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Quantum Dots as Acceptors in FRET-Assays Containing Serum*

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ABSTRACT

Quantum dots (QDs) are common as luminescing markers for imaging in biological applications because their optical properties seem to be inert against their surrounding solvent. This, together with broad and strong absorption bands and intense, sharp tuneable luminescence bands, makes them interesting candidates for methods utilizing Förster Resonance Energy Transfer (FRET), e. g. for sensitive homogeneous fluoroimmunoassays (FIA). In this work we demonstrate energy transfer from Eu^{3+}-trisbipyridin (Eu-TBP) donors to CdSe-ZnS-QD acceptors in solutions with and without serum. The QDs are commercially available CdSe-ZnS core-shell particles emitting at 655 nm (QD655). The FRET system was achieved by the binding of the streptavidin conjugated donors with the biotin conjugated acceptors. After excitation of Eu-TBP and as result of the energy transfer, the luminescence of the QD655 acceptors also showed lengthened decay times like the donors. The energy transfer efficiency, as calculated from the decay times of the bound and the unbound components, amounted to 37%. The Förster-radius, estimated from the absorption and emission bands, was ca. 77 Å. The effective binding ratio, which not only depends on the ratio of binding pairs but also on unspecific binding, was obtained from the donor emission dependent on the concentration. As serum promotes unspecific binding, the overall FRET efficiency of the assay was reduced. We conclude that QDs are good substitutes for acceptors in FRET if combined with slow decay donors like Europium. The investigation of the influence of the serum provides guidance towards improving binding properties of QD assays.

Keywords: Quantum Dot, Luminescence, Serum, Europium, Immunoassay, Energy Transfer, FRET

1. INTRODUCTION

Quantum dots (QDs) are gaining more and more interest for biological applications. In that context they are chiefly used as luminescing markers since their optical properties seem to be inert against their surrounding solvent. This, together with broad and strong absorption bands and intense, sharp tuneable and luminescence bands, makes them interesting candidates for Förster Resonance Energy Transfer (FRET). The use of QDs as donors in FRET has already been reported,[1a-d] whereas their employment as acceptors has proven to be difficult,[2] and was only recently unequivocally demonstrated.[3] The FRET technique is useful for diagnostic methods like sensitive homogeneous fluoroimmunoanalysis (FIA)†. With their unique and robust photophysical properties, QDs are suitable for FIA optimization and offer the potential for multiplexing. (For further description of immunoanalysis techniques see Ref.4.)

FIA is a diagnostic tool for detecting small amounts of specific molecules in a 'heterogeneous sea' of proteins, fibres, etc. The non-ambiguous answer of the assay is ensured by the specific reaction between antibody and antigen. With homogeneous assays, it is possible to detect proteins in the picomolar range in blood serum without washing and

† FIA, referring to *fluorescence* in immunoassays, is commonly used throughout the literature. We prefer the term *luminescence* as it is a more general notion for radiative transitions.

separating steps. This is achieved by the 'sandwich' architecture, with two antibodies bound to the same target antigen (see Figure 1A). The donor luminophore of one antibody is excited by laser light. FRET towards the acceptor luminophore of the second antibody only occurs when both antibodies are bound together via the antigen. Thus, the emission of the first luminophore is quenched while the latter is then able to luminesce, working as a measure for the antigen concentration.

The donor comprises an Eu^{3+} ion surrounded by the so called cryptand trisbipyridin (TBP) resulting in the cryptate Eu-TBP.[5,6] Due to an internal energy transfer from the cryptand to the lanthanide, a spectral shift of about 300 nm is achieved between absorption and luminescence. The Eu^{3+} luminesces with a long decay time (hundreds of microseconds). This allows delay of the detection until the short lived autofluorescence from the surrounding matrix has faded away. Then only the photons from the donors and the bound acceptors are seen. Calculating the ratio of bound and non-bound antibodies provides the concentration of antigen. This technique is already applied in commercially available systems (such as Kryptor™, Cezanne S.A., Nîmes, France).

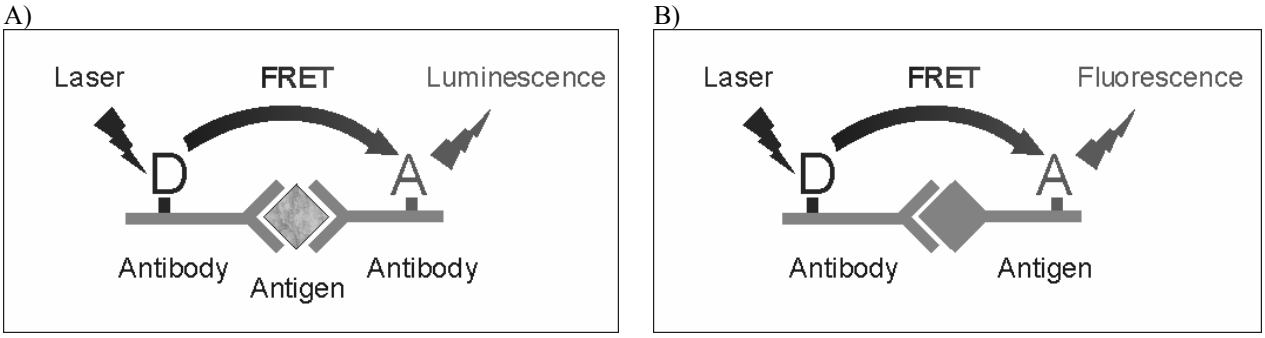

Figure 1 A) Scheme of Förster Resonance Energy Transfer (FRET) in an immunoassay. The antibody forms a 'sandwich' complex with the searched for antigen. The donor D absorbs the laser light and transfers it to the acceptor A nearby, which luminesces instead of the donor. B) Scheme of FRET in the test-assay. The donor attached to the antibody and the acceptor attached to the antigen are directly coupled.

In this work we demonstrate energy transfer from Eu-TBP donors to QD655 acceptors. The experiments were performed in solutions with and without serum to evaluate the influence on the FRET.[7] After the excitation of Eu-TBP with a N_2-laser and as result of the energy transfer, the luminescence of the QD655 acceptors also showed increased decay times. Instead of the whole immunoassay (Figure 1A), a simpler test scheme (Figure 1B) was used: the Eu-TBP donors were conjugated with streptavidin (Eu-TBP-Strep) and could directly couple to the QD655 acceptors conjugated with biotin (QD655-Biot) (see Figure 2). The intensities and the decay times were measured. The FRET efficiency, η_{FRET}, is the efficiency of the energy transfer in the coupled FRET pair and was calculated by:[8]

$$\eta_{FRET} = 1 - \tau_{DA} / \tau_{D} \quad (1)$$

Here, τ_D and τ_{DA} are the decay times for the uncoupled donor and for the donor coupled to the acceptor.

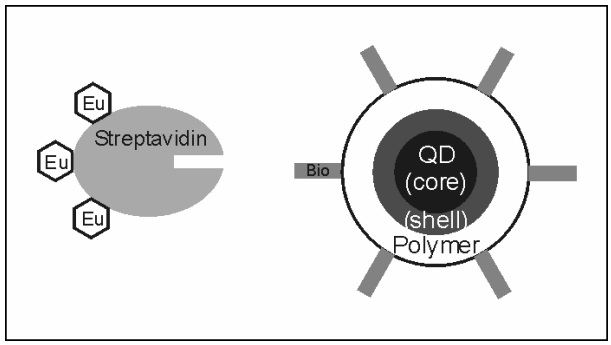

Figure 2 Eu-TBP coupled to streptavidin (Eu-TBP-Strep) is able to form FRET pairs with polymer-coated core-shell QDs which are marked with biotin (QD655-Biot). Streptavidin forms a very tight binding with biotin. In the experiments, ca. 3.1 Eu-TBPs are conjugated with one streptavidin and ca. 6 biotins with one QD655.

2. MATERIALS AND METHODS

The FRET donor molecule is the cryptate Eu^{3+}-trisbipyridin (Eu-TBP). For the binding assays, it is coupled to streptavidin (Eu-TBP-Strep). For the non-binding assays, the cryptate was coupled to tPSA (total prostate specific antigen). To improve the luminescence of Eu-TBP-Strep, KF was added resulting in a final concentration of 0.4 M in the assays. The serum assays contained 33% bovine serum. All cryptates, KF and serum were provided by Cezanne SA (Nîmes, France). The FRET acceptors were CdSe-ZnS core-shell quantum dots emitting at 655 nm (QD655), coupled to biotin (QD655-Biot) with a conjugating ratio biotin/QD = 6. They were supplied by Quantum Dot Corp. (Hayward, CA, USA). The concentration series was measured with varying concentrations of Eu-TBP-Strep between 0 M and $3.0 \cdot 10^{-8}$ M which results in concentrations of streptavidin up to $9.6 \cdot 10^{-9}$ M (i.e. a conjugating ratio Eu-TBP/Strep = 3.1) and constant QD655-Biot concentrations of $4.0 \cdot 10^{-10}$ M (biotin concentration $2.4 \cdot 10^{-9}$ M). This yields concentration ratios c(Strep)/c(Biot) between 0 and 4.
The FRET measurements were done with a commercial FIA reader system (KRYPTOR™, Cezanne SA, Nîmes, France) utilizing two single photon counting channels at 655 nm (channel A, acceptor signal, QD channel) and 620 nm (channel B, donor signal, Eu^{3+} channel) with 2 µs integration steps over 8 ms (see Figure 3). For the spectrally resolved measurements, a spectrometer was coupled with an ICCD camera (iStar DH720, Andor Technology, Belfast, Northern Ireland). The spectra were taken with a 200 µs delay from the laser pulse and with a measurement window of 1000 µs. The signal was integrated over 40 pulses. The light source in both cases was a N_2-laser emitting at 337 nm with a 20 Hz repetition rate.

The data were fitted by a two-step global fitting procedure using a set of monoexponential decay functions: first, the decay time of Eu-TBP-Strep without acceptors was determined, then, the signals of all concentration measurements at both channels were fitted with this decay time and a global second decay time for the FRET-signal. This avoids falsely enlarged FRET decay times for the weak signals when a strong signal of uncoupled Eu-TBP-Strep is present. Thus for each concentration one gets a fourfold set of amplitudes A_i and decay times τ_i: two pairs for the luminescence contribution of uncoupled Eu-TBP-Strep in both channels and two pairs for the FRET signal in both channels as the donor (at 620 nm) and the acceptor (at 655 nm) both take part in the FRET decay (see Figure 4). From these decay times and amplitudes the intensity of the contributions were calculated by:

$$I_i = \int_0^\infty A_i \cdot \exp(-t_i / \tau_i) dt_i = A_i \cdot t_i \tag{2}$$

As Eu-TBP emits with 1000 µs decay times and the FRET signal with decay times over 600 µs, the total intensity can be calculated from the delayed signal, omitting the intense short-time autofluorescence by neglecting the first 200 µs.

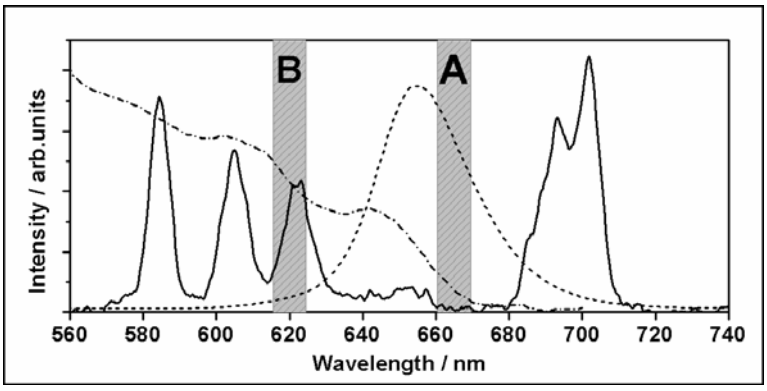

Figure 3 Emission spectra of donor Eu-TBP-Strep (solid line) and acceptor QD655-Biot (dotted line). The excitation spectrum of QD655-Biot is also included (dash-dotted line). The grey bars indicate the spectral detection regimes of the acceptor channel A (665 nm) and the donor channel B (620 nm).

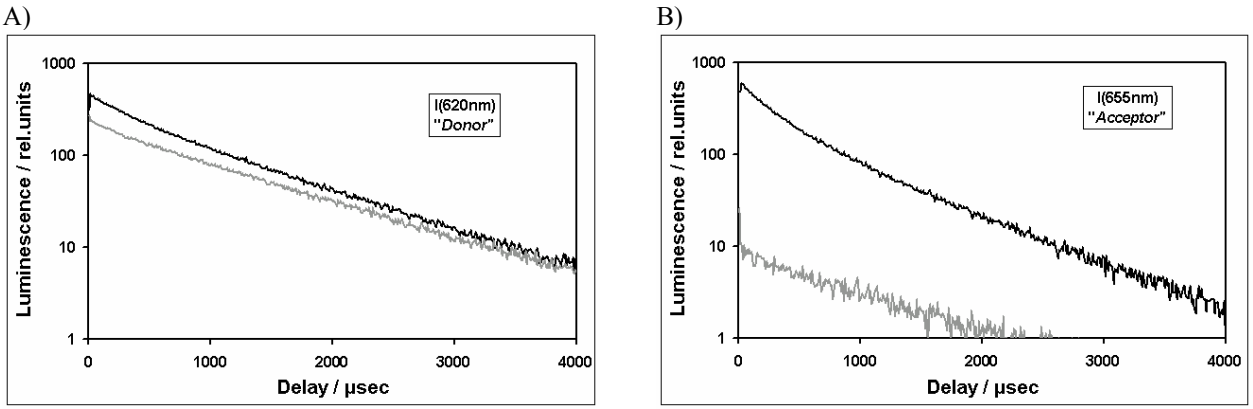

Figure 4 Decay measurements of pure donor (grey) and donor-acceptor mixtures (black) A) in the 620 nm channel and B) in the 665 nm channel (c(QD655-Biot) = $4.0 \cdot 10^{-10}$ M; c(Eu-TBP-Strep) = $3.0 \cdot 10^{-8}$ M; no serum). The intense FRET signal can be detected over the whole long-time range. The curves can be analyzed as biexponential decays ($\tau_{Eu} \approx 1000$ µs and 600 µs < τ_{FRET} < 800 µs).

3. RESULTS AND DISCUSSION

The spectra of the assays containing Eu-TBP-Strep and that with the Eu-TBP-Strep/QD655-Biot mixture of show significant differences in the spectral region around 665 nm (see Figure 5). This means that the QD655-Biots, which usually have a short-time emission, show now long-time luminescence, which is clearly detectable after 200 µs. This is due to an energy transfer from the excited Eu-TBP to the QD655 if they are coupled via streptavidin and biotin. No difference can be seen between the emission spectra of the uncoupling Eu-tPSA/QD655-Biot mixture and the solutions without acceptors in the long-time range as the luminescence decay of QD655-Biot is too fast for this detection window. Therefore, a diffusion enhanced energy transfer can be excluded for the used concentrations. An energy transfer is only detectable between coupled FRET partners. No spectral displacement of bands were detected between assays with and without serum.

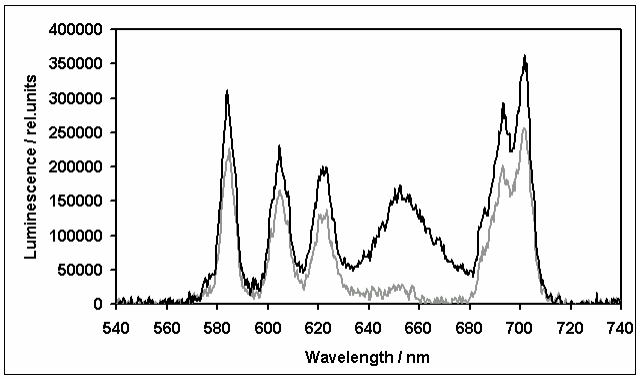

Figure 5 Emission spectra of Eu-TBP-Strep ($c = 1.6 \cdot 10^{-9}$ M; grey line) and of the Eu-TBP-Strep/QD655-Biot mixture (black line) c(Eu-TBP-Strep) = $1.6 \cdot 10^{-9}$ M; c(QD655-Biot) = $2.3 \cdot 10^{-9}$ M). Both assays contained serum. The detection window had a delay of 200 µs and a duration of 1000 µs.

The FRET luminescence intensity (channel A) of the serum-free FRET assay rises with increasing concentration of Eu-TBP-Strep until it gets saturated at c(Strep)/c(Biot) ≈ 3 (see Figure 6). The signal of the serum containing assay shows the same tendency with lower intensities ending at 60% of the saturation intensity of the serum-free assay. The onset of

saturation at higher concentration in the serum solutions indicates the unspecific binding concurring with the specific binding of streptavidin with biotin has previously been described.[7]

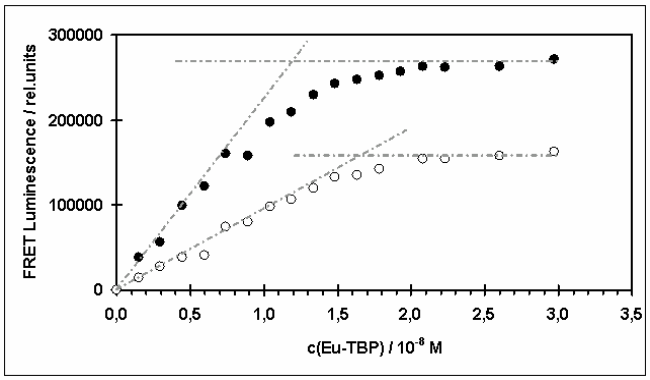

Figure 6 FRET luminescence (channel A; 665 nm) at different Eu-TBP-Strep concentrations (c(QD655-Biot) = $4.0 \cdot 10^{-10}$ M). Full circles indicate signals in assay without serum, open circles in serum assays. The dark dash-dot lines are just to guide the eye.

The Eu-TBP-Strep luminescence intensity (channel B) shows a similar behaviour: it rises with increasing Eu-TBP-Strep concentration (see Figure 7). Until c(Strep)/c(Biot) ≈ 1,2, the slope is steeper than at higher ratios when the intensity grows with the same slope as that from acceptor-free Eu-TBP-Strep. The difference between the Eu-TBP-Strep luminescence in the FRET assay and that of the pure Eu-TBP-Strep solution shows that the additional increase behaves like the FRET signal (channel A). The gain in luminescence is saturated at about the same Eu-TBP-Strep concentrations as in channel A. This means that there is not only an increase due to growing Eu-TBP-Strep concentration but also an increase in intensity due to the presence of the QD655-Biots. 'Crosstalk' luminescence can be excluded from the spectra as the luminescence of Eu^{3+} is equally enhanced in all bands (see Figure 5). This means that the gain is not due to luminescence from the FRET induced QD band centred at 655 nm 'shining' into channel B (Eu^{3+} channel; 620 nm).

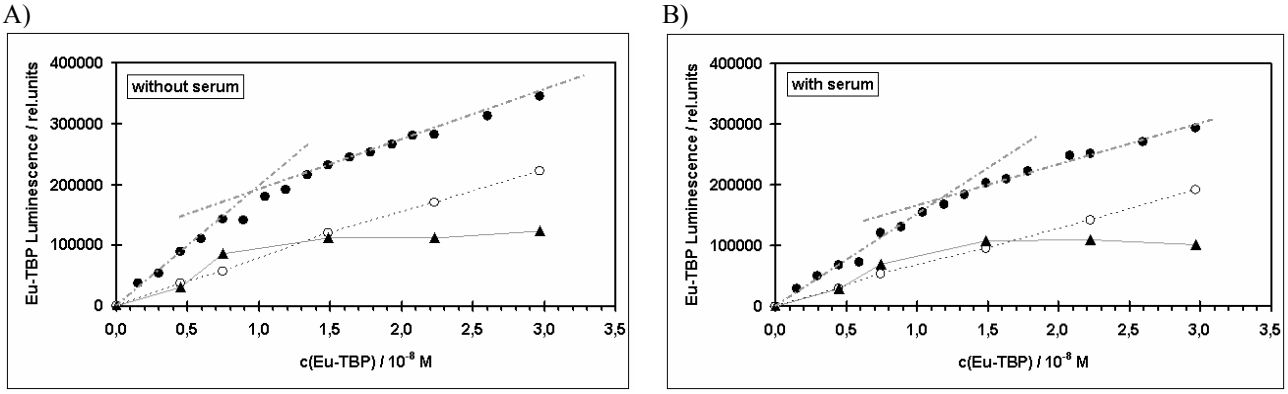

Figure 7 Eu-TBP-Strep luminescence (channel B) measured at different Eu-TBP-Strep concentrations. Full circles indicate signals in FRET assay (c(QD655-Biot) = $4.0 \cdot 10^{-10}$ M), open circles in acceptor-free assays and triangles the difference between both. The grey dash-dot lines are to guide the eye. A) Assay without serum. B) Assay with serum.

A look at the decay times evaluated from the measurements with concentration dependence gives a more detailed picture (see Table 1): the FRET decay times in solutions with and without serum differ significantly by 13%. This results in a maximum FRET efficiency of 37% from Eu-TBP-Strep to QD655-Biot (serum free assays) whereas the FRET efficiency in the serum solution is 27%. Thus, the serum reduces the FRET efficiency by ca. one third. This means that the serum does not only influence the availability of free binding places allowing FRET pairs, it also influences the properties of the energy transfer.

Assays ...	τ_{Eu}	τ_{FRET}	η_{FRET}	R
without serum	1080 µs ± 40 µs	690 µs ± 50 µs	0.36 ± 0.02	85 Å ± 1 Å
with serum	1060 µs ± 30 µs	790 µs ± 40 µs	0.25 ± 0.04	91 Å ± 3 Å

Table 1 Luminescence decay times of Eu^{3+} (τ_{Eu}) and FRET (τ_{FRET}) and the resulting FRET-efficiencies (η_{FRET}) with their maximum errors. The distances R between donor and acceptor were calculated with a Förster-radius of 77 Å (see text).

The FRET efficiency is connected with the distance R between donor and acceptor by the Förster distance R_0:[8]

$$\eta_{FRET} = \frac{R_0^6}{R_0^6 + R^6} \tag{3}$$

The Förster distance R_0 of the Eu-TBP-Strep and QD655-Biot pair was calculated from the overlap integral J of the donor emission band and acceptor absorption band (see Figure 3):

$$R_0 = (8.79 \cdot 10^{-5} \cdot Q_D \kappa^2 n^{-4})^{1/6} J^{1/6} \text{ in Å} \tag{4}$$

with the donor luminescence efficiency Q_D, the orientation factor κ^2 and the refraction index n. J was calculated to $6.2 \cdot 10^{16}$ $M^{-1}cm^{-1}nm^4$.[8] The donor luminescence efficiency is that of the Eu^{3+} in the cryptate $Q_D = Q_{Eu} = 0.2$, not of the whole cryptate Eu-TBP-Strep ($Q_D = 0.02$).[9,10] With $\kappa^2 = 2/3$ (isotropic orientation) and $n = 1.4$ (biomolecules in aqueous solutions)[11] the Förster distance amounts to 77Å. According Equations (3) and (4), one reason for a change of η_{FRET} is a different distance R. Due to the same donor decay times τ_D, no change in the quantum efficiency of the donor Q_D can be expected. The orientation of the molecules are possibly influenced by the additional molecules. The refractive index does not change for the biomolecule concentrations considered.[11]

The binding efficiency η_{Bind} can be calculated from the different decay contributions and is defined here as the numbers of bound donors n_b divided by the sum of bound and unbound donors $n_b + n_{nb}$. If FRET is the only distinguishing property between n_b and n_{nb} the assay efficiency can be calculated according to:

$$\eta_{Bind} = \frac{n_b}{n_b + n_{nb}} = \frac{I_{DA}/[Q_{Eu}(1-\eta_{FRET})]}{I_D/Q_{Eu} + I_{DA}/[Q_{Eu}(1-\eta_{FRET})]} = \frac{I_{DA}}{I_D(1-\eta_{FRET}) + I_{DA}} \tag{5}$$

The intensities I_D and I_{DA} are the intensities of the uncoupled donor and of the donor coupled to an acceptor molecule respectively. This can be understood as the absorbed energy in the bound molecules divided by sum of absorbed energy in the bound and the unbound molecules. The term $I_{DA}/(1-\eta_{FRET})$ is the non-transferred emitted light of the coupled donors divided by the 'non-transfer efficiency'. Thus, the binding efficiency can be calculated using only the luminescence contributions of Eu^{3+} (channel B).

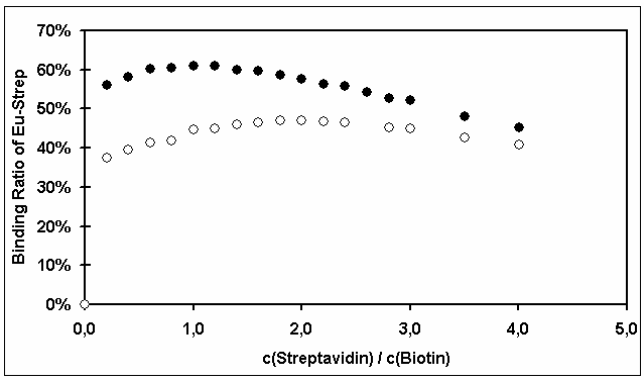

Figure 8 Binding ratio of Eu-TBP-Strep over the concentration ratio. Full circles indicate binding ratios in serum free samples, open circles in serum samples.

The maximum binding ratio is 60% at c(Strep)/c(Biot) ≈ 1 for the serum free assay and 47% at c(Strep)/c(Biot) ≈ 2 for the serum containing assay (see Figure 8). With growing excess of Eu-TBP-Strep the binding efficiency decreases. Serum promotes unspecific binding, such reducing the overall FRET efficiency of the assay.

The origin of the gain in luminescence in channel B can be found looking at the intensities of the uncoupled Eu-TBP-Strep. The intensity of the unbound Eu-TBP-Strep in acceptor free assays and in QD655-Biot containing assays is shown in Figure 9. One can see that the luminescence of the free Eu-TBP-Strep is not reduced in FRET assays as one would expect but has the same intensity. In serum assays, the Eu-TBP-Strep luminescence is even higher in the presence of QD655-Biot (not shown). This shows that in the presence of the QD655-Biot acceptors, the Eu-TBP-Strep luminescence is enhanced. The reason for this enhancement is subject of ongoing research.

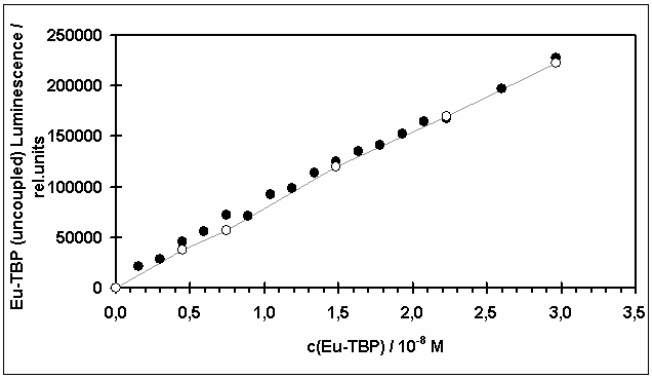

Figure 9 Luminescence of the uncoupled Eu-TBP-Strep (these are the components at 620nm with decay times at about 1000 µs) at different Eu-TBP-Strep concentrations. Full circles indicate signals in FRET assays (c(QD655-Biot) = $4.0 \cdot 10^{-10}$ M), open circles in acceptor free assays. (All assays were serum-free.)

4. CONCLUSION

We have shown FRET between Eu-TBP and QD655, if they are coupled by use of streptavidin and biotin. The FRET signal is intense in the measurement window thus enabling good signal-to-noise ratios not only for the Eu-TBP-Strep donor signal bat also for the FRET signal. Using serum assays, the FRET signal is reduced in comparison to serum free assays. The reason for this decrease is twofold: the unspecific binding of serum molecules occupies coupling sites of the FRET partners, and the serum reduces the FRET efficiency probably due to steric hindering effects which separates the FRET partners further than without serum. The conditions for this effect should be further investigated in order to get reliable assays with QDs as acceptors. Nevertheless, QDs are very promising substitutes for organic acceptors in FRET as their stability and their narrow emission bands provide the chance for new immunoassays, e. g. enabling multiplexing.

ACKNOWLEDGEMENT

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