Supporting Information

Photophysical evaluation of a new functional terbium complex in FRET-based time-resolved homogenous fluoroassays

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Table of Contents

1. Calculation of Förster Radius, Energy Transfer Efficiency, and Donor-Acceptor Distance

The spectral overlap between the acceptors absorption spectra and the donor emission spectrum implies high probability for RET to occur. According to the Förster's theory, the Förster radius R_0 is the distance between two luminophores (a donor and an acceptor) at which the transfer efficiency η is equal to 50%. The Förster radius was calculated using the following equation,

$$R_0 = \sqrt[6]{\frac{9\varphi_0(\ln 10)\kappa^2}{128\pi^5 n^4 N_A} J(\lambda)} \tag{3}$$

where φ_0 is the donor quantum yield equal to 0.3κ^2 is the dipole orientation factor taken as equal to 2/3 for the case, when a random donor-acceptor dipole orientation is assumed, N_A is the Avogadro's number (6.0221415 × 10^{23} mol^{-1}), and n is the refractive index of water at 615 nm (1.333). Due to the high dilution of the sample, the influence of nanoparticle concentration on both the refractive index and the dipole orientation factor has been assumed to be marginal.

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

where $F_D(\lambda)$ is the donor luminescence emission, $\varepsilon(\lambda)$ the extinction coefficient of the acceptor and λ is the wavelength. The overlap $J(\lambda)$ was determined to be equal to 1.7 and 1.6 L mol^{-1} cm^{-1} nm^4.

The luminescence emission decays were monitored for the donor biotin-Tb-LLC in the absence and presence of an acceptor as the change in the donor decay time indicates successful FRET. The decays $I(t)$ were fitted to a tri-exponential function to obtain respective components and the emission decay times for the calculation of the FRET efficiency,

$$I(t) = \sum_{i=1}^{3} A_i e^{\left(-\frac{t}{\tau_i}\right)} \tag{5}$$

where t is time, τ_i is the emission decay time for a component i, and A_i is the emission intensity at time 0 for a component i. The FRET efficiencies were calculated from amplitude-averaged decay times. The transfer efficiency η and the donor-acceptor distance r were calculated for FRET from biotin-Tb-LLC to an acceptor using following equations:

$$\eta = 1 - \frac{\tau_{DA}}{\tau_D} \tag{6}$$

$$r = R_0 \sqrt[6]{\frac{1-\eta}{\eta}} \tag{7}$$

where τ_{DA} and τ_D are decay times for a donor in the presence and absence of the acceptor. We chose to calculate τ_{DA} for FRET in the absence of an acceptor and τ_D in the presence of an acceptor. Noticeably, we used the standard Förster's theory considering energy transfer to be a consequence of interactions between two dipoles. We are aware of the fact that for multiple donors and acceptors transfer mechanism has not been fully understood and the application of the classical Förster's theory may lead to certain calculation discrepancies.

2. Fitting of the experimental results

In order to fit experimental points $I_{FRET}(A)$ from Figure 7 following function has been used:

$$I_{FRET}(A) = \frac{[I_{FRET}(A_0) - I_{FRET}(A_{max})] \cdot c_0}{c_0 + c} + I_{FRET}(A_{max})$$

where $I_{FRET}(A_0)$and $I_{FRET}(A_{max})$is the integrated FRET signal calculated in the acceptor absence and at full saturation, respectively; c is an acceptor concentration and c_0 is the acceptor concentration at which 50% of biotin-Tb-LLC is bound, i.e. produces FRET.

3. A full synthesis route to prepare biot-Tb-LLC

a) Synthesis of compound 2

To a solution of compound **1** (614 mg, 1.29 mmol) dissolved in acetonitrile (60 mL), dry K_2CO_3 (717 mg, 5.16 mmol) was added and the mixture was refluxed for 30 min. *Tetra*ethylimino*bis*-(methanephosphonate) (900 mg, 2.84 mmol) was added and the mixture was refluxed for 48 h. The remaining potassium carbonate was removed by filtration and the solvent was evaporated under

vacuum. The resulting oily residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH, 100/0 to 90/10, R_f = 0.13 at 95/15) affording compound **2** (730 mg, 0.80 mmol) as a yellowish oil (yield: 62 %). ^{1}H NMR ($CDCl_3$, 300 MHz):δ 8.44 (d, J = 2.6 Hz, 2H), 7.80 (s, 2H), 6.50 (d, J = 2.6 Hz, 2H), 4.13 (m, 20H), 3.24 (d, $^2J_{P\text{-}H}$= 10 Hz, 8H), 1.30 (t, J = 7.0 Hz, 24H). ^{31}P NMR ($CDCl_3$, 162 MHz):δ 23.98. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 153.2, 150.4, 136.53, 128.0, 112.2, 109.1, 62.06 (d, J = 6.8 Hz), 53.91 (t, J = 8.8 Hz), 50.01 (dd, J = 159.4 Hz), 16.51 (d, J = 6.0 Hz). ESI$^+$/MS (CH_2Cl_2): *m/z* = 949.3 ([**2** + H]$^+$, 100 %). Anal. Calc d for $C_{33}H_{58}BrN_7O_{12}P_4.H_2O$: C, 41.00; H, 6.26; N, 10.14. Found: C, 40.83; H, 6.44; N, 9.95.

b) Synthesis of compound 3

Compound **2** (1.12 g, 1.2 mmol), 6-heptynoic acid (90%, 182 mg, 1.44 mmol), and [$Pd(PPh_3)_2Cl_2$] (42 mg, 0.06 mmol) were dissolved in distilled THF (80 mL) and distilled Et_3N (24 mL) and the solution was degassed with nitrogen for 30 minutes. CuI (22.9 mg, 0.1 mmol) was added and the mixture was stirred for 14h at 60°C. The solvent was removed under reduced pressure and the resulting oil was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH, 95/5, R_f = 0.13 at 95/15) to afford compound **3** (1.01 g, 1.02 mmol) as a brown oil (yield: 85%). ^{1}H NMR ($CDCl_3$, 300 MHz):δ 8.45 (d, J = 2.6 Hz, 2H), 7.82 (s, 2H), 6.50 (d, J = 2.6 Hz, 2H), 4.17 (m, 20H), 3.26 (d, $^2J_{P\text{-}H}$= 10 Hz, 8H), 2.43 (m, 2H), 2.40 (m, 4H), 1.98 (m, 2H), 1.33 (t, J = 7.0 Hz, 24H). ^{31}P NMR ($CDCl_3$, 162 MHz): δ 22.20. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 206.94, 175.38, 152.06, 150.0, 137.37, 127.67, 111.35, 108.82, 96.63, 62.21 (d, J = 7.0 Hz), 58.33, 50.07 (dd, J = 159.9 Hz and J = 9.4 Hz), 27.19, 24.04, 19.39, 19.11, 16.46 (d, J = 6.1 Hz). ESI$^+$/MS (CH_2Cl_2): *m/z* = 994,4 ([**3** + H]$^+$, 60 %).

c) Synthesis of compound 5

To a solution of compound **3**(293 mg, 0.29 mmol) and compound **4** (144 mg, 0.29 mmol) in DMF (5 mL), was added EDCI (68 mg, 0.35mmol) and DMAP (43 mg, 0.35 mmol) dissolved in CH_2Cl_2 (5 mL). The mixture was stirred for 20 hrs at r.t. and was concentrated under reduced pressure. The resulting residue was dissolved in dichloromethane, washed with water and brine, dried over Na_2SO_4, filtered and concentrated to dryness under vacuum. Purification of the crude product was performed by

column chromatography over silica gel (CH_2Cl_2/MeOH gradient from 100:0 to 90:10) yielding compound **6**(338 mg, 85%) as an oil.

^{1}H NMR ($CDCl_3$, 300 MHz): δ8.40 (d, J = 2.4 Hz, 2H), 7.66 (s, 2H), 6.79 (s, br, 1H), 6.63 (s, br, 1H), 6.46 (d, J = 2.7 Hz, 2H), 6.40 (s, br, 1H), 6.00 (s, br, 1H), 4.44 (s, br, 1H), 4.26 (s, br, 1H), 4.09 (qt, J = 7.2 Hz, 16 H), 4.03 (s, 4H), 3.49 (m, br, 4H), 3.37 (s, br, 4H), 3.26 (s, br, 4H), 3.17 (d, $^2J_{P\text{-}H}$= 10.2 Hz, 8H), 3.07 (s, 1H), 2.92-2.60 (m, 2H), 2.43 (t, J = 6.8 Hz, 2H), 2.26-2.11 (m, 4H), 1.90-1.50 (m, 8H), 1.50-1.30 (m, 2H), 1.26 (t, J = 7.2 Hz, 24H).^{31}P-NMR ($CDCl_3$, 162 MHz): δ24.50.^{13}C NMR ($CDCl_3$, 75 MHz): δ173.4, 173.0, 159.8, 152.5, 150.0, 137.3, 127.8, 111.1, 108.7, 96.8, 78.5, 70.2, 70.1, 69.9, 69.7, 62.2 (d, J = 6.8 Hz), 61.9, 60.3, 55.7, 55.6, 50.0 (dd, J = 159.0 Hz, J = 8.5 Hz), 40.5, 39.2, 39.1, 36.4, 35.8, 35.0, 29.7, 25.7, 25.6, 25.5, 25.0, 16.5 (d, J = 6.0 Hz).ESI+/MS (CH_2Cl_2): m/z = 1372.6 ([**5**+Na], 89%).

d) Synthesis of biot-L

In a 50 mL rounded bottom flask, compound **5** (385 mg, 0.29 mmol) was dissolved in dichloromethane (10 mL). 2.6-dimethylpyridine (1.5 mL, 12.9 mmol) and TMSBr (1.5 mL, 11.4 mmol) were added. The solution was stirred at r.t. for 14 hrs. The solvent were removed under reduced pressure, the resulting residue was taken up with MeOH (10 mL) and stirred for 30 minutes at r.t.. The evaporation, dissolution procedure was repeated with MeOH (10 mL) and the solution was stirred for 2 hrs at r.t. The solvent was removed under vacuum and the residue was washed with MeOH and CH_2Cl_2 and the solid was collected by centrifugation and dried under vacuum to afford biot-L in 64% yield (206 mg).

^{1}H NMR (D_2O, 400 MHz): δ8.71 (d, J = 2.1 Hz, 2H), 7.82 (s, 2H), 6.86 (d, J = 2.2 Hz, 2H), 4.85 (s, 4H), 4.51 (m, 1H), 4.30 (m, 1H), 3.62 (m, 14H), 3.54 (t, J = 5.2 Hz, 2H), 3.40 (t, J = 5.4 Hz, 2H), 3.29 (t, J = 5.1 Hz, 2H), 3.16 (m, 1H), 2.92-2.82 (m, 1H), 2.70 (s, 1H), 2.57 (t, J = 6.8 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 2.14 (t, J = 7.5 Hz, 2H), 1.81 (m, 2H), 1.68 (m, 2H), 1.65-1.35 (m, 4H), 1.24 (m, 2H). ^{31}P-NMR (D_2O, 162 MHz): δ7.15.^{13}C NMR (D_2O, 75 MHz): δ176.5, 165.1, 148.8, 144.6, 137.5, 129.9, 112.0, 110.8, 98.7, 78.0, 69.5, 68.9, 62.0, 60.2, 55.3, 53.2, 52.0, 50.3, 39.7, 39.0, 38.9, 35.4, 35.3, 27.9, 27.6, 27.1, 25.1, 24.9, 18.7, 18.6.Anal. Calcd. for $C_{40}H_{63}N_{11}P_4O_{17}S.2H_2O$: C, 41.35; H,

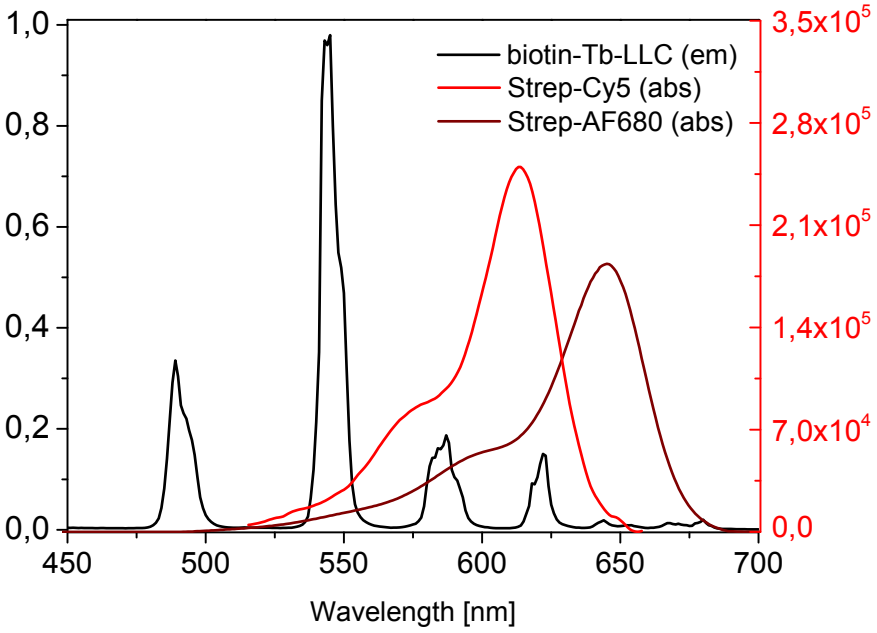
5.81; N, 13.26. Found: C, 41.54; H, 5.56; N, 12.76.IR (cm^{-1}, ATR): ν3278 (w, br), 2929 (w), 1611 (m), 1552 (m), 1460 (m), 1390 (m), 1328 (w), 1195 (s), 1047 (s), 987 (m), 938 (s), 775 (m, br).

e) Synthesis of biot-Tb-LLC

To a solution of the ligand (40.3 mg, 0.035mmol) in 20 mL of water a solution of $TbCl_3.H_2O$ (12.3 mg, 0.035 mmol) in 5 mL of water was added dropwise. The mixture was left for equilibration for 3hours and then a diluted NaOH aqueous solution was added to raise the pH to 7.0. The mixture was concentrated under vacuum and addition of methanol allows the precipitation of the **biot-Tb-LLC** (42 mg).ESI-/MS (H_2O): m/z = 1400.38 ([**biot-Tb-LLC**H_4]$^-$, 11%).

4. The overlap between donor emission and acceptor absorption

(a)



(b)

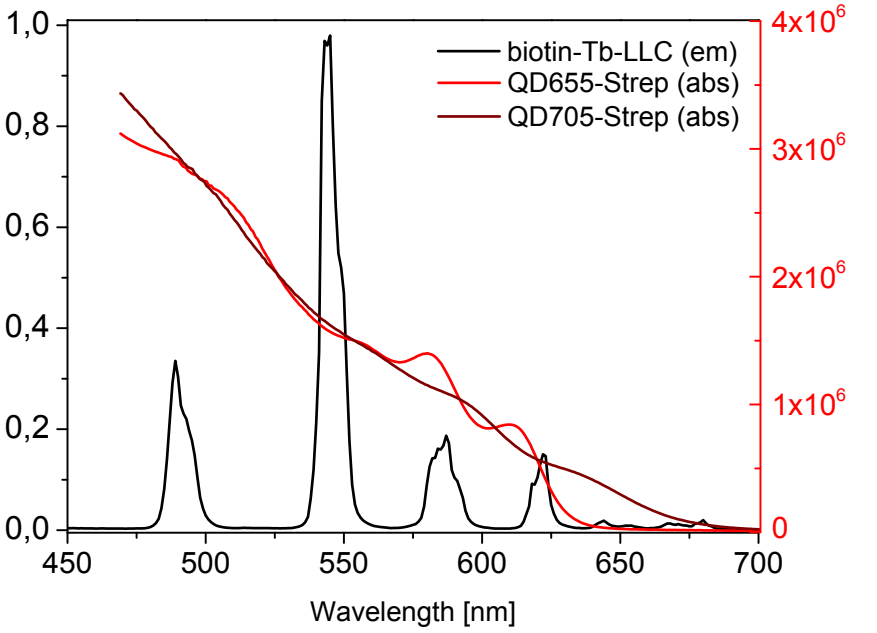


Figure S1. The spectra to demonstrate the overlap between donor emission and acceptor absorption. (a) Biotin-Tb-LLC emission spectrum (black line) together with absorption spectra for Strep-Cy5 (red line) and Strep-AF680 (brown line). (b) Biotin-Tb-LLC emission spectrum (black line) together with absorption spectra for QD655-Strep (red line) and QD705-Strep (brown line).

5. Time-gated steady-state luminescence spectra

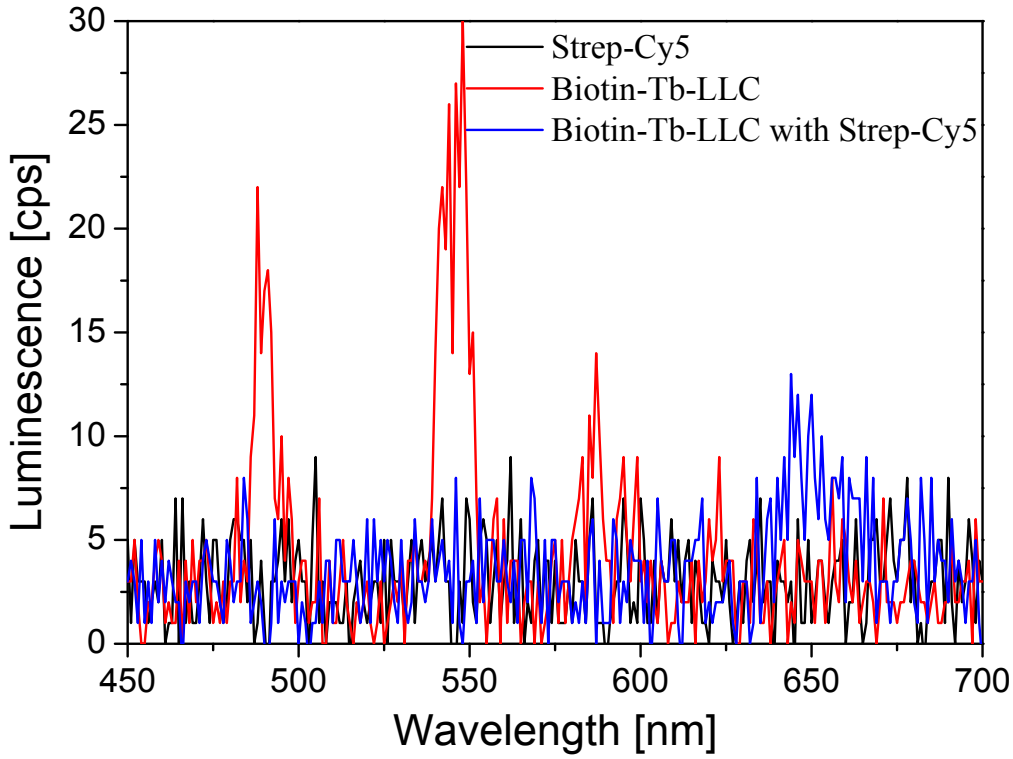


Figure S2. Time-gated steady-state luminescence spectra depicting Biotin-Tb-LLC to Strep-Cy5 energy transfer. The spectra were taken in the range 50-1000μs, with 20s acquisition time for each point. Long decaying acceptor emission (blue curve) can be readily seen at wavelengths above 600nm.