Direct conjugation of antibodies to the ZnS shell of quantum dots for FRET immunoassays with low picomolar detection limits

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Electronic Supplementary Information

1. Materials and Methods

1.1 Materials

1.1.1 Chemicals

D-Penicillamine, phosphate-buffered saline solution (PBS; 1 pellet was dissolved in 200 µL pure H_2O yielding 1× (10 mM) PBS at pH 7.4), tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP), tris(hydroxymethyl)aminomethane (TRIS/Cl) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. QD CdSeTe/ZnS 705 in organic solvent was purchased from Life Technologies/Thermo Fisher Scientific and terbium NHS-activated complex (Lumi4-Tb) was provided by Lumiphore in lyophilized form. Bovine calf serum, monoclonal primary antibodies ("PSR222" and "PSS233") against prostate specific antigen (PSA) and PSA were provided by Cezanne/Thermo Fisher. IgGs were fragmented to F(ab')$_2$ and F(ab) using a Pierce™ Mouse IgG F(ab') F(ab')$_2$ preparation kit and following the instructions provided by the supplier (Thermo Fisher Scientific). PSR222 was labelled with Lumi4-Tb and whole PSS233 as well as their fragments were conjugated to the QDs.

1.1.2 Instruments

Absorption spectra were acquired using a Lambda 35 UV/Vis spectrophotometer from Perkin Elmer. Photoluminecence (PL) spectra were recorded on a FluoTime 300 lifetime fluorescence spectrometer from PicoQuant using as excitation source a continuous-wave Xe lamp for spectra acquisition. Homogeneous FRET immunoassays were measured on a modified "KRYPTOR compact plus" clinical fluorescence plate reader using 500 detection bins of 2µs integration time from Cezanne/Thermo Fisher Scientific and a fluorescence plate reader from Edinburgh Instruments (EI) using 4000 bins of 2µs integration time for FRET decay curves. Both fluorescence plate readers used a pulsed nitrogen laser operating at 20 Hz for excitation.

1.2 Methods

1.2.1 Phase transfer

The phase transfer protocol of the QDs from organic solvent to aqueous medium with penicillamine ligand has been described in a previous study by Mattera *et al. Nanoscale,* 11275-11283 (**2016**).

1.2.2 Preparation of Tb-antibody conjugates

Lumi4-Tb in lyophilized form was dissolved to 8 mM in anhydrous DMF. 1 mg/mL of "PSR222" IgGs were buffer exchanged from PBS buffer to 100 mM carbonate buffer pH 9.0 using a 30 kDa molecular weight cut-off (MWCO) spin column from Millipore. The buffer exchange was performed 4 times by centrifuging at 14,000 g for 2 minutes each. A 30 times molar excess of Lumi4-Tb was mixed with the recovered IgGs after buffer exchange. The mixtures were incubated for 2 h at room temperature while rotating at 30 rpm using an ELMI Intelli-Mixer. The IgG-Tb conjugate was purified and washed 4 times with 100 mM TRIS/Cl pH 7.4 using a 50 kDa MWCO spin column from Millipore, to remove the unbound Tb-complex with a final volume of *ca.* 250 µL. The purified conjugate was stored at 4 °C.

Tb concentrations were calculated using Beer-Lambert's law with a molar absorptivity (extinction coefficient) of 26,000 $M^{-1}\cdot cm^{-1}$ at the absorption maximum of 340 nm. Antibody (AB) concentration was determined at 280 nm using a molar absorptivity of 210,000 $M^{-1}\cdot cm^{-1}$. **Table S1** represents the Tb and antibody concentrations of the Tb-antibody conjugate with a labeling ratio of 11 Tb per "PSR222" IgG.

1.2.3 Preparation of QD-antibody conjugates

A 20-fold molar excess of the "PSS233" antibodies (IgG, $F(ab')_2$ & F(ab)) were reacted with QD-Pen in 1× PBS (pH 7.4). A stock solution of 200 mM of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was freshly prepared by dissolving 0.0057 g of TCEP (molecular weight: 286.65 g/mol) in 100 µL of pure H_2O. The solution was vortexed until all the TCEP was dissolved. 3.75 µL of TCEP was then added to each 150 µL of "PSS233" antibodies (concentration of antibodies in 150 µL of 1× PBS : IgG, $F(ab')_2$=3.2 µM and F(ab)=7 µM) Disulfide bonds (S-S) on the antibodies were reduced to sulfhydryls (S-H) using 30 min of incubation (rotating at 30 rpm) with 5 mM of TCEP in 1× PBS buffer (pH 7.4). ABs-SH were purified using Zeba 7K desalting columns to remove excess reducing agent. The desalted solutions were used for conjugation with QD-Pen. For conjugation of IgGs and $F(ab')_2$ with QD-Pen : 150µL of 161 nM QD-Pen was mixed with 150 µL of 3.2 µM of each IgGs and $F(ab')_2$. For F(ab) conjugation, 150 µL of 349 nM QD-Pen was mixed with 150 µL of 7 µM of F(ab). The mixtures were incubated for 4 h at room temperature in the dark while rotating at 30 rpm. Unbound $F(ab')_2$ and F(ab) were separated from the conjugation mixture using a 100 kDa MWCO spin column from Millipore by washing four times with 1× PBS (pH 7.4). QD-IgG conjugate was washed with 1× PBS following the same procedure. Purified conjugates (*ca.* 200 µL) were centrifuged at 4000 g and supernatants were taken and stored at 4 °C.

QD concentrations were calculated using the QD molar absorptivity at 405 nm, ε_{405}(QD705) = 8.3 × 10^6 $M^{-1}{\cdot}cm^{-1}$ and summarized in **Table S1**.

Table S1. Overview of the concentration of the FRET-pairs and labelling ratio of the Tb-IgG conjugate.

Tb-AB	[Tb] (µM)	[AB] (µM)	Tb/AB	QD-AB	[QD] (nM)
Tb-IgG	2.6	27.6	11±2	QD705-IgG	60
				QD705-F(ab')$_2$	11
				QD705-F(ab)	132

2. Photoluminescence decay curves of Tb-AB donors and AB-QD acceptors within the PSA immunoassays

PL decay curves of Tb-AB and AB-QD conjugates within the PSA immunoassays were measured on a fluorescence plate reader from EI. **Figure S1** shows the PL decay curves for the Tb-to-QD-IgG/F(ab) systems where we can distinguish in the QD channel the appearance of new decay components in the µs range and higher PL intensities with increasing PSA concentration as compared to the 0 nM black curve, for which only Tb-QD FRET-pairs (µs decay component from QD and long decay component from Tb originating from PL background in the QD detection channel) are present, which indicates sensitization of the QDs by FRET. Fitting of the FRET-sensitized acceptor was performed by a four exponential fit function using "FAST" software from EI, where the 4th exponential component was fixed to the long lifetime of Tb (2.4 ms) to account for unquenched Tb emission. We followed a similar decay time fitting as described in Hildebrandt, N. *FRET – Förster Resonance Energy Transfer*, pp. 105–163 (Wiley-VCH Verlag GmbH & Co. KGaA, 2013).

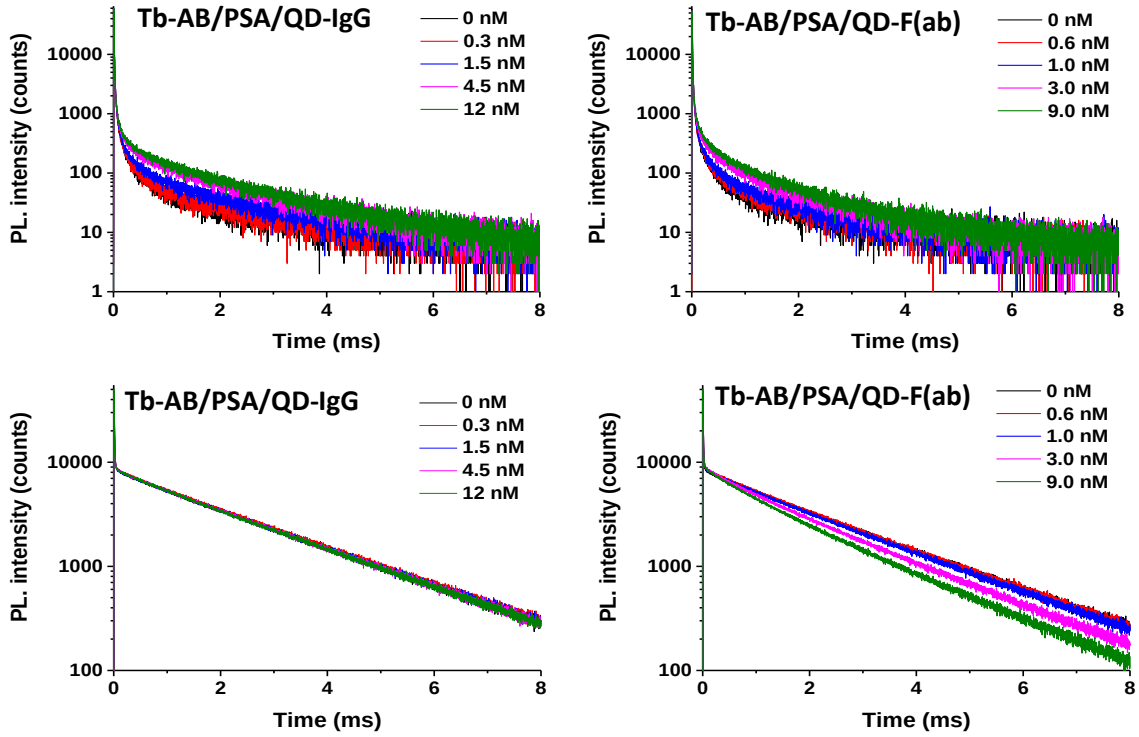

Figure S1. PL decay curves of the IgG and F(ab) system. Top curves correspond to acceptor channel showing sensitization of QDs due to FRET and bottom curves correspond to the respective Tb decay curves in the donor channel. Concentrations given in the graphs correspond to the concentrations of PSA inside the sample.

After corrections to account for unquenched donor emission in the QD channels, a FRET-efficiency (η_{FRET}) of 34 and 38 % with an estimated donor to acceptor distance of 12.3 and 11.9 nm for the IgG and F(ab) systems respectively, were determined. **Table S2** summarizes the η_{FRET} and donor to acceptor distances calculated from the decay times of the acceptor sensitization.

Table 2. FRET efficiencies and donor-acceptor distance calculated from the decay times of the acceptor sensitization. $<\tau_D>$ corresponds to the average lifetime of Tb in absence of QD and $<\tau_{AD}>$ is the average lifetime of QD in presence of Tb.

FRET system	η_{FRET} (acceptor channel)	D-A distance (acceptor channel)	$< \tau_D >$	$< \tau_{AD} >$
Tb-to-QD-IgG	34%	12.3 nm	2.20 ms	1.45 ms
Tb-to-QD-F(ab)	38%	11.9 nm	2.19 ms	1.36 ms

3. Immunoassay calibration curves for the estimation of LODs

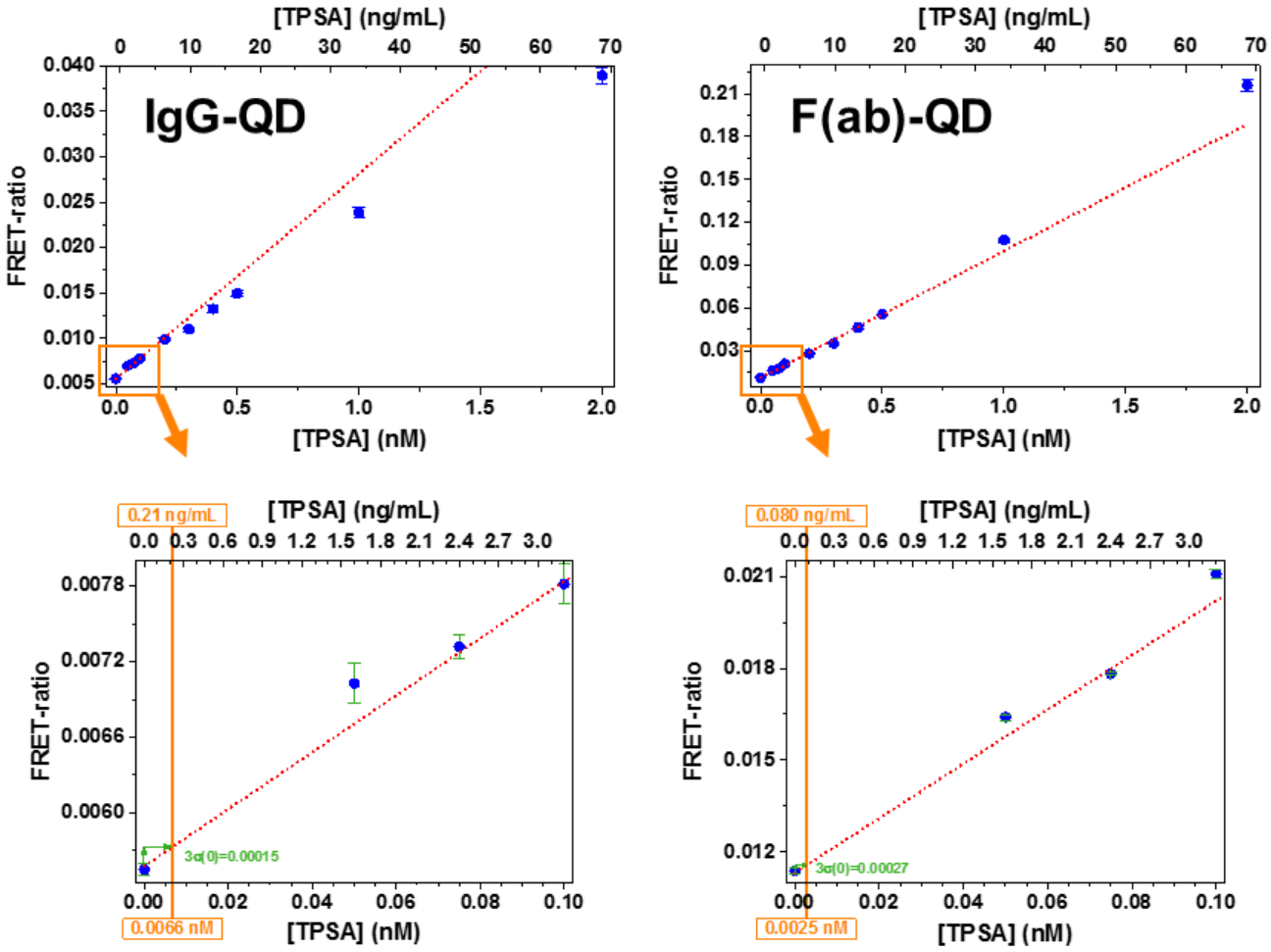

Figure S2. Immunoassay calibration curves for IgG-QD (left) and F(ab)-QD (right). Red lines are linear fits between 0 and 0.2 nM for IgG-QD and between 0 and 0.5 nM for F(ab)-QD. The bottom graphs were used to determine the LODs as zero concentration FRET ratios plus three times their standard deviation ($3\sigma(0)$).