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Terbium(III) Chelate as an Efficient Donor for Multiple-Wavelength Fluorescent Acceptors

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Abstract The emission spectra of the lanthanide chelates enable them to act as a donor for several acceptors emitting at different wavelengths. Fluorescence resonance energy transfer between terbium(III) chelate labeled antibody Fab fragment (donor) and a 17β-estradiol conjugated to Alexa Fluor 488, 555, 594 or 680 (acceptor) was employed to study the functionality of the terbium(III) chelate as an efficient donor for several acceptors emitting from green to far-red. During measurement, the sensitized emission of the acceptor was measured at acceptor specific wavelength. All the tested dyes proved to be efficient acceptors, and they were successfully used in the competitive homogeneous E2 assay. The highest signal to background ratio and the best assay performance was obtained with Alexa Fluor 680, due to the very low donor emission background at the far-red area. In addition, the sensitized emission of both Alexa Fluor 488 and 680 could be measured simultaneously without significant cross talk.

Keywords Lanthanide chelate · Homogeneous · Immunoassay · FRET (fluorescence resonance energy transfer)

Introduction

Fluorescence resonance energy transfer (FRET) is defined as a non-radiative energy transfer from an excited donor molecule to an acceptor molecule. [1] For FRET to occur, short distance between donor and acceptor as well as spectral overlapping between the emission of the donor and the excitation of the acceptor are required (Förster theory). [2] Lanthanide chelates are interesting donors in FRET due to their many beneficial characters. [3] They have an unusually large Stokes' shift and the peaks of the emission spectra are narrow and well separated from each other. Moreover, the slow decay of the lanthanide luminescence extends the observed lifetime of the fluorescent acceptors. Thus, the sensitized emission of the acceptor can be measured with a temporal delay in order to reduce the interference of the background fluorescence. [4, 5]

Organic fluorophores are the most often used group of donors. They have typically only one emission peak and can act as a donor for only one wavelength acceptors. [6] Lanthanide chelates, however, have several emission maxima and minima (for terbium(III) chelate, see Fig. 1) and they can theoretically act as donors for multiple-wavelength acceptors. For example in the case of terbium (III) chelate, fluorescein, green fluorescent protein and rhodamine have been used. The sensitized emission of fluorescein and green fluorescent protein has been measured at 520 nm [7, 8] utilising the first emission peak of terbium(III) chelate. The second emission peak has been utilised with rhodamine in a homogeneous βhCG assay (measured at 570 nm). [9] To our knowledge, the other emission peaks of terbium(III) chelate have not been used in FRET-based assays.

The purpose of our research was to study whether terbium(III) chelate could be used as an efficient donor for multiple-wavelength acceptors in homogeneous assay. The performance of four different fluorescent acceptor dyes (Alexa Fluor (AF) 488, 555, 594 and 680) in a homogeneous E2 assay was tested. In addition, the cross talk from other acceptors during measurement was examined to test the ability

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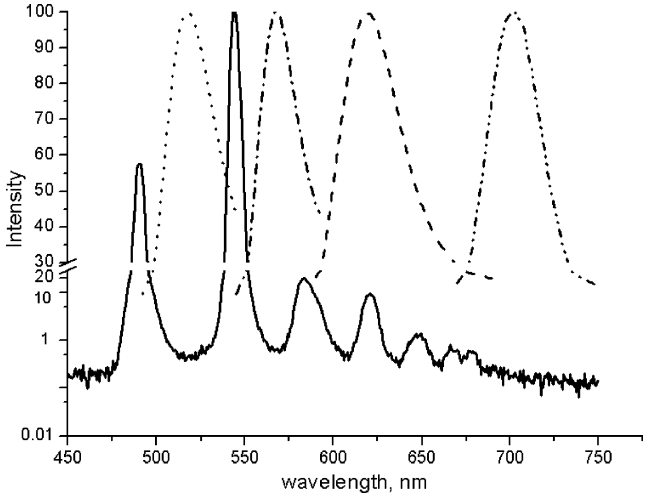

Fig. 1 The emission spectra of terbium(III) chelate (*solid line*), Alexa Fluor 488 (*dotted line*), Alexa Fluor 555 (*dot dashed line*), Alexa Fluor 495 (*dashed line*) and Alexa Fluor 680 (*dot dot dashed line*)

of terbium(III) chelate to work as a single donor for multiple-wavelength acceptors in a simultaneous measurement.

Materials and methods

Assay buffer and instruments

Buffer for all the assays and dilutions contained 0.05 M Tris-HCl, pH 7.75, 0.9% (*w*/*v*) NaCl, 0.05% (*w*/*v*) NaN_3, 0.01% (*v*/*v*) Tween 40, 0.05% (*w*/*v*) bovine-γ-globulin, 20 μM diethylenetriaminepentaacetate and 0.5% (*w*/*v*) bovine serum albumin (BSA) and was purchased from Innotrac Diagnostics (Turku, Finland). The fluorescence of the terbium(III) chelate was measured using Victor 1420 Multilabel counter (Wallac, Perkin-Elmer Life and Analytical Sciences, Wellesley, MA, USA) and the terbium measurement protocol. In all the E2-assays, excitation wavelength was 340 nm, and measurement wavelength varied depending on the emission maximum of the acceptor. Delay time and measuring time were 75 and 400 μs, respectively. Sensitized emission of AF 488, 555 and 594 were measured using time-resolved detection with Victor 1420 Multilabel counter. Emission filter for AF 488 had a 520-nm band-pass with 10-nm bandwidth (Chroma Technology Corporation, Rockingham, VT). The sensitized emissions of AF 555 and 594 were measured at 572 and 615 nm, respectively. The emission filters were installed by the instrument manufacturer. The sensitized emission of the AF 680 was measured at 730 nm with a modified 1234 Delfia Research Fluorometer (Wallac, Perkin-Elmer Life and Analytical Sciences) equipped with a 730-nm band-pass emission filter with 10-nm bandwidth (Coherent Inc, Santa-Clara, CA), a red-sensitive R2949 photomultiplier tube (Hamamatsu Photonics, Shimokanzo, Japan), and a 340-nm DUG11 excitation filter (Perkin-Elmer Life and Analytical Sciences).

BSA-treated microtitration plates

All assays were performed in Low Fluorescence 96-well Maxisorp microtitration plates purchased from Nunc (Roskilde, Denmark). Wells were treated with BSA prior to the assays to prevent non-specific binding by incubating with 0.1% (*w*/*v*) BSA (Bioreba, Switzerland), 0.1% (*w*/*v*) Germall II (ISP, Wayne, NJ, USA) and 3% (*w*/*v*) trehalose (Sigma-Aldrich, St. Louis, MO, USA) in 0.05 M Tris–HCl buffer, pH 7.2, for 1 h, in room temperature with low shaking. After incubation plates were aspirated, dried and stored in +4 °C until used.

Terbium(III) chelate labeled Fab

E2-specific antibody Fab fragment was produced and purified as described by Kokko et al. [10] Fab fragment was labeled with a long lifetime and intrinsically fluorescent isothiocyanate derivative of a stable 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) [11]). For the labeling reaction 300 μg of Fab and tenfold molar excess of terbium(III) chelate, dissolved in water, was used. pH was adjusted to 9.8 by adding 5 μL of 1 M carbonate buffer. The total reaction volume was adjusted to 100 μL with water. Reaction was incubated over night at room temperature. After the incubation, Tb(III)-Fab was purified using NAP5 and NAP10 gel filtration columns from GE Healthcare (Chalfont St. Giles, UK). The buffer in the purification was TSA, pH 7.75 (0.05 M Tris–HCl, 0.9% (*w*/*v*) NaCl, and 0.5% (*w*/*v*) NaN_3, all purchased from Sigma-Aldrich, St. Louis, MO, USA). The amount of terbium(III) chelate in the labeled Tb(III)-Fab was measured by comparing the fluorescence of the terbium(III) chelate against fluorescence of a known standard. The amount of Fab was measured by absorbance at 280 nm.

E2 conjugation to acceptor dyes

Amino-modified E2 (6-oxoestradiol 6[*O*-(6-aminohexyl) oximel]) [12] was conjugated to Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 594 and Alexa Fluor 680 carboxylic acid succinimidyl ester fluorescent dyes (all from Molecular Probes, Invitrogen, Carlsbad, CA, USA) to create E2-acceptor conjugates. For each reaction approximately 300 μg of amino-modified E2 was dissolved in 12 μL of ethanol. Thereafter, 5 μL of 1 M carbonate buffer, pH 9.0, was added. Fluorescent dyes, 100–300 μg, were dissolved into 20 μL of

N,*N*-dimethylformamide (Sigma-Aldrich) and added immediately to the reaction. Finally, reaction volume was adjusted to 100 μL with water. Reactions were protected from light and incubated for 4 h in +37 °C with slow rotation. After incubation, reactions were frozen until purification.

Reactions were purified using an reverse phase high-performance liquid chromatography (HPLC) technique (instrumentation from Thermo Electron Corp., Waltham, MA, USA) using a Genesis C18 column from Jones Chromatography (Grace Vydac, Hesperia, CA, USA). Gradient purification was done using 0.02 M triethylammonium acetate (TEAA; Fluka Biochemica, Buchs, Switzerland) in water and 0.02 M TEAA and 50% (*w*/*v*) acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) as buffers. The amount of acetonitrile was increased from 30% to 50% in 34 min. After purifications the amount of E2 and fluorescent acceptor dyes was measured with absorbance. The appropriate wavelengths and molar absorptivities for the acceptors were provided by the manufacturer.

Homogeneous competitive assay for E2

Four homogeneous competitive assays for E2 were designed utilising the various emission peaks of the terbium(III) chelate for energy transfer. In the assays Tb (III)-Fab was used as a donor and different fluorescent dyes as acceptors. First, 10 μL of E2-standards 0–25 μM (final concentrations in the total well volume of 100 μL were 0–2.5 μM), with three replicas, were added to BSA-treated wells. Then 10 ng of Tb(III)-Fab in 40 μL of assay buffer was added and wells were incubated for 30 min in room temperature and with low shaking. Thereafter, 50 μL of 50 nM E2-acceptor conjugate was added, wells were protected from light and incubation was continued for 15 min in room temperature, with low shaking. After the incubation, sensitized emission of the fluorescent acceptor dyes were measured with time-resolved mode using either Victor 1420 Multilabel counter (AF 488, 555 and 594) or modified 1234 Delfia Research fluorometer (AF 680).

Terbium(III) chelate as a simultaneous donor for multiple wavelength acceptors

The functionality of the terbium(III) chelate as a single donor for multiple wavelength acceptors was tested by measuring all the acceptors at all the wavelengths. The E2 assays were performed as described in the previous chapter. After the incubation of the E2-acceptors all the E2-assays were measured at 520, 572, 615 and 730 nm. AF 488 and AF 680 were used as the acceptors when testing the actual performance of a simultaneous measurement of both acceptors. Since both of the assays were E2-assays, the assay components had to be incubated in different wells. At first, competitive E2 assay was performed using either AF 488 or AF 680 as an acceptor as described in the previous chapter. After the measurement, 50 μL from one assay well was mixed with 50 μL of the other assay, which contained no free E2. Therefore, during the measurement the other acceptor created maximum interference. Wells were shaken for 1 min. Thereafter, the sensitized emission of AF 488 and 680 was measured with Victor 1420 Multilabel counter (at 520 nm) or modified 1234 Delfia Research fluorometer (at 730 nm), respectively.

Results and discussion

Reagents

E2 specific Fab-fragment was labeled with intrinsically fluorescent terbium(III) chelate. The labeling degree obtained after the purification was 4.0. The amino-modified E2 analogue was conjugated to four different fluorescent acceptors. The HPLC-purified E2-acceptor dye conjugates were characterized with absorbance measurements. Fractions that contained both acceptor dye and E2 were selected.

The principle of the homogeneous competitive E2 assay

The homogeneous competitive assay for E2 was based on FRET between the terbium(III) chelate (donor) and four different fluorescent dyes (acceptor). The purpose of our research was to study whether terbium(III) chelate could be used as a single efficient donor for multiple-wavelength acceptors. Instead of opting the acceptors only based on their spectral overlapping with terbium(III) chelate, the acceptors were chosen to have an emission maximum at the local emission valley of terbium(III) chelate. This minimized the background caused by the direct emission of donor (Fig. 1). All acceptors were tested using the same competitive assay for 17β-estradiol (E2), in order to eliminate the variations caused by the affinity of the antibody. Thus the variations in the assay performances were caused by the energy transfer properties of the dye and measurement conditions.

The assays were performed according to the back-titration principle. [13] First, the E2 standards were allowed to bind to the terbium(III) chelate labeled E2 specific antibody Fab fragment. Thereafter, the competing E2-acceptor conjugate was added. Since the dissociation of the E2 from the Fab is slow [14], the E2-acceptor conjugate could only bind to those Tb(III)-Fab complexes that are not bound to the E2 of the standards. During measurement, the chelate was excited at 340 nm and the sensitized emission was measured at acceptor specific wavelength (Fig. 2). The

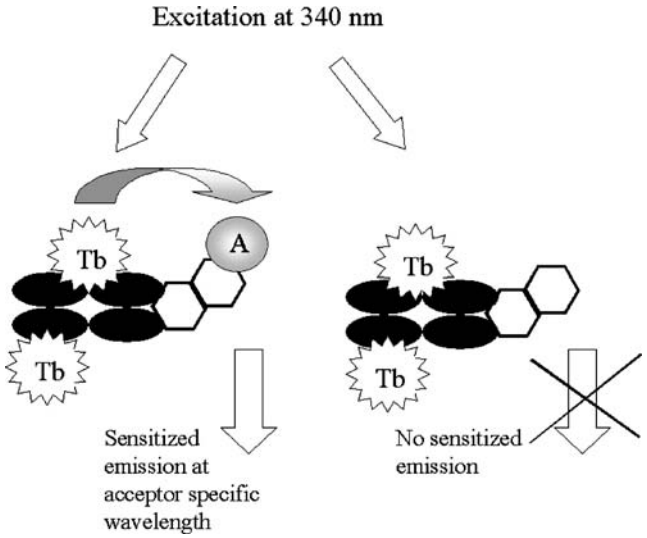

Fig. 2 The principle of the assay: The anti-E2 Fab labeled with terbium(III) chelate was bound to either the E2-acceptor conjugate or E2 standard. Sensitized emission was observed at the acceptor specific wavelength when the Tb(III)-Fab was bound to the E2-acceptor conjugate

measurement wavelengths for the acceptors were 520 nm (for AF 488), 572 nm (for AF 555), 615 nm (for AF 594) and 730 nm (for AF 680).

Homogeneous competitive assay with different acceptors

A standard curves for the homogeneous competitive E2 assays were done using different concentrations of E2 while the amount of Tb(III)-Fab was 10 ng per well. Four different acceptors with emission maximum at the local emission valley of the terbium(III) chelate were tested. The key parameters of the E2 assays are presented in Table 1. The lowest measurable concentration and the highest measurable concentration were calculated as the concentrations equaling 90% and 10% of the specific signal, respectively. Specific signal (Signal) was calculated by subtracting the signal of the highest E2 standard from the maximum signal (no E2 present). The signal to background ratio (S/B) was calculated by dividing the maximum signal with the background signal.

The specific signal of the AF 680 was lower than that of the other acceptors, even though the quenching efficiency was not that different from the other acceptors. Due to the instrumental limitations, the sensitized emission of AF 680 was measured with different instrument and at 730 nm. The emission maximum of the acceptor is at 700 nm, and thus the measurement wavelength was not optimal to the acceptor. At this measurement wavelength, however, there was very little background interference from the emission of terbium(III) chelate. Thus, the best S/B ratio was obtained with AF 680. The poor ratios of AF 555 and 594 can be explained by rather narrow emission valley of terbium(III) chelate at 572 nm and 615 nm, thus some of the fluorescence of the chelate could pass through the emission filter and increase the background. The lowest measurable concentrations were similar with all the acceptors. However, assays utilising AF 680 and 488 had somewhat better range than the other acceptors.

The Förster radii (Table 1) of the different donor-acceptor pairs were calculated from normalized absorption and emission spectra using equations:

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

$$\text{where } J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad (2)$$

Where 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. [15] The absorption spectrum of AF 488 overlaps only with the first emission peak of the terbium(III) chelate, thus the Förster radius of the AF 488 is the shortest. The best overlapping of the emission spectrum of the donor and the absorption spectrum of the acceptor, and thus the highest Förster radius, was obtained whit AF 555.

Efficiency of the energy transfer can also be estimated by comparing the fluorescence of the donor in the absence

Table 1 The performance parameters of the assays: Lowest measurable concentration (LMC), Highest measurable concentration (HMC), Specific signal (Signal), Signal to background ratio (S/B), Förster radii (R_0) and Quenching efficiency (Q%)

Acceptor	LMC (pM)	HCM (nM)	Signal	S/B	R_0 (Å)	Q%[a]
AF 488	300	4.5	38,000	24	46	29
AF 555	315	3.3	42,000	11	61	52
AF 594	310	3.3	48,800	6	53	46
AF 680	295	5.6	9,400	45	52	42

[a] Maximum achievable Q% was approximately 60% due to the large portion of inactive Fab-fragment

and in the presence of the acceptor (quenching efficiency) [15]:

$$Q\% = 1 - \frac{F_{DA}}{F_D} \quad (3)$$

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. The observed quenching efficiencies are presented in Table 1. The quenching results are compatible with our previous results. Only ~60% of this particular Fab-fragment is active and thus capable of binding antigen. Therefore, the maximum attainable quenching percentage is about 60%. [16] The observed energy transfer efficiencies (quenching percentage) followed the theoretical transfer efficiencies (Förster radii) nicely.

The E2-specific Fab-fragment has also been used as a binder in a homogeneous competitive assay utilising europium(III) chelate as a donor and AF 680 as an acceptor. [16] Compared to that assay, assays utilising terbium(III) chelate as a donor had better lowest measurable concentrations with all the acceptors.

Terbium(III) chelate as a simultaneous donor for multiple-wavelength acceptors

In order to test whether the emission of the acceptors interfere with each other, the sensitized emission of each acceptor was measured at all the acceptor specific wavelengths. The specific signals were compared as a percentage of the maximum signal of the appropriate acceptor (Table 2). At the measuring wavelength of 520 nm, no interference from the other acceptors was detected. In the case of 572 nm and 615 nm, the other acceptors emitting at lower wavelengths caused slight interference (~10%). The highest interference was from AF 594 when measured at 730 nm. In fact, due to the very low donor emission background at 730 nm, the signal to background ratio of AF 594 was higher at 730 nm (S/B=15) than at 572 nm (S/B=6).

Based on these results, the actual simultaneous measurement of the sensitized emission of AF 488 and 680 was tested. The procedure mimicked multiplex assay, where the concentration of one of the analytes changes and the concentration of the other is such that maximum interference is created. The standard curves (Fig. 3) from both single and simultaneous measurements were almost identical with both acceptors. Only a small increase in the background in the simultaneous measurement was detected, which slightly inflicted the range of the assays.

Table 2 The amount of interference compared to the signal of the theoretically suited acceptor: measurement wavelength

Acceptor	520 nm	572 nm	615 nm	730 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%

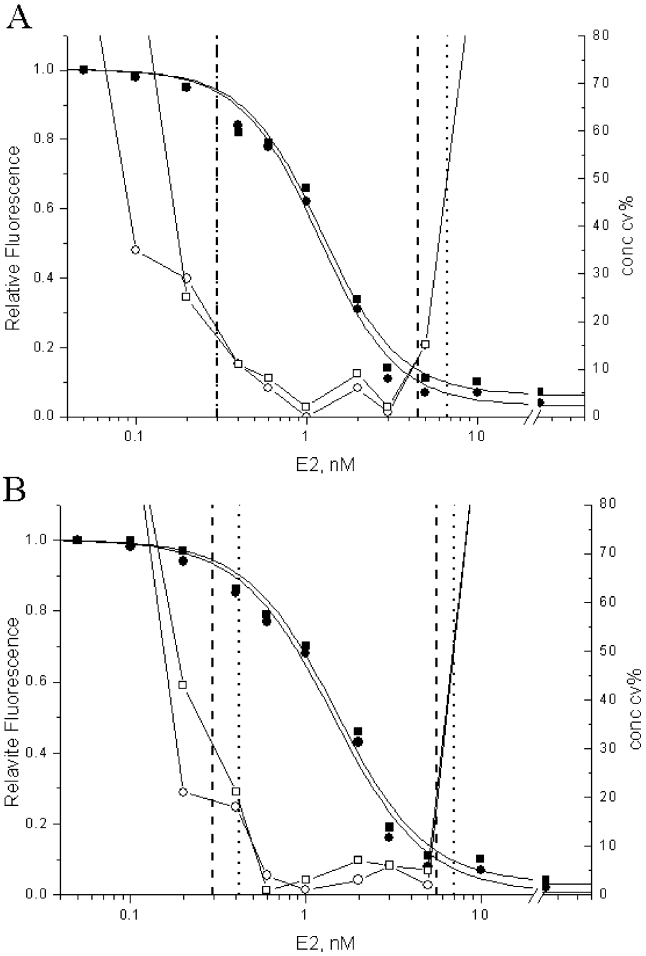

Fig. 3 The standard curves from the homogeneous single (*filled octagons*) and simultaneous (*filled squares*) measurements with cv-profile (*hollow symbols*) when using **a** Alexa Fluor 488 and **b** Alexa Fluor 680 as an acceptor. The ranges of the single and simultaneous measurement assays are illustrated with *dashed* and *dotted lines*, respectively

Conclusions

In conclusion, we have shown that terbium(III) chelate can be used as a donor for various acceptors emitting at green to far-red. Due to a very low donor emission background at the far-red area, the best signal to background ratio and assay performance was obtained with Alexa Fluor 680. If the acceptors are chosen appropriately, terbium(III) chelate could act as a single donor for a simultaneous measurement

of multiple acceptors in a homogeneous immunoassay without compromising the assay performance. In the future, the possibility of using terbium(III) chelate as a single donor in actual homogeneous multi-parameter assay will be tested.

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