Antibody Binding at the Liposome−Water Interface: A FRET Investigation toward a Liposome-Based Assay

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ABSTRACT: Different signal amplification strategies to improve the detection sensitivity of immunoassays have been applied which utilize enzymatic reactions, nanomaterials, or liposomes. The latter are very attractive materials for signal amplification because liposomes can be loaded with a large amount of signaling molecules, leading to a high sensitivity. In addition, liposomes can be used as a cell-like “bioscaffold” to directly test recognition schemes aiming at cell-related processes. This study demonstrates an easy and fast approach to link the novel hydrophobic optical probe based on [1,3]dioxolo[4,5-f][1,3]benzodioxole (DBD dye mm239) with tunable optical properties to hydrophilic recognition elements (e.g., antibodies) using liposomes for signal amplification and as carrier of the hydrophobic dye. The fluorescence properties of mm239 (e.g., long fluorescence lifetime, large Stokes shift, high photostability, and high quantum yield), its high hydrophobicity for efficient anchoring in liposomes, and a maleimide bioreactive group were applied in a unique combination to build a concept for the coupling of antibodies or other protein markers to liposomes (coupling to membranes can be envisaged). The concept further allowed us to avoid multiple dye labeling of the antibody. Here, anti-TAMRA-antibody (DC7-Ab) was attached to the liposomes. In proof-of-concept, steady-state as well as time-resolved fluorescence measurements (e.g., fluorescence depolarization) in combination with single molecule detection (fluorescence correlation spectroscopy, FCS) were used to analyze the binding interaction between DC7-Ab and liposomes as well as the binding of the antigen rhodamine 6G (R6G) to the antibody. Here, the Förster resonance energy transfer (FRET) between mm239 and R6G was monitored. In addition to ensemble FRET data, single-molecule FRET (PIE-FRET) experiments using pulsed interleaved excitation were used to characterize in detail the binding on a single-molecule level to avoid averaging out effects.

INTRODUCTION

Fluorescence-based techniques are the working horses in life sciences, especially when it comes to optical microscopy. Here, technical developments complemented with optimized fluorescence probes have paved the road for the investigation of many biological processes on a (sub)cellular level, e.g., using single-molecule spectroscopy or STED microscopy.1,2 In order to adapt fluorescence techniques on new topics and/or to optimize them, novel fluorescence dyes with tailored optical properties are needed. Here, [1,3]dioxolo[4,5-f][1,3]-benzodioxoles (DBD dyes) have shown many promising properties. The photophysical and chemical properties of the DBD dyes can be tuned (decay time, quantum yield, spectral position of emission maximum, as well as Stokes shift and their functional groups for bioconjugation) and can be excited by two-photon absorption, and they show a high photochemical stability.3–5 Förster resonance energy transfer (FRET) is a well-established concept to monitor the binding interaction between antigen and antibody with a very high sensitivity and is successfully applied in clinical diagnostics.6–9 DBD dyes in combination with coumarins were also characterized with respect to FRET parameters, with special emphasis on the

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orientation factor.\textsuperscript{10,11} Recently, DBD compounds have been also characterized in the context of membrane probes.\textsuperscript{10,12} Hydrophobic motifs like oligospirothioketal groups\textsuperscript{10} or alkyl chains\textsuperscript{10} were used as an anchor to the probes in membranes and vesicles. The length of the anchor was also directing the DBD compound to laterally ordered membrane domains. Due to the presence of a maleimide moiety, further biofunctionalization, e.g., to proteins, was successfully carried out.\textsuperscript{12}

For studying and understanding the interactions and signal pathways on cell membranes (e.g., of receptor−ligand interactions), liposomes are frequently used as model systems.\textsuperscript{9,13} Liposomes can also be seen as a class nanomaterial with a surface that can be designed to be biocompatible. They are adjustable in size, and more importantly for the investigation of ligand−receptor interactions their surface can be tailored (e.g., by the insertion of proteins), mimicking certain properties of biomembranes.\textsuperscript{14−16} Liposomes form small compartments (μm to subμm scale) consisting of a hydrophobic bilayer with polar surfaces on the in- and outside of the compartment. Because of these unique properties, they are used in many different analytical methodologies.\textsuperscript{17,18} In the hydrophobic bilayer, nonpolar molecules can be trapped. In contrast, inside the liposome, hydrophilic compounds can be located. The combination of hydrophilic and hydrophobic regions in a well-defined volume can be used for labeling (parts) of the liposome and for the development of assays.\textsuperscript{19,20}

A major feature of liposome-based assay formats was the potential for signal amplification because the number of markers “hosted” in the vesicle or on the surface of vesicles can be much higher than the number directly conjugated to a biomolecule (e.g., antibody). Enzyme-linked immunosorbent assays are reported with hundreds of enzyme molecules per vesicle yielding a significant signal amplification.\textsuperscript{21−24} Homogeneous as well as heterogeneous assay formats using liposomes are on hand, and often the signal enhancement is based on a liposome lysis (complement-mediated or melittin-mediated) upon biorecognition.\textsuperscript{25,26}

In our studies we used the liposomes in a 2-fold way: as a mediator phase to directly interface conjugation of hydrophobic biomolecules with hydrophobic probes and as a potential scheme for signal amplification. Here, we present the first results with respect to (i) the direct labeling of biomolecules to hydrophobic dye molecules and (ii) the proof-of-principle for a binding interaction on the surface of the liposomes. The hydrophobic part of the liposomes was used to host a hydrophobic fluorophore (DBD mm239), which subsequently was attached to an antibody. In our experiments, an antibody directed against rhodamine (DC7-Ab) was used. An advantage of using DC7-Ab is that rhodamine derivatives for labeling purposes of biomolecules are commercially available, and it has therefore a broad spectrum of possible “modified” antigens which could be disseminated in future applications. The binding interaction of rhodamine and DC7-Ab was characterized in detail before using fluorescence techniques with special emphasis on Förster resonance energy transfer (FRET) to evaluate the success of the binding.\textsuperscript{27}

## Results and Discussion

Some spectroscopic properties of the mm239-DTT in DMSO as well as immobilized in liposomes are shown in Table 1 (for mm239 only and in other solvents see ref 12). The DBD derivative is characterized by a large Stokes shift (∆λ ~ 90 ± 5 nm) and long fluorescence lifetime (τ ~ 15 ns) in both samples.

According to Figure 1 (left), before the reaction with DTT, the fluorescence intensity of mm239 dye (in liposomes) was very weak due to the quenching effect of the maleimide group, which is a result of a photoinduced electron transfer.\textsuperscript{12} After reaction with DTT, the fluorescence intensity increased significantly by a factor of about eight.

Like the fluorescence intensity, the fluorescence decay kinetics was also changed. While for mm239 in DMSO and also in liposomes a distinct quenching (biexponential decay, see Figure 1, right) was observed, upon modification with DTT the fluorescence decay became monoexponential, showing a fluorescence decay time of 15 ns in liposomes (see Table 1). The long fluorescence decay time and the large Stokes shift of about ∆λ = 90 nm in combination with the DTT for further functionalization made mm239-DTT very intriguing as an optical probe in biosensing. However, the successful incorporation of mm239-DTT into the liposome phase could not be concluded directly from the data.

### Incorporation of DBD Dye in Liposomes

Previously, the incorporation of mm239 into GUV membranes has been shown indirectly by fluorescence microscopy.\textsuperscript{12} Here, additional fluorescence depolarization experiments were performed to monitor the incorporation of dyes (and the conjugation of antibodies to liposomes, vide infra) directly. A full depolarization of the fluorescence was found in DMSO, and subsequently the steady-state fluorescence anisotropy r was calculated as zero. On the other hand, in the presence of liposomes (in PBS) the fluorescence anisotropy r increased up to r = 0.16 for mm239-DTT (Figure 2, right).

Complementary results from time-resolved fluorescence anisotropy data analysis yielded, in the case of mm239-DTT in DMSO, a fast, single-exponential decay kinetics (Figure 2, left) with a short rotational correlation time Φ = 0.3 ns, which is typical for small organic molecules in neat solvents.\textsuperscript{25} In the presence of liposomes, the rotation of mm239-DTT was distinctly slower (Figure 2, left). In the data analysis the “wobble-in-a-cone” model was used.\textsuperscript{29−31} Based on this model the rotational correlation time calculated for the anisotropy decay kinetics increased to Φ = 1.42 ns, and a semicone angle of θ = 48° was found. The increase of the steady-state anisotropy as well as the rotational correlation time of the dye in the presence of liposomes showed once more that the movement of the dye was restricted and underlined its incorporation into the lipid phase of the liposome.

### Binding of R6G (Antigen) to Liposome-Im mobilized Ab

In the next step, the binding of an antibody (Ab) to the liposome surface was investigated. In principle the maleimide group in DBD derivatives could react directly with thiol groups which are frequently present in proteins as cysteine residue under reducing conditions. However, in the case of Ab, thiols are often found in oxidized form as disulfide bridges which play an important role in keeping the tertiary structure and activity of antibodies. Direct labeling would require cleavage of

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**Table 1. Spectroscopic Properties of mm239-DTT in Liposomes**

| probe             | solvent | λ_{abs} (nm) | λ_{em} (nm) | ∆λ (nm) | r (ns) |
|-------------------|---------|--------------|-------------|---------|--------|
| mm239-DTT         | DMSO    | 370          | 456         | 86      | 14.3   |
| mm239-Liposome-DTT| PBS     | 369          | 464         | 95      | 15.4   |
disulfide bonds by using reducing agents which in some cases cause the loss of Ab activity. Instead, in this study an indirect labeling using dual functional cross-linker SMPH was applied, which also aimed to increase the efficiency of the conjugation of the Ab to the liposome because the distance to the liposome surface was enlarged this way (see Figure 7). The successful binding of the DC7-Ab to the liposome loaded with mm239-DTT was tested in fluorescence depolarization experiments (see Figure 2). For liposomes after conjugation with DC7-Ab the fluorescence anisotropy $r$ of mm239-DTT increased further up to $r = 0.19$, and the rotational correlation time was increased to $\Phi = 2.42 \text{ ns}$. The increase of both parameters is a very strong indication of DC7-Ab conjugation to the liposome surface. Only the semicone angle of $\theta_k = 52^\circ$ (calculated based on the wobble-in-a-cone model) did not change significantly. The almost unchanged cone angle indicates that the location of the dye in the lipid phase of the liposome is not altered by the attachment of the antibody, which could be an effect of the increased distance due to the cross-linker.

To show that the labeling as such and the presence of the liposome did not interfere with the function of the antibody, the binding of the respective antigen was investigated. The DC7-Ab is directed against R6G. Because of the spectral overlap between the emission spectrum of mm239-DTT and the absorption spectrum of R6G (see Figure 3), a potential FRET pair is on hand. The calculated Förster radius of $R_0 = 5.5 \text{ nm}$ is large enough to monitor the binding of R6G to DC-7-Ab bound to mm239-DTT in liposomes but on the other hand small enough to show a cross sensitivity between different liposomes of different mm239 dyes in one liposome at low to medium loading. Consequently, upon binding between the partners, FRET is expected to yield a decrease in the donor emission (quantum yield as well as fluorescence decay time) and a simultaneous increase of the acceptor emission, if the distance $R$ between mm239 and R6G in the complex is in the range of $0.5R_0 < R < 2R_0$ (with $R_0$ being the Förster radius), which should be the case for the liposomal system investigated.

Therefore, in order to monitor the binding between the mm239-DTT-DC7 and R6G, the fluorescence (intensity as well as decay kinetics) of both fluorophores was measured. The binding experiments were performed by preparing samples,
which had the same concentration of mm239-DTT-DC7-labeled liposomes but different amounts of R6G. According to the intensity spectra shown in Figure 4 (left), the intensity of the donor part (mm239 moiety) decreased when the concentration of acceptor R6G was increased, and at the same time the acceptor-related fluorescence increased. Both observations point to a FRET which proved the successful binding of DC7-Ab to the liposome surface.

Complementary to the steady-state emission spectra the fluorescence decay kinetics of the donor (mm239) and acceptor (R6G) of DC7-Ab-conjugated mm239-labeled liposomes were measured and analyzed. In Figure 4 (right) the decay kinetics of the donor and acceptor emissions are shown at different donor−acceptor ratios. The acceptor fluorescence was measured under donor-specific excitation conditions in order to obtain the energy transfer related decays. The donor fluorescence decay time decreased from about 16 ns (for DC7-Ab-conjugated mm239-labeled liposomes) to ~5 ns for the highest R6G concentration investigated (see Figure 4). At the same time the fluorescence decay kinetics of R6G became biexponential, showing a steady increase of the fluorescence decay. Because R6G was also excited directly to a small extent at the donor excitation wavelength of $\lambda_{ex} = 375$ nm, a combination of direct excited and via FRET excited emission kinetics was detected. As a trend from the analysis of the decay kinetics an increasing fraction of FRET-related R6G emission was found. For that fraction the determined decay time was $13 \pm 1$ ns, which was in agreement with the nonquenched donor fluorescence decay time and further stressed the active FRET in the liposome-based immune complex.

Single-molecule FRET experiments (PIE-FRET) were also carried out in order to avoid the averaging out effect, contribution from free donor emission, and the influence of directly excited acceptor emission, which often obscure ensemble FRET measurements yielding the wrong FRET parameters (see Figure 5). The distance between the donor and acceptor was determined to be $R \sim 5.1$ nm ($0.94R_0$).

In Table 2 the results of the ensemble-based (intensity as well as decay time related) and PIE-FRET results of the calculated FRET efficiencies are compared. For the calculation of the ensemble-related FRET efficiencies the direct excitation of the acceptor and its contribution to the signal had to be
Table 2. Comparison of FRET Efficiency Based on Different Techniques

|                | FRET efficiency (%) |
|----------------|---------------------|
| by intensity   | 64                  |
| by lifetime    | 68                  |
| by PIE-FRET    | 59                  |

The labeling of biomolecules with optical probes, that are often highly hydrophobic molecules, requires a compromise in the solvent polarity. However, many biomolecules are very sensitive to organic solvents and may be irreversibly altered (or even destroyed) in their function in organic solvents (mixtures). DBD dyes but also other classes of dyes can be used in the proposed labeling scheme without the necessity of adding additional (often) charged groups to the dyes in order to improve their water solubility. Using liposome as a mediator phase such labeling can be nicely accomplished. Here, the liposomes (or their surface) can be seen as “small” reaction vessels offering the hydrophobic environment for the probes and their hydrophilic surface for the attachment of the biomolecules. The full reaction scheme (see Figure 7) could be demonstrated step by step based on the change in the fluorescence parameters of mm239 in liposomes. First, the modification with DTT was indicated by a significant increase in the fluorescence intensity as well as the fluorescence decay time. The successful incorporation and the subsequent labeling to an Ab were demonstrated by the distinct changes of the fluorescence depolarization of the DBD dye mm239. The connection of the Ab to the surface of the liposome was also stressed by the anisotropy data. Finally, the binding activity of the Ab (when connected to the liposome) was demonstrated in FRET experiments. Here, R6G was used as antigen. Depending on the specific needs it can be envisaged that after the successful conjugation the liposomes may be destroyed, releasing the labeled biomolecules, or can be developed into a signal amplification scheme as has been shown before for different liposome-based assays.

Labeling of biomolecules often leads to the conjugation of more than one dye molecule per antibody (unless there is only one specific functional group such as R–SH per biomolecule), which then can lead to unwanted effects like self-quenching or loss of binding activity. With the proposed labeling scheme using liposomes as a mediator phase, the possibilities of multiple labelings of the biomolecule are distinctly reduced due to steric considerations, and at the same time an efficient, relatively simple strategy for signal amplification (on a single liposome level) is on hand. The assay response can be easily scaled with respect to binding sites per liposome via the loading of the vesicle with DBD dye derivative. This can be controlled, even on a single liposome level using laser fluorescence microscopy techniques. Since liposomes are “nanocontainers” the consumption of analytes/chemical can be minimized. In addition, even a multianalyte detection on one liposome can be envisaged, if different Abs are attached to the liposome surface using tailored DBD dyes, which allow an excellent tuning of the absorption/emission parameters.

Work is in progress to evaluate the sensitivity of the liposome-based assay relative to enzyme-based conventional ELISA assays. Although the experiments shown here were carried out in PBS buffer, additional experiments and fine adjustments are needed to transfer this test scheme to real-world samples.

## MATERIALS AND METHODS

DBD derivative (mm239) was synthesized by the group of Prof. P. Wessig (Institute of Chemistry, University of Potsdam). The chemical structure of the compound is depicted in Figure 6.

![Figure 6. Chemical structure of DBD derivative mm239.](image)

Succinimidyl-6-(b-maleimidopropionamido)hexanoate (SMPH), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), chloroform (CHCl₃), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany. The GC71-DC7 anti-TAMRA-antibody (DC7-Ab) was synthesized by Hybrotec GmbH, Am Mühlenberg 11, Potsdam, Germany. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and rhodamine 6G were purchased from Avanti Polar Lipids, Alabama, and Sirah Lasertechnik GmbH, Grevenbroich, Germany, respectively.

The fluorescence spectra were measured in DMSO and PBS. The experiments with samples containing antibodies were carried out in PBS. All chemicals and solvents were of analytical grade.

**Liposome Preparation.** The incorporation of mm239 into the phospholipid vesicles (liposomes) was carried out based on a rehydration technique (see Figure 7). The mixture of phospholipid and mm239 was first dissolved in CHCl₃; subsequently, CHCl₃ was evaporated by applying a gentle stream of nitrogen gas, followed by using vacuum overnight to fully remove the remaining organic solvent residues. The PBS solution was added to dry lipid film and incubated at 50 °C for 1 h. Freeze-and-thaw cycles (×5) were performed by immersion in liquid nitrogen followed by thawing in a water bath (50 °C). Finally extrusion through the 100 nm pore size polycarbonate membrane (Whatman, United Kingdom) was carried out, and homogeneous mm239-labeled large unilamellar vesicles (LUV) were obtained. The size of the liposomes was determined using dynamic light scattering (Zetasizer NanoZS, Malvern Panalytical) to 70 ± 5 nm (with a PDI of 0.13).
In order to couple DC7-Ab to liposomes, DTT was added to the mm239-labeled liposome sample solution with the molar ratio of 10:1. The mixture was then incubated for 1 h at RT, followed by removing the excess amount of DTT by using Amicon Ultra 0.5 mL centrifugal filters with a molecular weight cut off (MWCO) of 3 KDa (Merck, Germany) (centrifugation for 20 min twice).

DC7-Ab was treated with the hetero cross-linker SMPH (reaction molar ratio between DC7-Ab and SMPH = 1:20). The reaction mixture was incubated overnight at 4 °C, the NHS ester group of the SMPH cross-linkers reacted with primary amines in DC7-Ab to yield stable amide bonds. The mixture was then put in Amicon Ultra 0.5 mL centrifugal filters with MWCO of 10 kDa and centrifuged to remove excess amount of SMPH (twice for 20 min at 4 °C).

The DTT-mm239-labeled liposome solution was mixed with SMPH-labeled DC7-Ab (molar ratio 1:1) and incubated at RT for 1 h. The maleimide functional group in SMPH-labeled DC7-Ab reacted with the free thiol group on the DTT-mm239-labeled vesicle to form a thioether bond.

**Steady-State Fluorescence Spectroscopy.** The steady-state fluorescence measurements were performed using a Fluoromax 4 spectrofluorometer (Horiba Jobin Yvon, Japan) operated in the single photon counting mode. Excitation wavelengths of $\lambda_{ex} = 370$ nm were applied in the fluorescence experiments to record the emission spectra of the mm239 dye.

The fluorescence anisotropy excitation spectra were measured by exciting the mm239 with vertical polarized light, and the emission was detected with the polarizer set to vertical ($I_v$) and horizontal ($I_h$) orientation, respectively. The polarized emission was recorded at $\lambda_{em} = 470$ nm. The anisotropy $r$ was calculated according to equation (1):

$$ r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} $$  

(1)

Here, $G$ is the correction factor to take into account the different efficiencies of the optical components for vertical and horizontal polarized light. The $G$ factor value was calculated according to eq 2.

$$ G = \frac{I_{hv}}{I_{hh}} $$  

(2)

**Time-Resolved Fluorescence Depolarization.** To measure the fluorescence decay kinetics, a FL920 spectrometer (Edinburgh Instruments, UK) equipped with a multichannel plate (Europhoton, Germany) operated in the time-correlated single photon counting (TCSPC) mode was used. A laser diode (EPL-375, Edinburgh Instruments, $\lambda_{ex} = 375$ nm) with a pulse width of 55 ps and a repetition rate of 10 MHz was used as the excitation source. A supercontinuum source SC450-AOTF (Fianium, UK, $\lambda_{ex} = 520$ nm, pulse width of 50 ps, 20 MHz repetition rate) was applied for the investigation of the R6G fluorescence decay kinetics. The fluorescence decays were recorded with an emission polarizer (Glan Thompson prism, Thorlabs, Germany) set to magic angle conditions at $\lambda_{em} = 470$ nm for mm239 and at $\lambda_{em} = 580$ nm in the case of R6G. The time-resolved fluorescence anisotropy $r(t)$ was calculated based on eq 3. In the depolarization measurements photons were collected for 900 s.

$$ r(t) = \frac{I_{vv}(t) - GI_{vh}(t)}{I_{vv}(t) + 2GI_{vh}(t)} $$  

(3)

The $G$ factor was determined according to eq 4.

$$ G = \frac{I_{hv}(t)}{I_{hh}(t)} $$  

(4)

**Single-Molecule Spectroscopy (SMS).** The pulsed interleaved excitation FRET (PIE-FRET) SMS experiments were performed using a MicroTime 200 instrument.
After passing a 50 to a 200 Thermo Scientific counting mode. As the second laser source a picosecond by two avalanche photodiodes operated in the single photon excitation light by a dual band dichroic mirror (ZT 405/532rpc-UF1, AHF Analysentechnik AG, Tübingen, Germany). After passing a 50 μm pinhole, the emission light was detected by two avalanche photodiodes operated in the single photon counting mode. As the second laser source a picosecond pulsed diode laser (λex = 405 nm) was used to excite mm239. For the pulsed interleaved excitation the 532 nm laser pulse was electronically delayed by 23.5 ns with respect to the 405 nm laser pulse. The donor (mm239) and acceptor (R6G) emissions were separated using a beam splitter (FF552-Di02 – 25 × 36, Semrock, USA). Suitable bandpass filters (SP492 nm and LP560 nm in the donor and in the acceptor detection path, respectively) were inserted to further eliminate the excitation wavelength and reduce spectral crosstalk. All experiments were performed at room temperature (RT, T = 25 ± 1 °C). The data acquisition and the calculations of the autocorrelation curves were performed using the SymPhototime software (PicoQuant, Germany). All other data sets were further analyzed using Origin (OriginLab, Corp., USA). The calibration was carried out by measuring Rhodamine 6G in PBS with a diffusion coefficient of (4.14 ± 0.1) × 10−6 cm²/s at 25 °C to determine the focal volume (described by an optical plane w₀ and an optical axis z₀).

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Notes

The authors declare no competing financial interest.

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