et al., 2002). This could be a consequence of intramolecular collisions. If the distance between the quencher and the fluorophore is short enough for relatively high frequency of collisions, effective depression of the fluorescence might occur. (Yaron et al., 1979.) Streptavidin is relatively small molecule (~60000 MW) with four binding sites, thus the donor label and Dabcyl molecule could be close enough proximity for collisions. In conclusion, the non-fluorescent quencher dyes (Dabcyl, Atto612Q, QSY9 and QSY21) were the most effective at quenching the donor emission.

In the case of Tb(III) chelate, the selected acceptors were distributed more evenly through the emission spectrum of the donor (Fig. 16). However, the acceptor AF 555 (absorption spectrum covering most of the emission spectrum of Tb(III) chelate) had the longest Förster distance and also attained the highest quenching efficiency and, thus, had the most efficient energy transfer. In conclusion of the fluorescent dyes as acceptors for Tb(III) chelate, the observed results were perfectly in line with the calculated theoretical Förster distances.

The most efficient quenchers for Eu(III) chelate were also examined by using titration curve (Figure 17). For this study, SA-Eu(III) complex that had a labelling degree around four was selected. Streptavidin has four binding sites for biotin and the biotin/acceptor proportion was one, thus, when all SA-binding sites were occupied there was roughly one acceptor per one Eu(III) chelate.

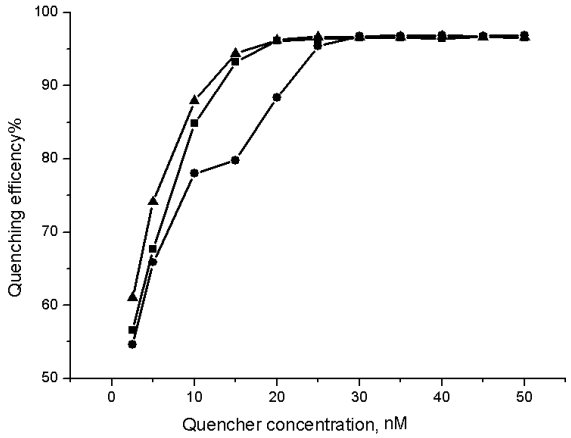


Figure 17 Titration of the quenching efficiencies of the Eu(III) chelate- acceptor pairs: QSY9 (■), Atto612Q (●) and CY7 (▲).

In order to obtain maximum quenching of the Eu(III) chelate emission 1.25-fold excess of Cy7 or QSY9 and 1.6-fold excess of Atto612Q quenchers per binding site of streptavidin was needed. In the case of one quencher being able to completely quench the fluorescence of one Eu(III) chelate, the theoretical quenching efficiency at 5 nM quencher concentration would have been around 30%. However, the observed quenching efficiencies were significantly higher. Since the quenching efficiency is relative to the distance between the D-A pair, the fluorescence lifetime of those Eu(III) chelates that were close to the quencher were diminished completely. The fluorescence lifetime of those chelates that were further from the quencher dye were only somewhat decreased and, thus, some fluorescence could still be measured. In conclusion, according to these results one quencher can affect the fluorescence of more than one Eu(III) chelate. Similar results have also been presented by Karvinen, 2004.

5.3 Cross-talk between acceptors (III)

In order to see whether the terbium(III) chelate could successfully be used as a single donor for multiple acceptors (AF 488, AF 555, AF 594 and AF 680) simultaneously, the cross-talk between different acceptors was tested. This was performed by measuring the sensitized emissions of each acceptor at all the measurement wavelengths (520, 572, 615 and 720 nm). The results were calculated as percentage of the emission of the interfering acceptor from the emission of the right acceptor for the given wavelength. Results of these cross-talk measurements are presented in table 6.

Table 6 Amount of interference compared to the signal of the theoretically suited acceptor.

	Measurement wavelength			
Acceptor	**520 nm**	**572 nm**	**615 nm**	**720 nm**
AF 488	--[a]	10%	n.d.[b]	n.d.
AF 555	n.d.	--	9%	2%
AF 594	n.d.	n.d.	--	19%
AF 680	n.d.	n.d.	n.d.	--

[a] The measurement wavelength of the acceptor
[b] Non-detectable, the percentage was less than 0.1%

At the measurement wavelength of 520 nm, no interference from the other acceptors was detected. In the case of 572 and 615 nm, the acceptors with emission maxima at lower wavelengths caused slight interference (~10%). The highest cross-talk was from AF 594 when measured at 720 nm. In fact, due to the very low background from donor emission at that wavelength, the signal to background ratio of AF 594 was higher at 720 nm (S/B=15) than at 615 nm (S/B=6). This indicates that if AF 594 is to be used as an acceptor for terbium(III) chelate, the longer measurement wavelengths (from 700 nm up) are more appropriate, even though the emission maximum of the acceptor is at ~617 nm. In conclusion, it appears that the cross-talk between different acceptors can be controlled by selecting the acceptors carefully.

The possibility to use AF 488 and AF 680 simultaneously was studied using competitive homogeneous E2 assay. First the assays were performed with E2 calibrators using one of the acceptors. After the incubation and just before the measurement, other solution containing Tb(III)-Fab and AF 680 or 488-E2 was added. (If AF 488 was used in the actual assay then AF 680 was used now and vice versa.) Thus, during the measurement the situation in the wells mimicked a dual-parameter assay, where the concentration of one analyte changes between wells and the other creates maximum interference to the measurement. The obtained standard curves for both acceptors are presented in figure 18. As can be seen, the standard curves for both single and simultaneous measurements are almost identical with both acceptors. Only a slight increase in the signal of the higher calibrators was observed, which slightly inflicted the range of the assays.

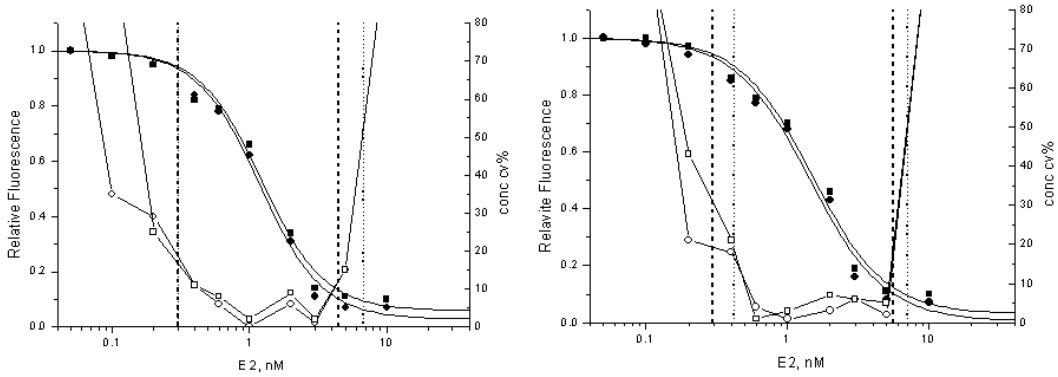


Figure 18 Standard curves from the homogeneous single (filled circles) and simultaneous (filled squares) measurements with cv-profile (hollow symbols) when using **left**: AF 488 and **right**: AF 680 as acceptors. The ranges of the single and simultaneous measurement assays are illustrated with dashed and dotted lines, respectively. (**III**)

Based on these results, it can be concluded that terbium(III) chelate can act as a single donor for multiple acceptors and the acceptors can be measured simultaneously without interfering cross-talk, provided that the acceptors were chosen appropriately. This also means that it could be possible to create a homogeneous multi-parameter FRET assay based on Tb(III) chelate as the donor and AF 488 and AF 680 as acceptors.

5.4 Performance of the developed homogeneous assays (I-IV)

5.4.1 Non-competitive assay for hapten (I, II)

Haptens are low molecular weight antigens, e.g. many drugs, steroids, metabolites and polluctants that are too small to be recognized simultaneously by two binders (for example antibodies). Therefore the developed assays for haptens are typically competitive assays, where the analyte to be measured competes with labelled analyte analogue for the binding sites of a single recognizing agent. However, due to the difference in the principles of the non-competitive and competitive assays (see chapter 2.1), the non-competitive assays are considered to have better sensitivity than competitive assays. (Saviranta, 2000.) Sensitivity is typically defined as the smallest amount of analyte that generates a distinguishable difference from the background signal (Ekins and Edwards, 1997, Ekins and Edwards, 1998). With non-competitive assays the signal level at zero calibrator is low, whereas with competitive assay it is high. It is considered to be more accurate to measure small changes in low signals than in high signals, thus non-competitive assay principles are regarded to be inherently more sensitive than competitive assays.

The developed homogeneous non-competitive assay principle was based on the quenching of europium(III) chelate using FRET and non-fluorescent dyes. First the assay principle was tested using biotin (analyte) – streptavidin (binder) as a model system (**I**). After the pre-testing, an actual homogeneous non-competitive assay was created for E2 using antibody Fab fragment as a binder (**II**). In both of these systems, the interaction between the analyte and the binder was strong and dissociation slow (Wilchek and Bayer, 1990, Lamminmäki et al., 2003). Therefore they were appropriate models for this principle, where one of the key features is the ability of the binder to clench to the analyte. The binder was labelled with europium(III) chelate and the analyte analogue conjugated to the non-fluorescent acceptor. First, the analyte to be measured and the Eu(III)-binder were let to interact with each other. Thereafter, the acceptor-analyte was added, thus it could bind to those binders that were still free from the analyte. During the measurement, FRET quenched the emission of those Eu(III)-binders that were bound to analyte-acceptor conjugates. Thus, the emission was created by those Eu(III)-binders that were bound to the measured analyte. Therefore, the signal increased when the amount of analyte increased. Thus the assay had a non-competitive nature.

For the biotin assay, three different quenchers were used as acceptors (QSY9, Cy7 and Atto612Q). Standard curves and kinetics of the assays are presented in Figure 19.

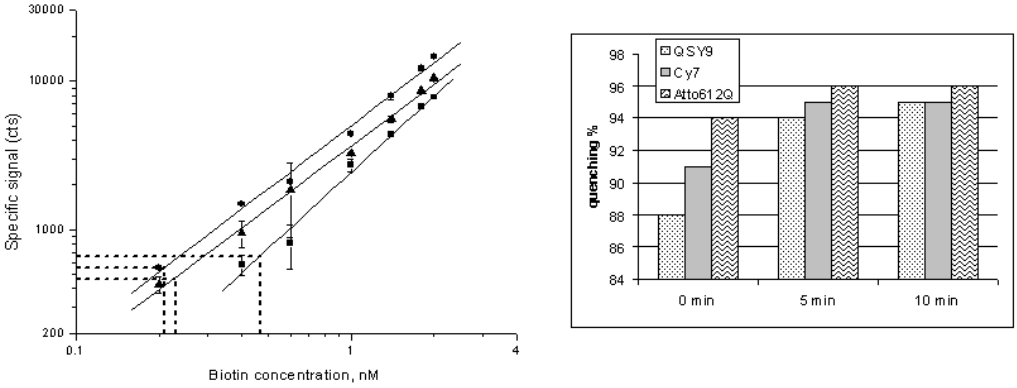


Figure 19 Left: Standard curves with standard error bars of the homogeneous non-competitive assay with different quenchers; QSY9 (■), Cy7 (●) and Atto612Q (▲). The dotted lines indicate the detection limits of the assays: 500, 200 and 250 pM for QSY9, Cy7 and Atto612Q, respectively. (**I**) **Right**: The kinetics of biotin-quencher incubation indicated as the quenching efficiencies at different time points. (Unpublished results)

One streptavidin has four binding sites for biotin. Furthermore, one quencher can quench the fluorescence of more than one chelate. Thus, rather significant increase in biotin concentration was required to alter the signal levels. Also, the proportion of the change in the signal level to the change in the concentration was not linear, and therefore the standard curves were not linear either. (Equations for the assays were for

QSY9: $y=2327x^{1.7}$, $R^2=0.85$; Cy7: $y=5005x^{1.4}$, $R^2=0.99$ and Atto612Q: $y=3372x^{1.5}$, $R^2=0.98$; unpublished data.) However, the kinetics of the assays were very fast. All the assays reached the maximum quenching efficiency within five minutes. Another, somewhat similar, homogeneous assay for biotin has been published. In that assay, the biotin replaces the quencher molecule from labelled avidin, thus the signal level increases with increasing biotin concentration. However, due to the replacement step, the lowest measurable concentration was only about 40 nM. (Batchelor et al., 2007.) In conclusion, it was shown that a homogeneous non-competitive assay based on the quenching of the donor emission could be created for hapten.

The actual hapten assay was created for E2 using europium(III) chelate as a donor and non-fluorescent QSY21 as an acceptor. Also the other, previously presented, acceptors were tested but the QSY21 gave the best results within this assay. The standard curves for the E2 assay using buffer and serum-based calibrators are presented in Figure 20.

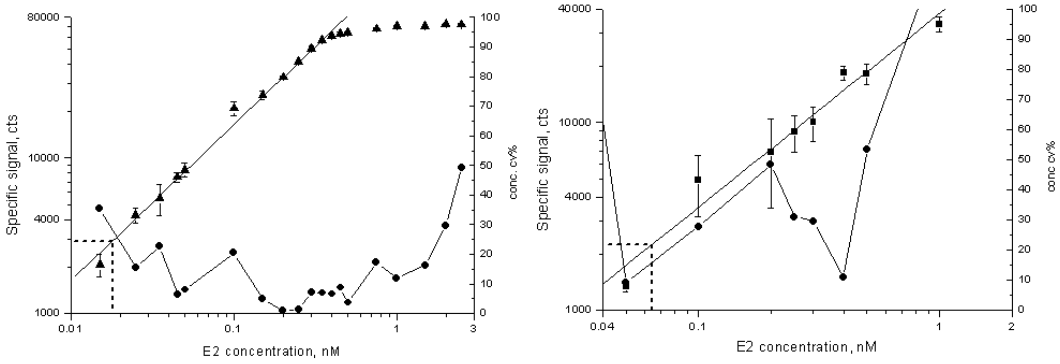


Figure 20 Standard curves for the E2 assays with standard error bars and cv-profile (●). Lowest limits of detection illustrated with dotted lines. **Left:** using buffer based calibrators ($y=172000x^{1.02}$, $R^2=0.994$). **Right:** using human serum-based calibrators ($y=38000x^{1.03}$, $R^2=0.963$). (**II**)

Since Fab fragments are monovalent, both of the assays gave linear response. The lowest limits of detection were 18 pM (4.9 pg/mL) and 64 pM (17 pg/mL) with buffer calibrators and serum calibrators, respectively. The performance of the buffer calibrator assay was tested against a homogeneous competitive assay utilizing the same components with AF680 as an acceptor conjugated to amino-modified E2 (for further information see **II**). The obtained competitive assay had a lowest limit of detection at 1.2 nM, thus the non-competitive assay had 67 times better detection limit than the competitive assay. Since the Förster radii for QSY21 and AF 680 were almost similar (see table 5), the efficiencies of the energy transfer could assumed to be similar in both assay principles. Therefore, the improvement of the detection limit was most likely a consequence of the non-competitive nature of the designed assay. Zhao et al. have developed a heterogeneous competitive assay for E2 using enzymatical signal amplification attaining detection limit of 5.4 pg/mL (Zhao et al., 2006). Thus, the

homogeneous non-competitive assay had equal performance, even though heterogeneous assays are generally regarded as more sensitive than homogeneous assays (Blomberg et al., 1999). However, when the same E2 specific Fab fragment was used to create a competitive heterogeneous assay, detection limit of 8 pM was attained (Lamminmäki et al., 2003). Thus, when the same binder was used, the heterogeneous competitive assay performed better than the homogeneous non-competitive assay. When serum calibrators were used, the detection limit increased 3.5 times from the detection limit of the buffer calibrators. This is most likely due to the endogeneous steroid binding proteins in serum, which impair the usability of E2 assays with serum samples (Brock et al., 1978, Slaats et al., 1987, Masters and Hahnel, 1989). Therefore, Mesterolone was added to the buffer to block these proteins. Mesterolone improved the reproducibility and decreased dispersion but had no effect to the detection limit. For further discussion of the matrix effect to FRET based assays see chapter 5.5.1.

Since the maximum attained quenching efficiency was only 50-60%, the properties of the Fabs16 were studied. The functionality of the Fab was tested using either streptavidin coated wells together with biotinylated estradiol or anti-mouse IgG coated wells. The results showed that only ~50% of the Fab was capable of binding to estradiol compared to the anti-mouse IgG treated wells. Thus, for some, reason a substantial amount of Fab was inactive and only contributing to the background signal.

In conclusion, a homogeneous non-competitive assay with very good detection limit was demonstrated. However, due to the presence of inactive Fab fragment, the acceptor was able to quench only approximately 50% of the donor emission. Thus by improving the quenching efficiency, to be precise by removing the inactive binder, even more sensitive assay could be created.

5.4.2 Homogeneous antibody assay (Unpublished)

The homogeneous hapten assay could be reversed to create a homogeneous antibody assay. In this assay principle, the binder was labelled with non-fluorescent acceptor and analyte analogue was conjugated to europium(III) chelate. E2 specific Fab fragment was used as a model antibody. First, the antibody to be measured was let to bound with E2-Eu(III). Thereafter, the Fab-Atto612Q was added and it was let to interact with those E2-Eu(III) that had not bound to the measured antibody. Thus, during the measurement emission of those E2-Eu(III) that are bound to Fab-Atto612Q is quenched and only the emission from antibody-E2-Eu(III) complexes could be measured. The obtained standard curve is presented in Figure 21.

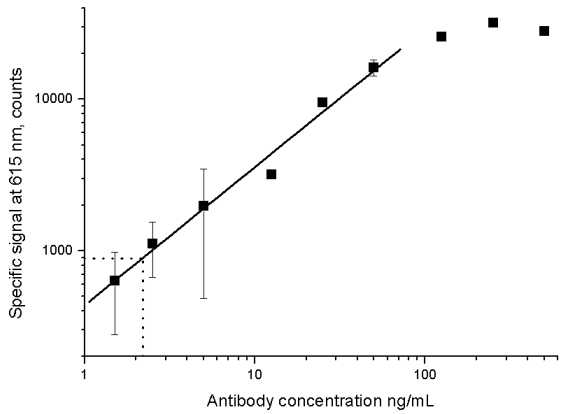


Figure 21 Standard curve of the homogeneous antibody assay with standard error bars. The detection limit is presented with dotted line ($y=440x^{0.91}$, $R^2=0.98$, detection limit 2.2 ng/mL). (Unpublished results)

The slope of the curve was rather small, thus increase of the antibody concentration created only small increase in the signal. This is, however, understandable since each of the antigenes can be conjugated to only one europium(III) chelate. Also the maximum quenching efficiency of the system was surprisingly low at 30%. Thus, the background of the assay was high, which impaired the lowest limit of detection. The low quenching efficiency was most likely due to the poor stability of the Fab fragment. Furthermore, the labelling with the non-fluorescent dyes requires the use of organic solvents, which presumably deteriorated the functionality of the Fab fragment even further. In fact, Atto612Q-Fab was the only quencher complex that could provide some quenching. The other tested quenchers apparently destroyed the activity of the Fab completely and, thus, no quenching was observed. However, the presented assay principle could be used to measure antibody concentration in situations where the sensitivity of the assay is not the key factor, for example during antibody production or purification.

5.4.3 Competitive homogeneous assay for E2 (III)

The homogeneous competitive E2 assay has been published previously by Kokko et al., 2004. In this work, the E2 assay was used to study the donor properties of the terbium(III) chelate; more precisely the possibility of using the localized emission valleys of the terbium(III) chelate to create homogeneous assays. The tested acceptors were AF 488, 555, 594 and 680 (for spectra, see Fig. 16). The obtained standard curves are presented in Figure 22.

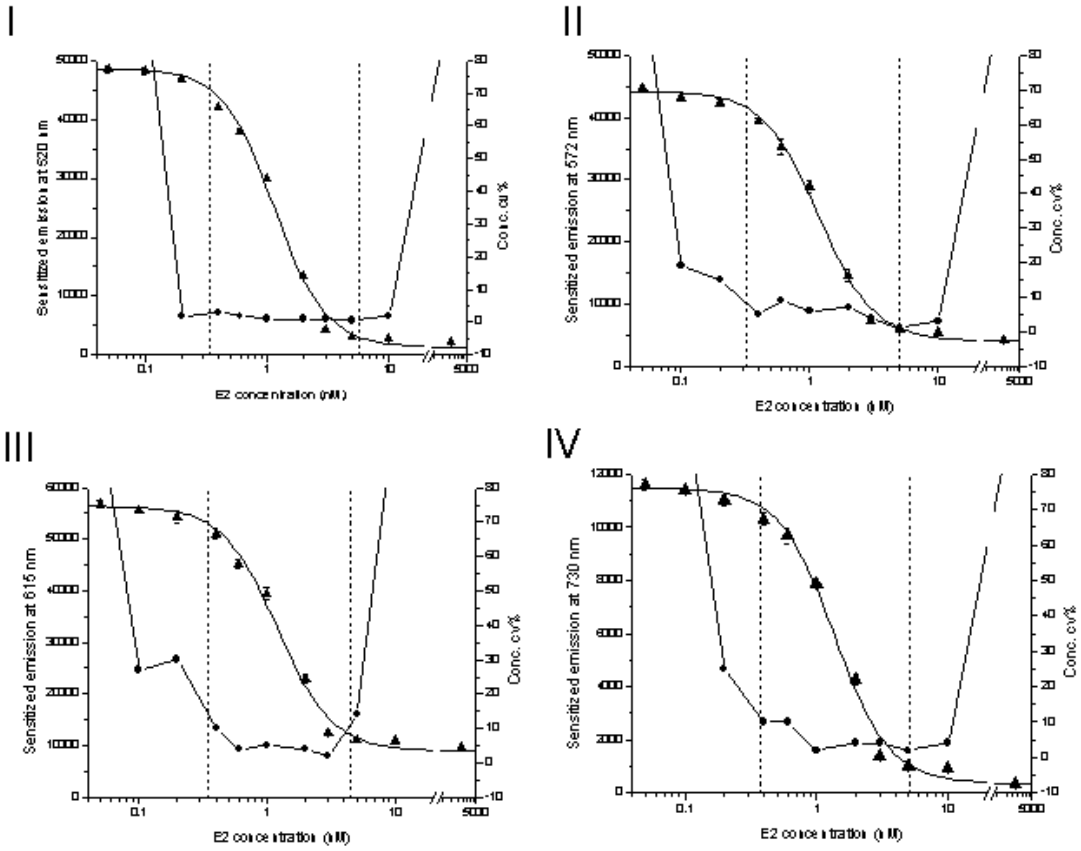


Figure 22 The standard curves with standard error bars for the homogeneous competitive assays using **I**: AF 488; **II**: AF 555; **III**: AF 594 and **IV**: AF 680 as acceptors. The practical ranges of the assays are presented with dashed lines. (Unpublished)

As can be seen, with all of the acceptors a standard curve for E2 was obtained. The lowest measurable concentration was ~300 pM for all of the assays. The highest measurable concentration varied from 3.3 to 5.6 nM. Signal to background ratios were 24, 11, 6 and 45 for AF 488, AF 555, AF 594 and AF 680, respectively. Thus the performances of the assays were rather similar, even though there were differences in the S/B ratios. In conclusion, all of the four emission minima of the terbium(III) chelate could be used to create a homogeneous assay.

*5.4.4 The Homogeneous dual-parameter assay for PSA (**IV**)*

Due to the overlapping of the emission spectra of the different lanthanides (see figure 7), developing a homogeneous multiplexed assay based on the measurement of the sensitized emission of the acceptors has been challenging. Since the emission maxima of the different lanthanides encompass the entire measurement area, selecting the appropriate measurement wavelengths could be difficult. However, previously it has

been shown that terbium(III) chelate can be used as a single donor for multiple acceptors (**III,** (Kupcho et al., 2007)). Total PSA (PSA-T) and free PSA (PSA-F) were used as model analytes to create an actual homogeneous dual-parameter assay where the sensitized emission of the acceptors is measured. PSA is an excellent model analyte for this type of assay principle because PSA has multiple epitopes (Nurmikko et al., 2000) and a large variety of PSA specific antibodies exists. The developed assay was based on FRET between terbium(III) chelate (labelled to PSA-T specific antibody) and two fluorescent acceptor dyes AF 488 and AF 680 (conjugated with PSA-F specific and PSA-T specific antibodies, respectively). Therefore, if PSA-F was present in the sample FRET could happen between terbium(III) chelate and AF 488 and sensitized emission could be measured at 520 nm. The amount of PSA-T could be determined from the sensitized emission of AF 680 at 720 nm. Thus, the assay could simultaneously measure the amount of both PSA-F and PSA-T. To study the possible interactions of the acceptors to each other, the assays were also performed as homogeneous single-parameter assays containing only one of the acceptors at time. The standard curves for the assays were performed using a recombinant PSA, which does not form complexes and, thus, remained in PSA-F form. (Rajakoski et al., 1997)(figure 23).

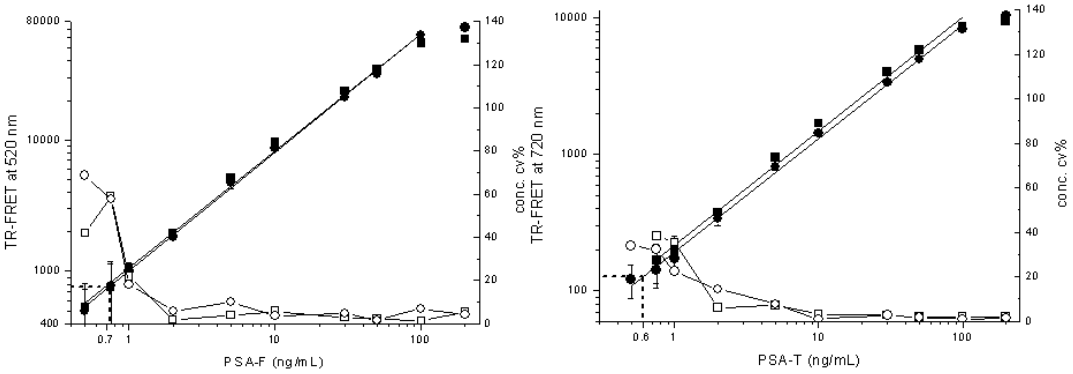


Figure 23 Standard curves for the homogeneous single (■) and dual-parameter (●) assays for **Left**: PSA-F and **Right**: PSA-T. The lowest limit of detection of the dual-parameter assay is illustrated with dotted lines. (**IV**)

As can be seen, the performance of the homogeneous dual-parameter assay was almost identical with the performance of the single-parameter assays. The analytical sensitivities (3 times the standard deviation of the background) for PSA-F and PSA-T were 0.74 ng/mL and 0.60 ng/mL, respectively. The highest calibrator still in the linear range was 100 ng/mL for both assays. The maximum obtained signal to background ratios (using 200 ng/mL calibrator) were 12 and 10 for PSA-F and PSA-T, respectively. The equations of the homogeneous dual-parameter assays were: $y=998x^{0.90}$ ($R^2=0.999$) for PSA-F assay and $y=195x^{0.83}$ ($R^2=0.997$) for PSA-T assay. The kinetics of the dual-parameter assay were relatively fast. When a 50 ng/mL

calibrator was used, the PSA-F assay had reach over 80% of the signal level within 10 min. PSA-T assay was slightly slower and reached the 80% signal level in 30 min. The performance of the homogeneous dual-parameter assay for PSA-F and PSA-T was comparable to the previously published heterogeneous dual-parameter assays (Leinonen et al., 1993, Mitrunen et al., 1995).

5.5 Limiting factors of the developed assays (II, IV)

5.5.1 Matrix effects (II, IV)

Due to the nature of the homogeneous assay principles, the sample is present during the measurement. Therefore, the sample matrix, especially biological matrixes e.g. human plasma and serum, can cause interferences to the assay results by, for example, interacting with the fluorescent label (Mathis, 1993). When lanthanide chelates are used as donors in homogeneous assays, whole blood cannot be used as a sample matrix. This is due to the fact that whole blood has a strong absorption of light at the ultraviolet region where the excitation of the lanthanide chelates also occur. Thus, the matrix prevents the excitation of the donor. (Kuningas et al., 2005.) However, other biological matrixes could be viable. In this research, the homogeneous non-competitive E2 assay principle was tested using spiked male serum and the homogeneous dual-parameter assay for PSA-F and PSA-T was tested using spiked female heparin plasma.

As can be seen in figure 20, the performance of the E2 assay was somewhat poorer when serum based calibrators were used. The lowest limits of detection were 18 pM and 64 pM for buffer and serum calibrators, respectively. The matrix effect to the actual FRET process was studied by adding un-spiked serum to the wells shortly before measurement. Therefore, serum could only affect FRET during the measurement and not interfere with the antigen antibody interactions during the incubation. In that experiment, the obtained signal levels for 5% and 10% serum additions (percentage of the serum in total well volume) were 98% from signal with no serum. Thus, the serum addition did not interfere with FRET measurement. Instead, the deterioration of the performance with serum calibrators was most likely due to the steroid binding proteins, which interfere with the interaction of E2 and antibody (Brock et al., 1978, Slaats et al., 1987, Masters and Hahnel, 1989).

Different situation prevailed when PSA assay was tested with spiked plasma samples. Plasma absorbs within the area where the FRET between terbium(III) chelate and AF 488 or AF 680 occurs. (Figure 24, left) Especially, the strong absorption between 300 nm and 500 nm could mean significant interference for the PSA-F assay (AF 488). Also the absorbance spectrum of the assay buffer was measured but the absorbance values were less than 0.04 between the area of 300 nm to 750 nm.

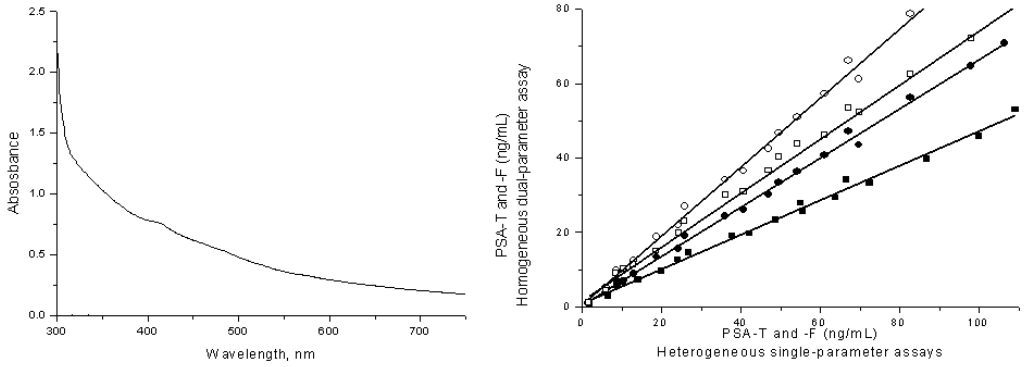


Figure 24 Left: The absorbance spectrum of 10% lithium-heparin plasma in water. (Unpublished) **Right:** The correlation of homogeneous dual-parameter assay with heterogeneous single-parameter assay using spiked plasma samples for PSA-F (square) and PSA-T (circle). Equations for the original results (filled symbols): PSA-F 0.46x+0.91 (R^2=0.994) and PSA-T 0.66x+0.33 (R^2=0.997). Equations for the corrected results (hollow symbols): PSA-F 0.73x+1.43 (R^2=0.994) and PSA-T 0.93x+0.46 (R^2=0.997). (**IV** and unpublished results)

When the performance of the homogeneous dual-parameter assay was tested against heterogeneous single-parameter assays, the effect of the plasma was clearly seen. (Figure 24, right) The results of the homogeneous assay were consistently lower than the results of the heterogeneous assay and as speculated earlier, the PSA-F results were poorer than PSA-T results. The effect of the plasma to FRET was estimated similarly than in the case of E2 by adding 10% plasma in to the wells shortly before measurement. From these results a corrective coefficient was calculated using equation:

$$coefficient = \frac{S(50ng/mL(10\%)) - S(0ng/mL(10\%))}{S(50ng/mL) - S(0ng/mL)}$$

Where the S(X ng/mL(10%)) were sensitized emission signals from the wells that contained 10% plasma and the other values were results from wells that contained only buffer. Using these coefficients (0.6 for PSA-F and 0.7 for PSA-T) the results were corrected (Figure 24, right, hollow symbols). After the correction the slopes of the curves improved, especially with PSA-T. Thus the absorbance of the plasma explained some of the deviations. However, even after the correction, the homogeneous assay still generated somewhat lower results than the heterogeneous assays. Despite these differences, the correlations of the homogeneous dual-parameter assay versus the heterogeneous single-parameter assays were good. In the review of literature, it was

shown that the sample interference can be decreased by using dual wavelength detection where both donor and acceptor emission is measured (Mathis, 1993). However, dual-wavelength concept is not feasible with this assay principle, since either one or two acceptors are gaining energy from the donor. The number of acceptors present during measurement depends on the ratio of PSA-F and PSA-T, thus the influence of the energy transfer to the donor emission varies with the sample composition.

5.5.2 Hook effect (IV)

The Hook effect occurs when a high excess of antigen saturates both capture and tracer antibodies, thus preventing the formation of detectable capture-antigen-tracer complexes (for example Wild, 2005). The Hook effect is a common problem especially within tumour markers since their concentration can vary from low to very high values (Basuyau et al., 2001). In the developed homogeneous dual-parameter PSA assay, all the antibodies are present simultaneously, thus the principle is susceptible to Hook effect. The severeness of the Hook effect was tested using high PSA concentrations. The obtained sensitized emissions and the calculated concentrations are presented in figure 25.

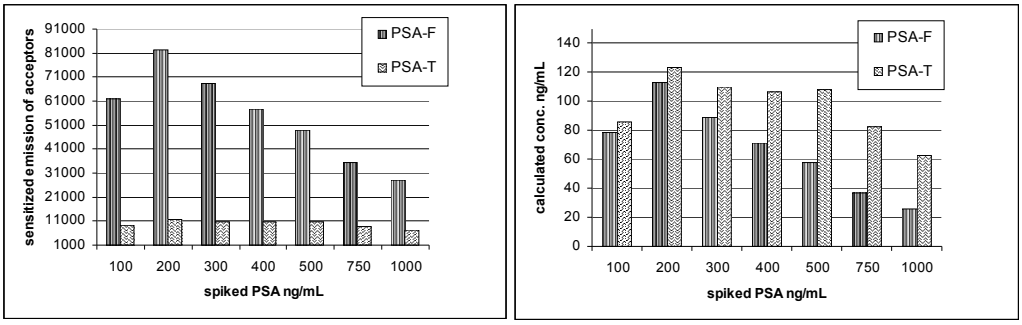


Figure 25 Hook effect studies using high PSA concentrations. **Left:** The measured sensitized emissions of the acceptors for different spiked PSA concentrations. **Right:** The calculated PSA concentrations from the sensitized emissions. (Unpublished)

The signal levels of both assays started to decrease after 200 ng/mL. However, when a very high PSA concentration (1000 ng/mL) was used, the calculated concentrations were ~60 ng/mL and ~25 ng/mL for PSA-T and PSA-F, respectively. Thus, the signal levels were still clearly elevated from the background level and above the clinically important cut off levels. It also appears that the Hook effect decreased the results of PSA-F more than the results of PSA-T. On the other hand, the amount of PSA-F in blood is small compared to PSA-T, thus the Hook effect might eventually be more severe in the case of PSA-T measurement.

6 CONCLUSIONS

During the past view decades FRET has gained popularity as the starting point for designing various homogeneous assays. Even though these techniques are predominantly based on energy transfer between two organic dyes, also lanthanide chelates have previously been used as donors due to their many beneficial characteristics. In this study, the objective was to design novel immunoassays that utilize those features.

The conclusions based on the original publications are:

I: When europium(III) chelate labelled streptavidin was used as a donor complex and biotin-organic dye as an acceptor to quench the fluorescence, quenching efficiencies up to 98% were achievable. Furthermore, one quencher dye molecule was able to quench the fluorescence of more than one europium(III) chelate. For maximal quenching 1.25 to 1.6–fold excess of the dye per streptavidin binding site was needed. It was also shown that a non-competitive assay can be designed using the quenchers and lanthanide chelates, even though the performance of the biotin assay was somewhat compromised by the quadrovalent nature of the streptavidin as a binder. This, together with the high quenching efficiency, deteriorated the linearity of the assay since the proportion of the response to the changing biotin concentration was not straightforward. However, this research provided a good foundation for the development of an actual homogeneous non-competitive assay for hapten.

II: The previously studied donor/quencher characteristics were utilized to develop the homogeneous non-competitive assay for 17β-estradiol. The assay was based on europium(III) chelate labelled antibody Fab fragment (the donor complex) and QSY21 conjugated to amino-modified estradiol (the acceptor/quencher complex). With this system, the maximum obtained quenching was unexpectedly only ~55%. This was found to be due to the presence of inactive Fab fragment, which was not capable of binding estradiol and, thus, only contributed to the background of the assay. However, even with this defect the assay principle was able to detect 18 pM concentrations of estradiol, which was 67 times better detection limit than with corresponding conventional competitive homogeneous assay. Furthermore, due to the monovalent binder, the response was impeccably linear with exponent of the equation of the standard curve at 1.02. However, it can be speculated that if the quenching efficiency could be improved further, the sensitivity of the assay might be even better. The developed assay principle can be applied to other haptens as well, provided that a high quality antibody (with low dissociation rate and good affinity) is available.

III: Terbium(III) chelate has several sharp emission peaks with clear emission minima between them. The possibility to use terbium(III) chelate as simultaneous, single donor for different acceptors emitting at the emission valleys was studied using conventional homogenous competitive assay for estradiol. Signal to background ratios from 6 to 45

were obtained; the major cause for the variation being the background from the terbium chelate emission. Furthermore, the obtained quenching efficiencies followed the theoretically calculated Förster radii perfectly. All of the tested acceptors could be used to create a functional estradiol assay. The leakage of the acceptor emission to the other measurement wavelengths was observed to be small; and, mainly, from the acceptor emitting closest at the smaller wavelengths. Thus, based on these results, the terbium chelate could potentially act as a single donor for multiple acceptors emitting at different wavelengths, provided that the acceptors are selected accordingly.

IV: The possibility to use terbium(III) chelate as a single donor for two acceptors simultaneously was studied by designing a homogeneous dual-parameter immunoassay for free and total PSA. The two acceptors did not interfere with each other and the performance of the dual-parameter assay was identical with the performance of corresponding homogeneous single parameter assays. Both free and total PSA was measurable using clinically important concentrations. The typical disadvantage of the homogeneous assays was observed when the performance of the assay was tested against heterogeneous single parameter assay; the sample material (plasma) interfered with the energy transfer and, thus, impaired the functionality compared to the heterogeneous assays. However, the correlation of both free and total PSA was good, even though the obtained concentrations were slightly less than with the heterogeneous assays. Furthermore, the developed assay had very simple protocol (mix and measure) and it is adaptable to other analytes that should preferably be measured simultaneously from one sample.

To conclude, it is possible to stretch the current applications of lanthanide chelates in FRET further and find new ways to design assay principles. The superior fluorescence characteristics of lanthanides (e.g. long lifetime, large Stokes' shift and narrow emission peaks) enable them to overcome many of the problems encountered by other donors (such as autofluorescence of the background or direct excitation of the acceptor) and contribute to the development of ever better assays. In this research, the sensitivity of hapten immunoassay was improved by designing a non-competitive assay format. In addition, the simultaneous measurement of total and free PSA was eased with a homogeneous, dual-parameter assay format.

7 ACKNOWLEDGEMENTS

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Finis coronat opus.

Turku, May 2009 Tiina Kobbo

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