Excitable Oil Droplets - FRET Across a Liquid-Liquid Phase Boundary

L. J. Gruner,[a] L. Bahrig,[b] K. Ostermann,[a] S. G. Hickey,[b] A. Eychmüller,[b] and G. Rödel [a]*

[a] Dr. L. J. Gruner, Dr. K. Ostermann, Prof. Dr. G. Rödel
Department of Genetics TU Dresden
Zellescher Weg 20b, 01217 Dresden (Germany)

[b] Dr. L. Bahrig, Dr. S. G. Hickey, Prof. Dr. A. Eychmiiller
Department of Physical Chemistry TU Dresden
Bergstraße 66b, 01062 Dresden (Germany)

**E-mail: alexander.eychmueller@chemie.tu-dresden.de*

ABSTRACT

FRET forms the basis for energy transfer in biological systems and organisms and it has become an investigative tool in the analysis of protein-protein interactions and in the study of semiconductors (SC). Until now, FRET has been restricted to the simultaneous presence of both components in the same phase. Here, we report on the first successful prototype demonstrating interfacial FRET. This innovative FRET between inorganic SC-nanoparticles and illuminating protein chimeras takes place across an oil/water interface. As a 'proof of concept' oil droplets were stabilized by hydrophobin-derivatives in aqueous solution. These proteins possess the ability to attach fused functional domains close to an interface. Moreover, an optically active nanostructure directly docks to the hydrophobin at the oil/ water interface. Due to its modular design, this signal amplification array has the potential to be exploited in numerous fields ranging from biosensors, biotechnology to medical applications.

Keywords: Energy Transfer, FRET, nanoparticles, proteins, self-assembly

Förster Resonance Energy Transfer (FRET) is a widely used quantum phenomenon for detection and sensor application on the nanometer scale in one phase systems. Excitation energy is transferred between two fluorescent species, from a donor to an acceptor, through a dipole-dipole interaction i.e. without the emission and reabsorption of a photon. Subsequently, the emission of the donor fluorophore is quenched and the acceptor fluorophore becomes excited. Such systems are extremely sensitive to changes in distance between donor and acceptor and the effect decreases as the sixth power of the distance separating the two. In common FRET cascades the donor and acceptor molecules are located in one phase so that the distance over which FRET occurs is of the dimensions of the Förster radius.[1, 2] When FRET occurs, the fluorescence intensity of the acceptor increases while a decrease in donor fluorescence intensity and fluorescence lifetime takes place.[1] In nature FRET is a prevalent concept to guide energy between biological molecules.[3] These well known systems are also used in molecular biology for example to detect protein-protein-interactions.[4] FRET is not only possible between biological proteins or molecules, it is also possible to use quantum dots (QDs) and transfer energy between different kinds of nanoparticles.[5–7] QDs are well suited for this task because they have a broad absorption spectrum and narrow emission. Furthermore, QDs show a high photostability and can be synthesized such that they possess a high quantum yield.[8] The combination of biological molecules and QDs has already been demonstrated and hold many advantages such as size tunable absorption and emission.[9, 10] Also their inherently high photostability and brightness result in an increase in the efficiency of the FRET process. Despite their many benefits, QDs are normally synthesized in organic solvents and thus have to be transferred to the aqueous phase in order to interact with biological samples.[11, 12] The hydrophobic ligands on the surface of the QDs must be capped with hydrophilic ligands or exchanged in

an additional post-synthesis step. Unfortunately, surface defects can potentially be introduced during this procedure, with the result that the quantum yield of the QDs decreases dramatically. Also the stability and photostability of the single nanoparticles decrease in aqueous media.

To prevent losses in quantum efficiency during phase transfer we developed a highly stable and sensitive construction kit to transfer energy via FRET across a phase boundary. This innovative construction consists of a QD in the organic phase and an optically active protein in the aqueous phase. Hence, we present for the first time a FRET system with the lipophilic donor QD residing in one phase while the hydrophilic acceptor proteins are located in the aqueous phase. As opposed to systems with a FRET across lipid membranes the newly developed system infers much larger flexibility and applicability in e. g. microfluidic systems. In the present case, turbo red fluorescent protein (tRFP) was fused to a phase mediating protein, which is a surface active protein, e. g. hydrophobins (Ccg2, HFBI). In nature, these surface active proteins tend to (self-)assembly and form highly ordered and robust structures. Finally, the system consists of three tuneable components: donor, acceptor and phase mediator, which makes it truly adjustable in comparison to common surface bound FRET systems, where the protein or dye components are attached directly to the QD surface or indirect via ligand molecules.[9, 13–18] In comparison to described systems in the literature, the system presented here is more dynamic conditioned by self-organization. Advantageously, the components are kept in their favourable media maintaining their optimized properties, with the consequence that phase transfer of single components is no longer required. Additional self-healing processes at the liquid-liquid interface are operative as a result of rearrangements and flexible self-assembly.

In contrast to common protein families, hydrophobins possess distinct hydrophobic and hydrophilic patches and so they perfectly stabilize the oil-water emulsion by driven assembly at the phase boundary. Due to their hydropathy pattern, hydrophobins are predestined to serve as interfacial anchor domains at various interfaces. Moreover, interfacial adhesion of hydrophobin-based fusion proteins allows targeted surface functionalization. In the present study, the ability of these synthetic proteins to self-assemble along oil/water interfaces was exploited to generate hydrophobin-stabilized oil/water emulsions. Therefore, mature forms of class I and class II hydrophobins (Ccg2 of Neurospora crassa and HFBI of Trichoderma reesei) were labelled with red fluorescent protein tRFP to visualize hydrophobin attachment to the oil droplet 's surface. With average diameters of about 2.5 nm in aqueous solution, hydrophobins are indeed very small proteins.[19, 20] Hence, they are able to attach to the fused protein domain very close to an oil/water interface. To avoid potential interferences between two protein domains, they are separated by a randomly structured (GGGGS)2 linker. When the flexible linker is completely stretched, it reaches a maximum length of 2.4 nm.[21] Furthermore, the fused tRFP domain allows continuous photometric monitoring of the long-term stability of excitable oil bodies (EOBs). The red fluorescent protein is characterized by a high photo- and pH-stability.[22] The protein-mediator-complex is within the size limitation of the Förster radius so that in this context, it is only necessary to locate another fluorophore on the opposite side of the oil/water interface, which offers a suitable overlap in its emission or excitation spectrum. QDs, due to their tunable optical properties, are suitable candidates as they meet many of the aforementioned requirements. II–IV group semiconductors especially, have been found to have high emission quantum yields and synthetic routes for their production are well known. Cadmium selenide (CdSe) QDs and

cadmium sulfide/zinc sulfide (CdS/ZnS) core-shell QDs were used as reliable representatives of this group.

The ability of hydrophobin mediated two-phase systems to support FRET at an oil/water interface were studied. CdSe QDs localized in excitable oil bodies were surrounded by tRFP-labelled class I and class II hydrophobins in such a hybrid system.

For Förster resonance energy transfer the freely moveable CdSe QDs must come close to the oil/water interface where the immobilized tRFP domains are located. In this dynamic system FRET only occurs when the distance between both components is decreased to reach within the Förster radius. Hence, the QDs with a diameter of 1.3 nm need to be located at a distance smaller than ~6 nm to the interface as, in its wide-stretched shape, the hydrophobin tRFP fusion protein has a maximum calculated size of 6.9 nm (see Figure 1). Due to the dynamic nature of the QD component within this FRET system the QD

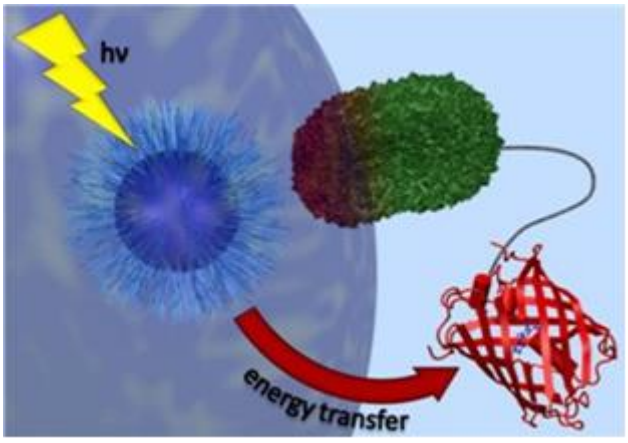

Figure 1. *Scheme of the hydrophobin mediated two–phase system to support FRET at an oil/water interface. tRFP-labelled class I and class II hydrophobins stabilize the oil/water interface and keep the tRFP localized at this position. The CdSe quantum dots are soluble in organic media and can absorb incoming light. The stabilizing ligand shell of the quantum dots has the ability to interact with the hydrophobin proteins. Below the Förster radius (˜10 nm) energy transfer from the quantum dots to the tRFP can occur.*

concentration will influence the number of QDs which fulfill the FRET conditions because of proximity effects. Additionally the stability of the EOBs is influenced by the QD/hydrophobin ratio through interactions between these components. The interaction locates the nanoparticles at the EOB interface and introduce a concentration gradient between the body of the EOB and the interface, where the interaction is stronger than the introduced force. We do not expect the QDs to penetrate through the mediator as this would quench the energy transfer to the fluorescent protein due to a collapse of the organic stabilizers in the hydrophilic phase. The energy transfer process from QDs to proteins should be detectable due to the emission spectrum of the CdSe QDs overlapping with the excitation spectrum of the tRFP as shown in Figure 2. Initially, the fluorophore components were independently localized using fluorescence microscopy. Because of their excitation/emission spectra, CdSe QDs and tRFP-labelled hydrophobins are detectable using the available DAPI or DsRed specific filters, respectively (Figure 2). The QD fluorescence is uniform and shows a homogeneous distribution of QDs in the oil phase. After a certain period of time, a pronounced fluorescent 'corona' was detectable at the oil/ water interface. That the QDs begin to accumulate at the oil/water interface may be due to their interaction with the protein chimeras. As expected the tRFP fluorescence was exclusively localized at the oil/water interface which confirms that the fusion protein containing hydrophobin and tRFP is attached to the surface of EOBs. Also shown in Figure 2 is the 'drop cap effect ' (B2.2) which is as a result of the applied EOB preparation technique. The specially adapted excitation/emission settings allow selective excitation of CdSe QDs as well as the simultaneous detection of the tRFP fluorescence emission. The fluorescence pattern of B1.3 and B2.3 images is identical to those resulting from protein specific excitation. In a control experiment without QDs (B3.3), a weak fluorescence signal can be observed.

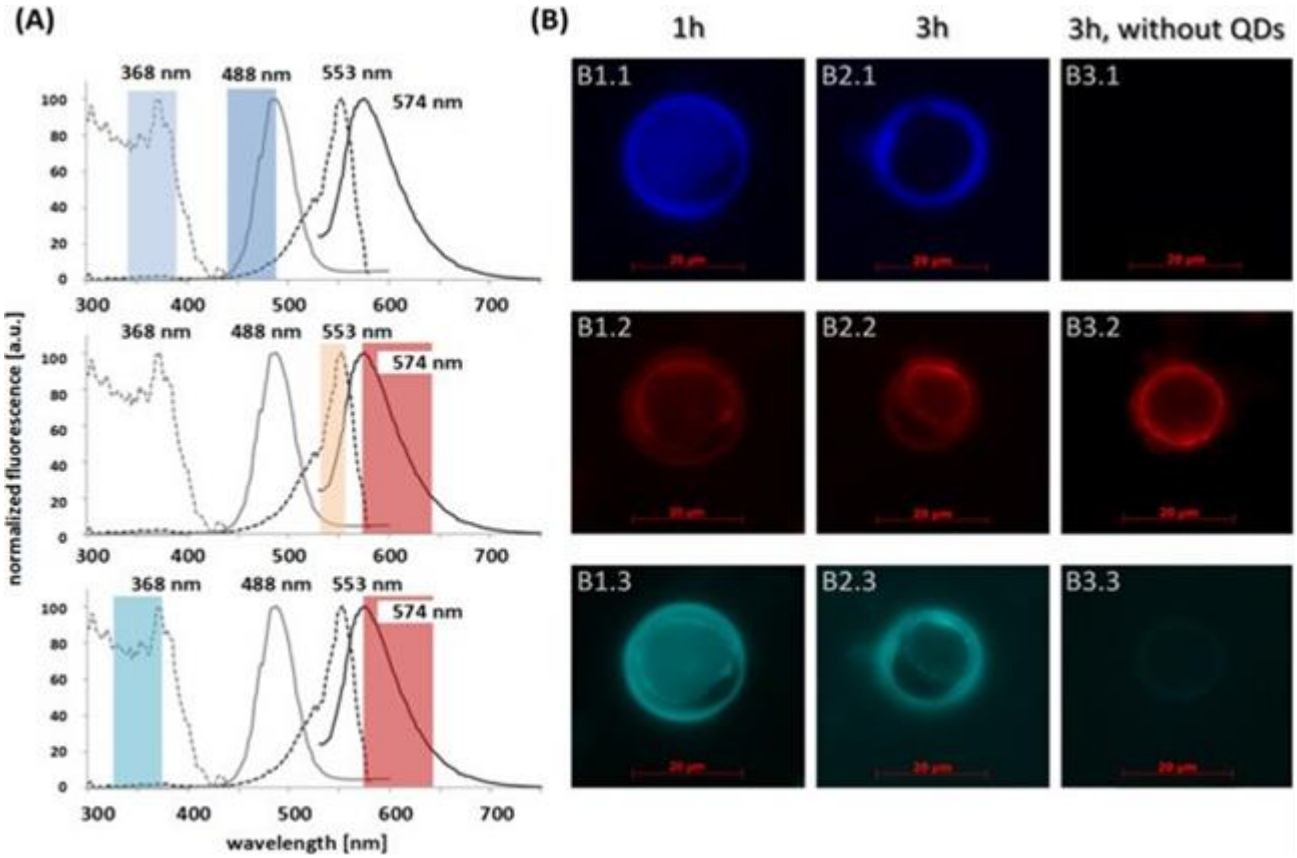

Figure 2. *Visualization of protein/QD interactions at an oil/water interface using fluorescence microscopy. (A) shows the absorption/emission spectra of the QDs (left) and proteins (right). Colored insets schematically illustrate the applied filter sets used to match the spectral excitation/emission characteristics of the CdSe QDs (upper section), tRFP (middle) and the FRET pair (lower section). (B) Images of 0.5 % (v/v) oil-in-water emulsions stabilized by Ccg2-tRFP (300 ng mL^{-1}). Samples containing QDs in the oil phase were observed after 1 h (B1) and 3 h (B2) using three different filter sets, as illustrated in (A). The bottom images are 'false-color' images. (B3.1-3.3) is the negative control with no QDs added. Samples were monitored using a Zeiss ApoTome1 confocal microscope with an exposure time of 500 ms.*

Quantitative analysis confirmed that the signal occurs as a result of weak tRFP excitation in the range of the QD excitation. Fluorescence microscopy images indicate a direct interaction between QDs and proteins which is shown by the increasing fluorescence intensity of the proteins during QD excitation.

The FRET, as presented in Figure 2, depends on a number of different parameters such as the character of the protein domain, concentrations of the protein chimera and QDs, pH-value and protein stability. The tRFP exhibits a constant fluorescence stability over the whole pH range. In combination with hydrophobins as fusion protein the fluorescence intensity decreases at lower pH (Figure S1) and additionally the fluorescence maximum is shifted to longer wavelengths. To generate protein-stabilized EOBs in the size range of 1–10 mm, the hydrophobin concentration is varied between 60 and 400 ng/ml. Depending on their hydropathy pattern, a critical hydrophobin concentration is required to induce self-assembly. Generally, above this threshold concentration, hydrophobins start to form compact, but well defined protein films which are semi-permeable to small molecules.[23–26] Only once this controlled assembly process is initiated the generation of protein stabilized oil/water spheres is possible. Time dependent fluorometric analysis with different protein concentrations was performed to determine the stability of the oil droplets (Figure 3). Generally, the fluorescence intensity of the acceptor protein increases rapidly to a concentration-dependent maximum, followed by a decline. With increasing protein concentration the presence of a steady-state becomes obvious. In addition the time frame of the stable-steady state is concentration-dependent. Furthermore, the QD concentration influences the EOB stability and the efficiency of the FRET process as is shown in Figure 4. The phenomenon of FRET manifests itself through an increase in acceptor fluorescence emission which is proportional to any increases in the donor concentration. To evaluate the FRET efficiency of the potential donor/acceptor system, hydrophobin stabilized 1-Octadecene-in-water emulsions were prepared containing equimolar concentrated solutions of tRFP-tagged hydrophobins and different CdSe QD concentrations (0.5–2.5 %(v/v)). It was

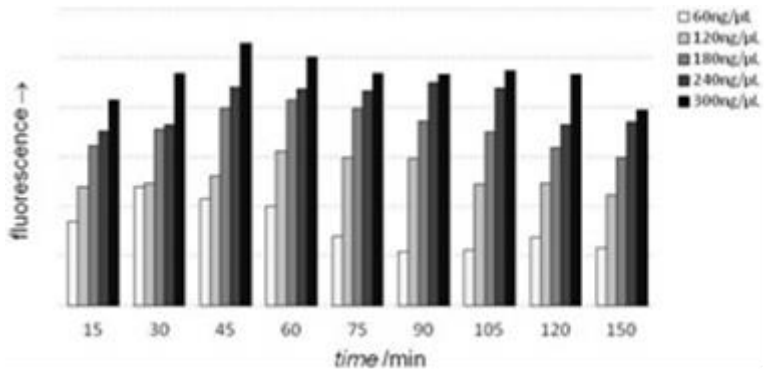

Figure 3. *Stability analysis of oil droplets at different protein concentrations. To observe the influence of the protein concentration on oil droplet stability, emulsions with different protein concentrations (in ng mL^{-1}: 60, 120, 180, 240, 300) were prepared. The oil phase contains CdSe QDs. For each sample, total fluorescence of 100 mL aliquots was determined with a microplate reader using excitation/emission wavelengths of 368/450-600 nm and a gain of 150. Graphical plots of the fluorescence maxima at 574 nm (due to acceptor) reveal time-dependent details concerning the system stability.*

observed that an increased QD concentration yielded an increase in the emission maxima of the tRFP-tagged hydrophobin. As a negative control, equimolar emulsions without QDs were prepared, which reveal the base signal. Detected fluorescence intensities of hybrid systems were usually higher than the negative control. In the first hour, fluorescence emission of the tRFP-tagged hydrophobins was discovered to increase with time and reaches a steady-state. The donor and acceptor fluorophore assemble during this period of time along the interface where, as is shown in Figure 2, it can be observed that an increased donor to acceptor ratio gives rise to higher maxima in the fluorescence intensity, as confirmed by the FRET study. However, an increased donor concentration affects the stability of the EOBs, which results in a decrease of the acceptor emission after a given period of time. It is also apparent that the stability of the EOBs suffers if too many hydrophobins are involved in the QD interactions. The interaction between QDs and hydrophobins reduces the effective area of the

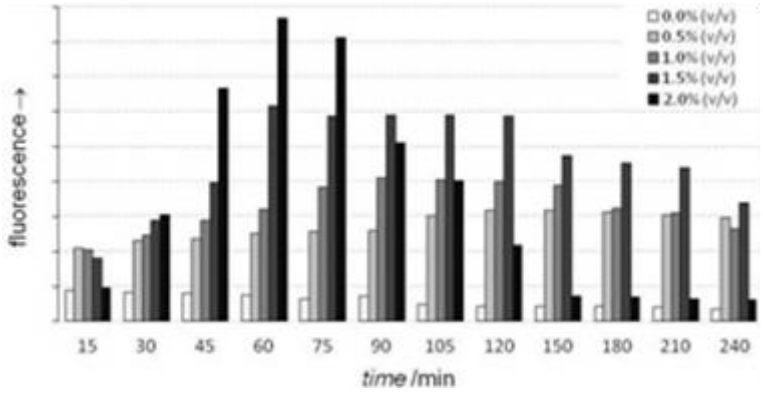

Figure 4. *Time dependent fluorescence intensity. To observe the effect of an increasing CdSe QD concentration on the fluorescence intensity of Ccg2-tRFP, equimolar protein (300 ng mL^{-1}) emulsions with different CdSe QD concentrations (in % (v/v): 0.0, 0.5, 1.0, 1.5, 2.0) were prepared. The fluorescence intensity at the emission maximum of the protein (574 nm) was plotted versus time. For each sample, the total fluorescence of 100 mL aliquots was determined using a microplate reader and excitation/emission wavelengths of 368/450-600 nm and a gain of 150. Graphical plots of the obtained fluorescence maxima at 574 nm (due to acceptor) reveal time-dependent details concerning the system stability.*

hydrophobic patch of hydrophobin resulting in an indirect destabilization of the oil/water interface. Figures 3 and 4 clearly show that the stability of the EOBs dramatically decreases at a protein concentration less than 120 ng/mL and at a QD concentration higher than about 15 % (v/v). The surface activity of hydrophobins depends on a protein self-assembly process accompanied by a conformational change of the molecules at the interface,[27] which does not appear to occur during QD interaction.

To show the existence of non-radiative energy transfer between the semiconductor QDs and the proteins, lifetime measurements were undertaken. The fluorescence lifetime is the time which is needed in order to decrease the fluorescence intensity to 1/e of the initial value. The decay of the excited state of high quality CdSe QDs normally follows a non-mono-exponential law which is in the range of nanoseconds. The relaxation process depends on

the chemical surroundings of the fluorophore hence lifetime measurements can be used to detect different interactions between the fluorophore and the chemical environment.

The as-synthesized CdSe and CdS/ZnS QDs have a high quantum yield and follow a non-monoexponential decay on the scale of nanoseconds (~ 11 ns) as it is shown in Figure 5. The QD relaxation processes increase drastically upon the addition of tRFP-tagged hydrophobin, the lifetime decreasing by a factor of 11 in magnitude, which indicates a non-radiative relaxation or energy transfer to another fluorophore. If the QDs were to act as an energy donor the emission intensity of the energy acceptor should increase. The exponential decays of the protein with and without QDs show the same results (data not shown) which indicates that the relaxation processes occurring in both are the same. However a time dependent measurement of both reaction systems shows the increased fluorescence of the protein in the presence of the QDs. The formation rate of the acceptor fluorescence is nearly the same as the degradation rate of the QD fluorescence which is an additional indication of FRET.

As a result it may be concluded that energy must be transferred from the QDs via a non-radiative process to the proteins (FRET) due to the fact that the fluorescence intensity of the protein increases while the lifetime of the QDs decreases.

The fluorescence quenching is a result of direct interactions between the quencher and the fluorophore. These effects can be dynamic, from particle collisions or static, from the formation of a ground state complex between the fluorophore and the quencher.[30]

For both the dynamic and static quenching a plot of fluorescence efficiency versus the quencher concentration shows a linear trend. In the particular case of the combination of QDs with tRFP-tagged hydrophobins we observe an upward curvature as is shown in Figure 6. This represents a mixture of interactions.

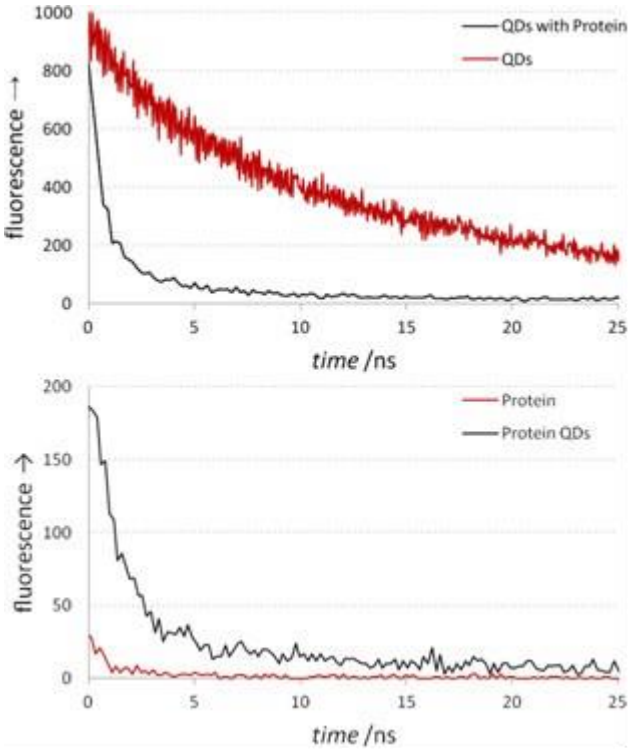

Figure 5. *Lifetime measurements. Emission decay traces of CdS/ZnS QDs (0.5 % (v/v)) with and without Ccg2-tRFP (300 ng mL^{-1}) in an oil/water emulsion (top). Lifetime of Ccg2-tRFP in the presence or absence of CdS/ZnS QDs (bottom) using a 403 nm laser for excitation.*

The diffusion-controlled dynamic quenching rate constant k_q is limited by the maximum value possible for quenching in water (~ 10^{10} L mol^{-1} s^{-1}). Smaller values are usually indicative of dynamic quenching processes while larger apparent values of k_q suggest some form of binding interaction.[29] At constant temperature (298 K), a KD value of 8.4 x 10^4 L mol^{-1} was calculated by using equation (7) and kq shows a value of 8.8 x 10^{12} L mol^{-1} s^{-1} when using formula KD/t_0.[29] Hence, the dynamic quenching rate constant implies the formation of a QD/ protein complex. According to equation (2) the apparent binding constant K_b was calculated as 1.21 x 10^{-6} L mol^{-1} and the binding site number, obtained from the slope observed in Figure 6 to be n = 2.3. The high binding constant is evidence for strong interactions between the CdSe QDs and the hydrophobin fusion proteins. Data evaluation implies the accessibility of more than one binding site on the QD surface.

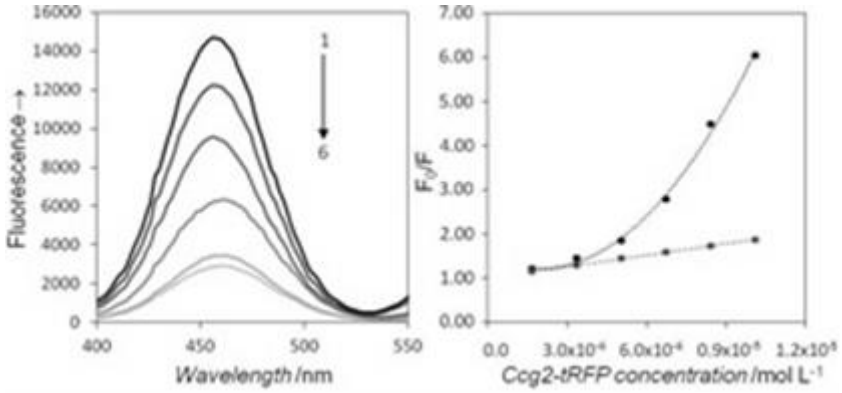

Figure 6. *Influence of HFBI-tRFP on emission spectra of CdSe QDs. Fluorescence emission spectra (left) of CdSe QD oil/water emulsions (concentration 0.5 % (v/v)) (lex = 462 nm) in the presence of different concentrations of HFBI-tRFP (in ng mL^{-1}: (1) 60, (2) 120, (3) 180, (4) 240, (5) 300 and (6) 360) at 308 K. For the steady-state quenching of CdSe QDs by HFBI-tRFP in water, quenching parameters were determined using the STERN-VOLMER plot (right).*

As previously mentioned, the EOB setup consists of three adjustable components that provide several advantages in comparison to the common setups. Applications in biological systems generally require water-soluble QDs. Therefore, a variety of techniques, often of a high degree of complexity, have had to be developed to transfer QDs into the aqueous phase. Unfortunately, this usually leads to a dramatic decrease in their fluorescence quantum yield. A phase transfer becomes unnecessary, when both donor and acceptor can be kept in their favored medium whilst at the same time fulfilling their required roles. The new setup introduced here allows the tailoring of EOBs to particular individual needs, e. g. by exchanging components to increase selectivity. Hence, tailored EOBs can be exploited as effective signal transducers for the dynamic monitoring of processes or environmental conditions (e.g. nutrition concentration, pH value or salt content). With regard to applications in bioanalytics, the possibility to interchange donor and acceptor could be applied for the in vitro quantification of micronutrients.

Another key advantage of EOBs is their long-term stability, which is necessary for near-line and long-term monitoring applications. Zampieri et al. [32] reported on the formation of highly stable hydrophobin coatings, which can be used to improve the biocompatibility of materials.[32] At the same time, encapsulation of EOBs in hydrophobin matrices inhibits the leakage of QDs from the oil phase, which is advantageous when using EOBs in an aqueous surrounding. More importantly, the toxicity of the QDs is locked away from the environment because they are highly stable in the organic media so that the risk of degradation products in the water phase tends to zero. For this reason, it is in principle conceivable to apply EOBs in biological systems, e.g. for a wide range of medical applications. Considering applications in biolabelling, this novel technique represents an efficient amplifier tool for photothermal cancer therapy[33, 34] using directed cell irradiation or focused cell heating.

Moreover, EOBs represent microstructured two-phase systems, which offer further advantages. The high surface-to-volume ratio of EOBs is advantageous in terms of high mass and heat transfer rates, as well as a narrow residence time distribution.[35] A faster system response time improves process control and product yields. Small-sized systems are also characterized by lower material and energy consumption,[36] which is important for technical applications in industry.

Development of artificial systems is an appealing strategy for producing sustainable fuels.[37] Unfortunately, biomimetic alternatives possess several disadvantages: first, synthetic electron mediators are often based on precious and water-unstable metal compounds.[38] Secondly, many synthetic materials have low electron transfer rates, which lead to relatively low efficiencies.[39] The lower the efficiency the larger the surface area required for light harvesting. EOBs can offer a promising alternative for efficient, cell-free hydrogen production. Based on artificial self-assembling peptides and proteins, size-

optimized oil-anchoring domains can provide a near-zero distance between donor and acceptor. Such customized setups enable relatively short electron transfer fluxes from reaction centres in aqueous solution to synthetic catalysts, located in the oil phase, and vice versa. Therefore, light could be harvested and concentrated by QDs of different size.[40] The captured radiant energy enables the separation of charges across the oil/water interface, whereby an excited electron is transferred to a reaction centre.[38] As an example, water could be oxidized by the accumulated positive charges of QDs and in addition the remaining negative charges of the reaction centre can reduce chemical compounds to generate sustainable fuels. Hence with such versatility and wide ranging applicability this new methodology has the potential to impart a significant and positive impact on many of today's important technological challenges.

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■ ACKNOWLEDGEMENT

This work was supported by the DFG Research Training Group ‘Nano- and Biotechniques for the Packaging of Electronic Systems’ (DFG 1401/2). We would like to express our thanks to Dr. Christian Waurisch from the Institute of Physical Chemistry, TU Dresden, Germany for support in nanoparticle synthesis.