

Nanobodies and Nanocrystals: Highly Sensitive Quantum Dot-Based Homogeneous FRET Immunoassay for Serum-Based EGFR Detection

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Semiconductor quantum dot nanocrystals (QDs) for optical biosensing applications often contain thick polyethylene glycol (PEG)-based coatings in order to retain the advantageous QD properties in biological media such as blood, serum or plasma. On the other hand, the application of QDs in Förster resonance energy transfer (FRET) immunoassays, one of the most sensitive and most common fluorescence-based techniques for non-competitive homogeneous biomarker diagnostics, is limited by such thick coatings due to the increased donor-acceptor distance. In particular, the combination with large IgG antibodies usually leads to distances well beyond the common FRET range of approximately 1 to 10 nm. Herein, time-gated detection of Tb-to-QD FRET for background suppression and an increased FRET range is combined with single domain antibodies (or nanobodies) for a reduced distance in order to realize highly sensitive QD-based FRET immunoassays. The “(nano)²” immunoassay (combination of nanocrystals and nanobodies) is performed on a commercial clinical fluorescence plate reader and provides sub-nanomolar (few ng/mL) detection limits of soluble epidermal growth factor receptor (EGFR) in 50 μ L buffer or serum samples. Apart from the first demonstration of using nanobodies for FRET-based immunoassays, the extremely low and clinically relevant detection limits of EGFR demonstrate the direct applicability of the (nano)²- assay to fast and sensitive biomarker detection in clinical diagnostics.

1. Introduction

Semiconductor quantum dots (QDs) continue to be of large interest for spectrally and temporally multiplexed biosensing

because they provide high photostability, bright fluorescence, size-tunable absorption and emission wavelengths and a large surface area for attaching several biomolecules of the same or different kind.^[1–5] Functional bioconjugation of QDs in particular, preserving both the QD photophysical properties and the binding properties of the biomolecule, has been widely investigated.^[6–8]

Antibodies are among the most applied tools for biomolecular detection and targeted therapy, and are therefore one of the most important biomolecules for QD-bioconjugation. However, the large size of full IgG antibodies (ca. 150 kDa molecular weight and $14.5 \times 8.5 \times 4$ nm dimension)^[9] and the different possible antibody attachment groups for bioconjugation^[10] are disadvantageous for labeling many antibodies in a controlled manner to a QD. To overcome this problem single domain antibodies (also called “nanobodies” or V_HH) have been proposed for QD conjugation.^[11–14]

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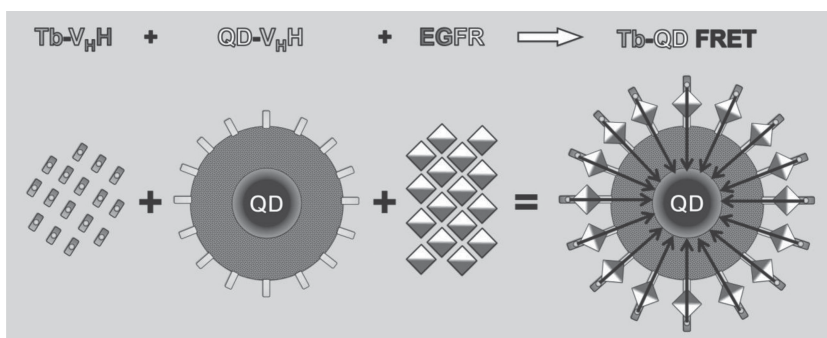
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V_HH are single domain antigen binding fragments of heavy chain antibodies (ca. 15 kDa and $4 \times 2.5 \times 3$ nm)^[15] that were first discovered in *Camelus dromedarius*.^[16] To date, they are established functional biomolecules that can be easily selected, produced, and manipulated using standard molecular biology techniques. V_HHs can be found in many antibody-based applications including the detection of different targets such as toxins, haptens, bacteria, viruses, and cellular biomarkers.^[12,15,17–20]

Despite the many advantages of nanobodies, they have never been applied in non-competitive homogeneous immunoassays, which do not require any washing or separation steps (simple mix-and-measure method) and are therefore highly important for rapid and sensitive biosensing.^[21] Förster resonance energy transfer (FRET) is one of the most sensitive and most common fluorescence-based techniques for such homogeneous immunoassays.^[21] As FRET is strongly distance dependent, the use of the small V_HHs for FRET sandwich immunoassays appears to be very advantageous. Nabiev et al. have recently developed compact QD-V_HH conjugates, which could be successfully used in flow cytometry and fluorescence immunostaining and were also proposed as promising probes for FRET-based biosensing.^[12]

Here we show for the first time that such QD-V_HH conjugates can indeed be efficiently applied for homogeneous FRET-based biosensing. We selected a challenging sandwich immunoassay, in which two V_HH bind non-competitively to different epitopes of the ectodomain of the epidermal growth factor receptor (EGFR) in order to detect soluble EGFR in serum samples. This is of high interest for the prognosis and diagnosis of cancer.^[22–24] The assay is not only challenging in the sense of high sensitivity (ng/mL limits of detection, LODs) and the measurement in serum (required for clinical diagnostics). As we use commercial QDs with a thick polyethylene glycol (PEG)-based coating (ca. 7 nm QD radius),^[25] two V_HH (ca. 4 nm length per V_HH, vide supra) and a large EGFR dimer (ca. 190 kDa; V_HHs specific against domain I and III at ca. 4 nm distance) the overall distance for FRET is expected to be larger than 10 nm (7 nm + 4 nm + 4 nm = 19 nm in the case of maximum elongation), which is beyond the distance range of conventional FRET pairs.^[26] We therefore used time-gated detection of FRET from Tb-complexes to QDs, which allows Förster distances (donor-acceptor distance for which FRET is 50% efficient) of up to 11 nm.^[27] Our assay, which we have coined “(nano)²”, is performed on a clinical fluorescence plate reader and provides sub-nanomolar (few ng/mL) LODs of soluble EGFR in 50 μ L buffer or serum samples. Our results demonstrate the suitability of QD-V_HH-based FRET for sensitive biosensing in challenging environments (serum) and the direct application to homogeneous serum-based biomarker detection in clinical diagnostics.



Scheme 1. Principle of the homogeneous (nano)²-immunoassay. The combination of Tb-labeled V_HH and V_HH-labeled QDs allows the formation of Tb-V_HH-EGFR-QD-V_HH sandwich complexes, which results in FRET from several Tb to the central QD. Time-gated detection of FRET-sensitized QD photoluminescence is used for a sensitive quantification of soluble EGFR in serum. Random labeling and orientation of the V_HH is not taken into account in the Scheme.

2. Results and Discussion

The principle of the (nano)²-immunoassay is shown in **Scheme 1**. 50 μ L of each Tb-V_HH and QD-V_HH conjugate solutions are mixed at constant concentrations to yield 100 μ L of assay solution. Two different anti-EGFR V_HH, which bind to two different non-overlapping epitopes on the ectodomain of EGFR, are used for Tb and QD. Commercial Tb-complexes (Lumi4®-Tb-NHS, Lumiphore, Inc.) and QDs (eFluor 650NC antibody conjugation kit, eBioscience) were used for bioconjugation. The broad spectral overlap of Tb-emission and QD-absorbance (**Figure 1**) leads to a large Förster distance calculated as $R_0 = 0.02108 (\kappa^2 \Phi_{Tb} n^{-4} J)^{1/6}$ nm = 11.0 ± 0.5 nm, with the FRET orientation factor $\kappa^2 = 2/3$, the Tb-centered luminescence quantum yield $\Phi_{Tb} = 0.67 \pm 0.5$, the refractive index $n = 1.35$ and the spectral overlap integral $J = \int F_{Tb} \epsilon_{QD} \lambda^4 d\lambda$ (from 450 to 700 nm). This very large

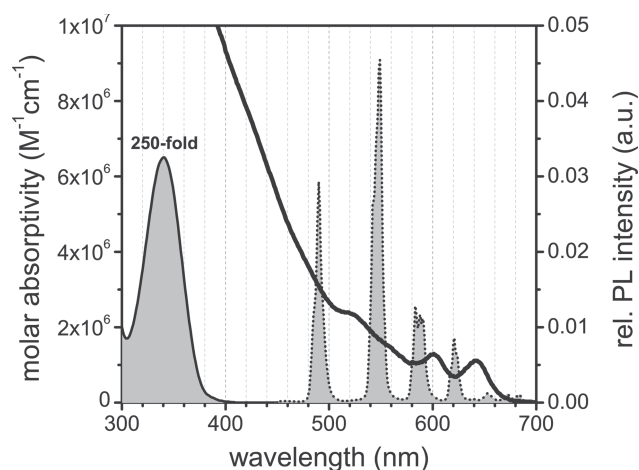


Figure 1. Molar absorptivity spectra of the Tb donor (ϵ_{Tb} , black curve with gray background, multiplied by 250) and QD acceptor (ϵ_{QD} , black curve). For the calculation of the overlap integral J between Tb-donor emission and QD-acceptor absorption, the area normalized Tb photoluminescence (PL) spectrum (F_{Tb} , black dotted curve with gray background) is also shown.

R_0 value allows for long-distance FRET beyond the common limit of ca. 10 nm, a necessary requirement for our large QD-based sandwich immunoassay system.

As a result of the small V_{HH} size, the labeling ratio of Tb per nanobody is relatively low (ca. 1.5 Tb/ V_{HH}) and the labeling ratio of nanobody per QD is relatively high (ca. 30 V_{HH} /QD). This conjugation feature has two main advantages: 1) the free V_{HH} can be easily separated from the large QDs by using convenient spin-column purification; and 2) many V_{HH} will be available on the QD surface for EGFR-binding. After addition of EGFR-containing buffer or serum samples (50 μL) to the assay solution, the specific recognition of EGFR by the two different V_{HH} leads to the formation of FRET sandwich immunocomplexes consisting of one QD and several Tb (Scheme 1, right). With so many Tb-donors surrounding, this configuration leads to an increased probability of FRET sensitization and therefore a higher FRET-sensitized photoluminescence (PL) intensity per QD.^[28]

Within the detection setup, pulsed UV light (337 nm) is applied and excites both the Tb-donors and the QD-acceptors (see Figure 1). Both fluorophores begin the process in an electronically excited state. However, the QD's PL decay is $\sim 100\,000\times$ faster than for the Tb emitters (PL lifetimes of ~ 20 ns vs ~ 2 ms). By simply delaying the collection window by 0.1 ms ($\sim 5000\times$ the QD PL lifetimes), all QDs have decayed to the ground state and no further observed QD-emission may originate from direct excitation. At the same time, more than 95 % Tb are still in their excited state after 0.1 ms (considering an exponential decay and a PL lifetime of ~ 2 ms) and efficient FRET can occur from the associated Tb donors to the QD as ground-state acceptor. Time-gated (nearly background fluorescence-free) PL of the QD-acceptor and the Tb-donor are simultaneously detected in two detection channels using transmission filters for wavelength separation (see Figure 2).

Measuring the QD-acceptor PL decays (Figure 3) and the time-gated PL intensities (integration in a time-window

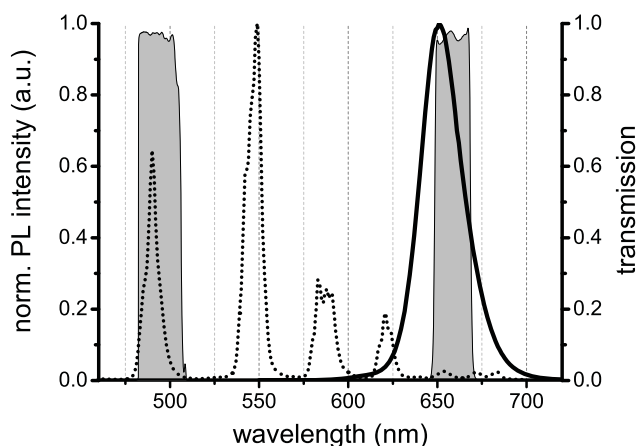


Figure 2. PL intensity spectra of Tb- V_{HH} (black dotted) and QD- V_{HH} (black) conjugates normalized to unity at their respective maxima. The gray spectra in the background define the transmission of the optical bandpass filters within the Tb (494 ± 20 nm) and QD (660 ± 13 nm) detection channels.

from 0.1 to 0.9 ms, **Figure 4**) shows significant FRET sensitization with increasing EGFR concentration. The QD-acceptor decay curves reveal some interesting aspects about the (nano)²-immunoassay, which are related to the use of V_{HH} . The mixture of Tb- V_{HH} and QD- V_{HH} (assay solution without any EGFR) already shows significant FRET. This becomes clear by comparing the thick black curve (50 μL Tb- V_{HH} + 50 μL QD- V_{HH} + 50 μL buffer, no EGFR) with the thick gray curve (mathematical sum of [50 μL Tb- V_{HH} + 100 μL buffer] and [50 μL QD- V_{HH} + 100 μL buffer], no EGFR) in Figure 3a. The gray curve contains only the pure QD component (dotted curve with a short decay time) and the pure Tb component (dotted curve with a long decay time). The black curve has a much stronger intensity (FRET-sensitization) and contains a new decay time component, which is significantly longer than the pure QD and shorter than the pure Tb component (strong evidence for FRET from Tb to QD). Although camelid V_{HH} s are known to have a higher solubility than human V_{H} fragments,^[29] our first assumption was that hydrophobic interaction or V_{HH} dimerization^[30] could lead to a partial V_{HH} aggregation and Tb-to-QD FRET when both Tb- V_{HH} and QD- V_{HH} are present in the same sample. However, addition of excess of free V_{HH} (in order to decrease the fraction of Tb- V_{HH} +QD- V_{HH} in close distance within such aggregates) and the addition of Tween-20 (in order to decrease hydrophobic interactions) did not lead to any significant changes (see Supporting Information). Another possibility of such “pre-FRET” is related to non-specific binding caused by the 0.5 % BSA in all samples. In fact, control experiments without BSA and different amounts of BSA revealed a significant influence of BSA on the “pre-FRET” signal (see Supporting Information). We assume that BSA-induced non-specific binding can bring a small fraction of Tb- V_{HH} and QD- V_{HH} in close proximity, which leads to this “pre-FRET” effect even without EGFR. As the (nano)²-immunoassay aims at biomarker detection in serum samples we retained the 0.5 % of BSA (ca. 76 μM), which saturates non-specific binding (e.g. to the walls of the microplate wells and to human serum albumin in the serum samples) and assures stable assay conditions.

Despite this “pre-FRET” in the mixture without EGFR, the addition of EGFR leads to further FRET sensitization (thin black curves in Figure 3a) showing another new decay time component. This decay time is longer than the component from the non-specific binding-induced “pre-FRET” as indicated in Figure 3b. Taking into account the smaller size of BSA (ca. 66 kDa vs. ca. 190 kDa for EGFR) and the fact that non-specific binding between V_{HH} and BSA can occur over the complete surface of the BSA (compared to the specific binding of the V_{HH} to two defined epitopes of EGFR), the longer decay time for specific EGFR binding is most probably caused by a larger distance (see **Scheme 2**). A larger distance r leads to a lower FRET efficiency η_{FRET} , which results in a longer decay time τ_{DA} [$R_0^6/(R_0^6 + r^6) = \eta_{\text{FRET}} = 1 - \tau_{\text{DA}}/\tau_{\text{D}}$]. It should be noted that the BSA concentration is ca. 7600 times higher than the V_{HH} concentrations (76 μM vs. 10 nM), which should lead to a low probability of QD- V_{HH} and Tb- V_{HH} binding. However, taking into account that the largest part of BSA will bind to the walls of the microplate

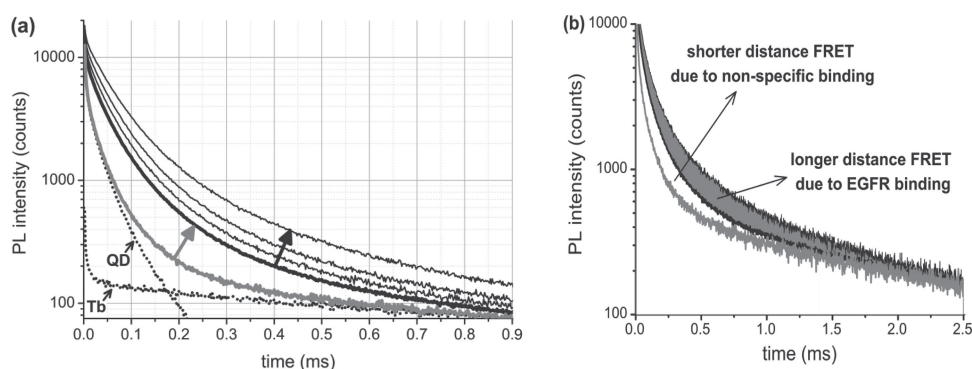


Figure 3. (a) QD detection channel PL decay curves of: gray = mathematical sum of (50 μ L Tb- V_{HH} + 100 μ L buffer, dotted Tb curve) + (50 μ L QD- V_{HH} + 100 μ L buffer, dotted QD curve); black from bottom to top = (50 μ L of 0 nM, 0.6 nM, 1.5 nM or 6 nM EGFR) + (50 μ L Tb- V_{HH}) + (50 μ L QD- V_{HH}). The gray and black arrows indicate FRET sensitization due to non-specific binding (“pre-FRET”) and EGFR binding, respectively. (b) Non-specific binding via BSA causes a faster decay (illustrated by the white area) compared to specific binding via EGFR (gray area) due to shorter Tb-QD distances for non-specific binding (see Scheme 2).

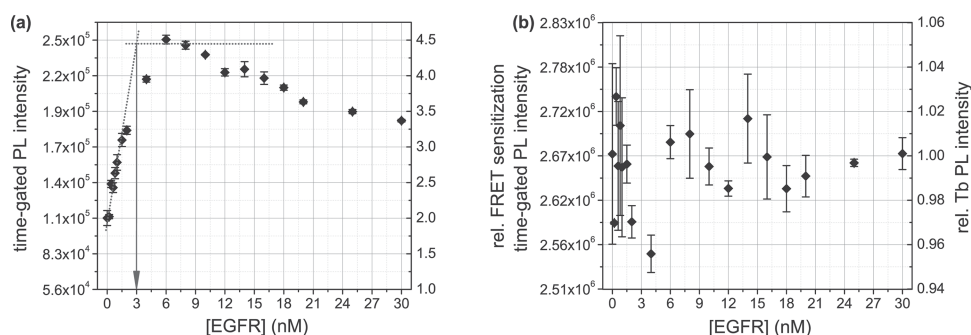


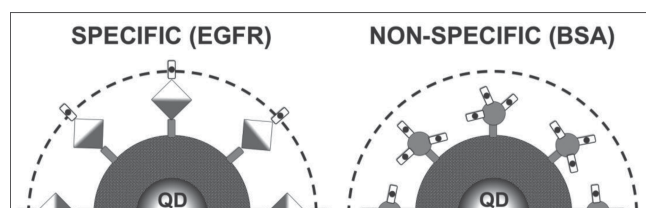
Figure 4. Time-gated PL intensity (integration from 0.1 to 0.9 ms) in the QD detection channel (a) and in the Tb detection channel (b) as a function of EGFR concentration. The QD PL shows FRET sensitization until binding saturation (indicated by the gray arrow) followed by a PL intensity decrease (hook effect). Tb-quenching is not detectable (maximum changes $\pm 6\%$ without specific direction) due to a large fraction of Tb- V_{HH} , which do not participate in FRET, and a relatively low FRET efficiency.

wells and that the “pre-FRET” sensitization is ca. 4.5-times smaller than FRET sensitization due to EGFR binding (Figure 4a), a small fraction of Tb and QD might still be brought into proximity as suggested in Scheme 2.

Although a detailed time-resolved study would be necessary to find the exact decay times of the FRET components, the FRET sensitization due to Tb- V_{HH} -EGFR-QD- V_{HH} binding displays significantly shorter decay time components (more efficient FRET) compared to sandwich assays realized

with full IgG antibodies or F(ab')₂ and F(ab) fragments, as we have demonstrated for prostate specific antigen immunoassays using the same Tb-to-QD FRET system.^[31] This more efficient FRET for the V_{HH} -based system demonstrates that the shorter nanobodies can indeed realize shorter distances within FRET immunoassays.

Another interesting aspect can be found in the time-gated QD PL intensities (Figure 4). The FRET sensitization increases linearly with EGFR concentration until it levels off at a concentration of approximately 3 nM of EGFR (in the 50 μ L sample), which corresponds to ca. 1 nM (1/3 of 150 μ L) in the total assay volume. The V_{HH} concentration is ca. 2.7 nM (1/3 of 8 nM V_{HH} in the 50 μ L Tb- V_{HH} solution) and 5 nM (1/3 of 15 nM V_{HH} in the 50 μ L QD- V_{HH} solution) for Tb and QD, respectively. Thus, the concentration of binding saturation is approximately 3 or 5 times lower than the V_{HH} concentrations. The V_{HH} concentration for the QDs was chosen higher than for Tb because there are ca. 30 V_{HH} on one QD and not all of them will be available for EGFR binding due to random orientation. Moreover, with this high labeling ratio (leading to a QD concentration of ca. 0.17 nM in the assay) it can be assumed that the large majority of QDs will take part in FRET at the saturation concentration of ca.



Scheme 2. Tb-donors (small black dots) to QD-acceptor distance comparison for specific (via EGFR, left) and non-specific (via BSA, right) binding, indicating a shorter Tb-to-QD distance for non-specific binding. Randomly oriented binding and multiple QDs per BSA (due to non-specific binding) are omitted for clarity.

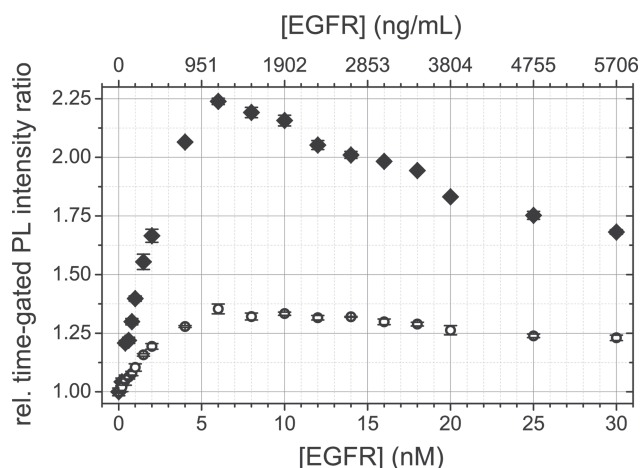


Figure 5. Calibration curves of the (nano)²-immunoassays using 50 μ L buffer (rhombi) or serum (circles) samples. The time-gated (0.1–0.9 ms) PL intensity ratios (normalized to unity for EGFR-free samples) increase with increasing EGFR concentration until the Tb-V_HH-EGFR-QD-V_HH binding is saturated and no further FRET sensitization occurs. Both curves were acquired using the same experimental conditions. The LODs (within the 50 μ L samples) are 0.12 nM (23 ng/mL or 6 fmol) and 0.18 nM (34 ng/mL or 9 fmol) for buffer and serum samples, respectively.

1 nM EGFR. On the other hand there is a large fraction (ca. 2/3) of the 2.7 nM Tb-V_HH, which do not take part in binding, possibly due to non-functional V_HH and non-specific binding. Taking into account a relatively low FRET efficiency (due to the large donor-acceptor distance) per Tb-donor on the one hand and a high fraction of Tb-V_HH per QD (see Scheme 1) on the other hand, this situation leads to minimal FRET-quenching of the Tb PL intensity (Figure 4b) but significant FRET sensitization of time-gated QD PL (Figure 4a). In fact this is one of the big advantages of measuring FRET sensitization (small increasing signal against low background) instead of FRET quenching (small decreasing signal against high background). Apart from this behavior (Figure 4) the assay is also showing the so-called hook effect, for which the FRET sensitization decreases after binding saturation because higher EGFR concentrations lead to the formation of single Tb-V_HH-EGFR or QD-V_HH-EGFR complexes and therefore less donor-acceptor pairs.

In order to evaluate the diagnostic performance of the (nano)²-immunoassay we determined the limits of detection (LODs) for soluble EGFR in buffer and serum. We therefore performed simultaneous time-gated (0.1–0.9 ms) detection of the Tb-donor and the QD-acceptor PL intensities on a KRYPTOR compact plus clinical fluorescence plate reader using 18 different EGFR concentrations ranging from 0.2 to 30 nM. The FRET ratio (time-gated PL from the QD-channel and the Tb-channel) was used for the acquisition of assay calibration curves in serum or buffer (Figure 5). Although the buffer-based measurements show a stronger FRET ratio increase compared to the serum measurements, both lead to typical immunoassay calibration curves with an increasing signal as a function of biomarker concentration followed by a decreasing FRET ratio after binding saturation (hook effect). The LODs were determined by using the

corresponding concentration of the average FRET ratio plus three times its standard deviation (30 measurements) for the EGFR-free samples. Both buffer and serum measurements resulted in sub-nanomolar LODs (0.12 nM or 23 ng/mL EGFR for buffer and 0.18 nM or 34 ng/mL EGFR for serum) within the 50 μ L samples (LODs within the total 150 μ L measuring volume are three times lower). These LODs are below the recommended cut-off level of 45 ng/mL and well in the concentration range for revealing relevant clinical information.^[23,24]

3. Conclusion

In conclusion we have demonstrated for the first time a homogeneous V_HH-based FRET immunoassay, which uses Tb-to-QD energy transfer. The (nano)²-immunoassay requires relatively short incubation times (ca. 1 h), does not need any washing and separation steps (simple mix-and-measure procedure), is measured quickly (5 s per sample) and ratiometrically (low coefficients of variation), and time-gated detection (void of autofluorescence background and directly excited QDs) allows sub-nanomolar detection limits of EGFR (in both buffer or serum), which is in a relevant clinical range of EGFR serum levels for cancer diagnostics. This proof-of-principle for very sensitive V_HH-based FRET biosensing in combination with the fast, easy and inexpensive production of ultra-small V_HH antibodies (compared to monoclonal IgG antibodies), the possibility of oriented V_HH conjugation, and the unrivaled optical properties of QDs for sensitive and multiplexed biomarker detection will make our (nano)²-immunoassay a valuable tool for future in vitro diagnostics and other FRET-based biosensing applications in imaging and spectroscopy.

4. Experimental Section

Nanobodies binding to EGFR (EgA1 and EgB4)^[32] were produced as described elsewhere.^[20] In brief, single bacterial clones were picked, grown in 2xTY containing 100 g/mL ampicillin and 0.1% (w/v) glucose and nanobody-expression was induced by the addition of IPTG. After four hours of induction, bacteria were harvested by centrifugation (2000 rpm, 15 min) and the pellet was frozen overnight at –20 °C. Cells were thawed, re-suspended in PBS and the nanobodies were purified from the periplasmic fraction by IMAC using TALON beads. The nanobodies show high affinity binding after fluorophore conjugation.^[19]

eFluor 650 Nanocrystal Conjugation Kit – Sulfhydryl Reactive (provided by eBioscience in lyophilized form) was used for QD conjugation. Prior to conjugation, protected sulfhydryl groups (N-Succinimidyl S-Acetylthioacetate) were introduced to V_HH (EgB4) at 5× molar excess using SATA kit (Thermo Fisher Scientific). The sulfhydryl groups were deprotected by diacylation according to the manufacturer's protocol. Sulfhydryl-activated V_HH solutions (in concentration excess compared to the QD solutions) were prepared in 1 × PBS and conjugated to QDs according to manufacturer's instructions. Unbound V_HH were separated by washing 3–6 times in 100 kDa molecular weight cutoff (MWCO) spin-columns (Millipore) using 100 mM sodium-tetraborate (pH

8.3) as the wash buffer. QD concentrations were determined by absorbance measurements using a molar absorptivity of $1.1 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ (at 641 nm) for QD650. $V_{\text{H}}\text{H}$ were quantified by absorbance measurements at 280 nm using a molar absorptivity of $35\,800 \text{ M}^{-1}\text{cm}^{-1}$. The labeling ratios were determined by linear combination of the respective absorbance values of QDs and $V_{\text{H}}\text{H}$ within the QD- $V_{\text{H}}\text{H}$ conjugates.

Lumi4-Tb-NHS^[33] (provided by Lumiphore in lyophilized form) was dissolved to 8 mM in anhydrous DMF and mixed (in concentration excess to the $V_{\text{H}}\text{H}$) with the $V_{\text{H}}\text{H}$ (EgA1) solutions in 100 mM carbonate buffer at pH 9.0. The mixtures were incubated rotating at 25 rpm (Intelli-Mixer, ELM) for two hours at room temperature. For Tb- $V_{\text{H}}\text{H}$ conjugate purification the samples were washed 4–6 times with 3 kDa MWCO spin-columns (Millipore) using 100 mM TRIS/Cl (pH 7.2) as the wash buffer. Tb concentrations were determined by absorbance measurements at 340 nm using a molar absorptivity of $26\,000 \text{ M}^{-1}\text{cm}^{-1}$ as provided by the manufacturer. The labeling ratios were determined by linear combination of the respective absorbance values of Tb and $V_{\text{H}}\text{H}$ within the Tb- $V_{\text{H}}\text{H}$ conjugates.

The Tb- and QD- $V_{\text{H}}\text{H}$ conjugates were each dissolved in 50 μL 10 mM TRIS/Cl-buffer (pH 7.4) containing 0.5 % bovine serum albumin (BSA, Sigma-Aldrich). The concentrations in these 50 μL solutions were approximately 8 nM $V_{\text{H}}\text{H}$ (12 nM Tb using the labeling ratio of 1.5 Tb/ $V_{\text{H}}\text{H}$) for the Tb- $V_{\text{H}}\text{H}$ and 15 nM $V_{\text{H}}\text{H}$ (0.5 nM QD using the labeling ratio of 30 $V_{\text{H}}\text{H}$ /QD) for the QD- $V_{\text{H}}\text{H}$. 50 μL buffer or serum samples containing soluble EGFR were prepared from a stock solution of 100 $\mu\text{g}/\text{mL}$ EGFR in sterile PBS (R&D Systems) by dilution with 10 mM TRIS/Cl-buffer (pH 7.4) containing 0.5 % BSA or purified new born calf serum (provided by Cezanne/Thermo Fisher Scientific) to yield the desired EGFR concentrations. These EGFR samples with different concentrations were added to the 100 μL assay solutions containing both the Tb- $V_{\text{H}}\text{H}$ and QD- $V_{\text{H}}\text{H}$ conjugates. Time-resolved and time-gated PL intensity measurements (Figures 3 and 4) were acquired on a KRYPTOR compact plus fluorescence plate reader (Cezanne/Thermo Fisher Scientific) using 475 detection bins of 2 μs integration time and nitrogen laser excitation. Optical transmission filter bandpass wavelengths were $(494 \pm 20) \text{ nm}$ (Semrock) for the Tb detection channel and $(660 \pm 13) \text{ nm}$ (Semrock) for the QD detection channel. Longer time-resolved PL intensity measurements (Figure 3b) were acquired on an EI fluorescence plate reader (Edinburgh Instruments) using 4000 detection bins of 2 μs integration time and nitrogen laser (VSL 337 ND, Spectra Physics) excitation (337.1 nm, 20 Hz). All FRET assays were measured in black 96-well microtiter plates with an optimal working volume of 150 μL . Each sample containing EGFR was prepared three times and the EGFR-free samples were prepared ten times. All samples were measured in triplicates. After sample preparation the microtiter plates were incubated for 60 min at 37 °C before measurements.

Absorption spectra (Lambda 35 UV-vis System, PerkinElmer) and emission spectra (FluoTime 300, PicoQuant) were recorded in TRIS/Cl (Sigma Aldrich) with a pH of 7.4 and sodium-tetraborate with a pH of 8.5 (Sigma Aldrich) for Tb and QD samples, respectively. PL quantum yields and Förster distances were acquired as described elsewhere.^[25] PL decay curves were acquired directly from the FRET immunoassay samples on the KRYPTOR and EI fluorescence plate readers.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgement

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