Fluorescent Probes Hot Paper

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A Rotational BODIPY Nucleotide: An Environment-Sensitive Fluorescence-Lifetime Probe for DNA Interactions and Applications in Live-Cell Microscopy

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Abstract: *Fluorescent probes for detecting the physical properties of cellular structures have become valuable tools in life sciences. The fluorescence lifetime of molecular rotors can be used to report on variations in local molecular packing or viscosity. We used a nucleoside linked to a meso-substituted BODIPY fluorescent molecular rotor (**dC^{bdp}**) to sense changes in DNA microenvironment both in vitro and in living cells. DNA incorporating **dC^{bdp}** can respond to interactions with DNA-binding proteins and lipids by changes in the fluorescence lifetimes in the range 0.5–2.2 ns. We can directly visualize changes in the local environment of exogenous DNA during transfection of living cells. Relatively long fluorescence lifetimes and extensive contrast for detecting changes in the microenvironment together with good photostability and versatility for DNA synthesis make this probe suitable for analysis of DNA-associated processes, cellular structures, and also DNA-based nanomaterials.*

Nucleic acids are essential molecules of living systems and are also intensively applied in life sciences as tools for specific delivery, molecular recognition, and biosensing.[1] In order to study relevant processes, a variety of methods have been developed for labeling and characterization of nucleic acids, with fluorescence standing in the midpoint. Fluorescence techniques, particularly imaging, allow highly specific characterization of molecules and local environment both in vitro and in vivo. Efficient in vivo investigation of cellular structures was enabled by recent dramatic development of advanced fluorescence microscopy techniques.[2] However, all these technologies require labeled substrates with appropriate fluorescence properties.

Fluorescent nucleoside analogues (FNAs) are widely used to probe the structure and dynamics of nucleic acids and their interactions.[3,4] The majority of environment-sensitive FNAs have been developed for fluorescence-intensity-based assays.[5] Detection of fluorescence lifetime (τ) instead of intensity has many intrinsic advantages for life-science applications. In particular, the independence of the signal on the probe concentration and illumination are beneficial for imaging of living cells or complex systems.[2c,6] To date, only a few examples of FNAs for lifetime sensing have been reported.[5a] 2-Aminopurine (2AP) and cyanine-dye-based nucleosides have been used in lifetime-based assays. While 2AP is excited with short-wavelength UV light incompatible with live cells, and has very low brightness in DNA,[7] cyanine dyes with excitation in the yellow-to-red region and bright fluorescence represent more attractive probes. When attached to nucleobases, cyanines exhibit microenvironment-modulated changes in intensity and lifetime.[8] This protein-induced fluorescence enhancement (PIFE) has been used for the studies of DNA–protein interactions.[9] Rather short lifetimes (less than 2 ns) and moderate photostability of these fluorophores set limitations for lifetime measurements.

The capacity of some dyes to respond to the local environment by changing their fluorescence properties has been employed to characterize cellular structures.[10] A number of parameters such as local viscosity, polarity, or electric potential can be inspected site-selectively when environment-sensitive probes are attached to specific structures in the cell. Fluorescent molecular rotors (FMRs) were established as tools to probe local viscosity.[11] These fluorophores undergo intramolecular rotation which effectively dissipates the excitation energy. The rate of the radiationless relaxation depends on local viscosity and influences the fluorescence of the probe. FMRs covalently conjugated to biomolecules were very recently used in biomolecular assays, particularly for the studies of lipid–lipid packing,[12] detection of abasic sites in DNA,[13] and peptide–protein interactions.[14] Recently, we labeled ssDNA with an aminobenzylidene-cyanoacetamide FMR nucleoside to observe an approximate fourfold increase in fluorescence intensity upon binding of the SSB protein.[15] However, these FMRs exhibit short lifetimes,[11a] which prevent their use in advanced lifetime assays. On the other hand, introduction of a rotor exhibiting a broad dynamic range of lifetimes suitable for sensing and imaging should give a new nucleoside-like probe of high utility for studying DNA interactions. FMRs based on meso-substituted

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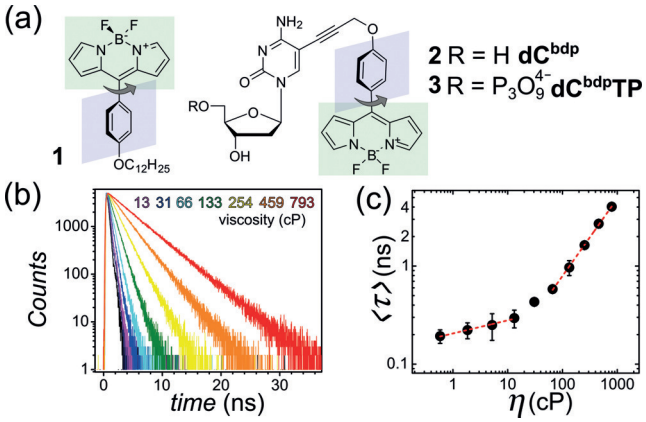

Figure 1. a) Previously reported BODIPY FMR membrane probe **1** (Ref. [12a]) and compounds studied in this work. b) Fluorescence decays of **dC^{bdp}** in solvent mixtures of different viscosities. c) Förster–Hoffmann plot; error bars show SE for $n=3$.

boron dipyrromethene (**1**, Figure 1a) exhibit excellent fluorescence properties and were successfully applied to probe local viscosity in different systems.[11a,12] Aiming to expand the benefits of these probes to the field of DNA sensing, we recently introduced nucleoside **dC^{bdp}** (**2**, Figure 1a) and showed that its triphosphate **3** is tolerated by DNA polymerases in vitro.[16] Herein we report the use of **dC^{bdp}** as a new environment-sensitive FNA for advanced fluorescence lifetime-based biophysical assays. We found that the nucleoside exhibits excellent properties for techniques such as time-correlated single photon counting (TCSPC), fluorescence correlation spectroscopy (FCS), and fluorescence lifetime imaging (FLIM). Such techniques, together with the capacity of the probe to be incorporated into DNA by polymerases,[16] and good photostability (Figure S1 in the Supporting information) can provide novel information on interactions of DNA with proteins and lipids but also lead to characterization of DNA microenvironment in a cellular context.

Sensitivity of **dC^{bdp}** to viscosity was tested by time-domain lifetime measurements in a gradient of viscosity (Figure 1b; see the Supporting Information for all experimental details). Plotting of the mean fluorescence lifetime ($\langle\tau\rangle$) versus viscosity (η) in a double-logarithmic scale, in a so-called Förster–Hoffmann plot, showed two linear correlations in regions 0.6–13 cP and 66–793 cP (Figure 1c). These results are consistent with the observations made by Kuimova and co-workers for probe **1**[12b] and indicate that **dC^{bdp}** behaves similarly to other BODIPY-based rotors. The broad range of fluorescence lifetimes (0.2 to 4 ns) underlines the excellent suitability of this probe for FLIM. We further tested the sensitivity of **dC^{bdp}** to molecular crowding, a factor which affects the behavior of nucleic acids in the cell,[17] but which has unknown effect on the lifetime of FMRs. We studied fluorescence decays of compound **3** in the presence of PEG, dextran, and bovine serum albumin (BSA) as model crowders and sucrose as a low-molecular-weight control. As a trend, the increase in lifetime of **3** does not depend on the molecular weight of the crowder (Figure S2). The largest increase was observed in the presence of BSA, where a 20-fold increase in fluorescence lifetime of **3** was caused by as little as 0.1 wt% BSA. This dramatic change was likely caused by the binding of the BODIPY rotor to a hydrophobic pocket of the protein, as was reported for other FMRs.[18] These observations suggest that the FMR nucleoside probe is more sensitive to short-distance changes in molecular environment than to mesoscopic properties of the media. These properties potentially make **dC^{bdp}** a suitable probe for DNA interactions.

Probe **dC^{bdp}TP** was used to prepare labelled DNA for further studies in vitro and in cellulo. We used in vitro primer extension (PEX)[16,19] with KOD XL DNA polymerase in the presence of **dC^{bdp}TP** and three remaining natural nucleoside triphosphates (dNTPs) to prepare 50 bp dsDNA with two modified nucleosides in the central region of the duplex, referred to here as **DNA^{bdp}** (Figure 2a and Scheme S1). The modification did not change the B conformation of the duplex as concluded from circular dichroism spectra (Figure S3). The

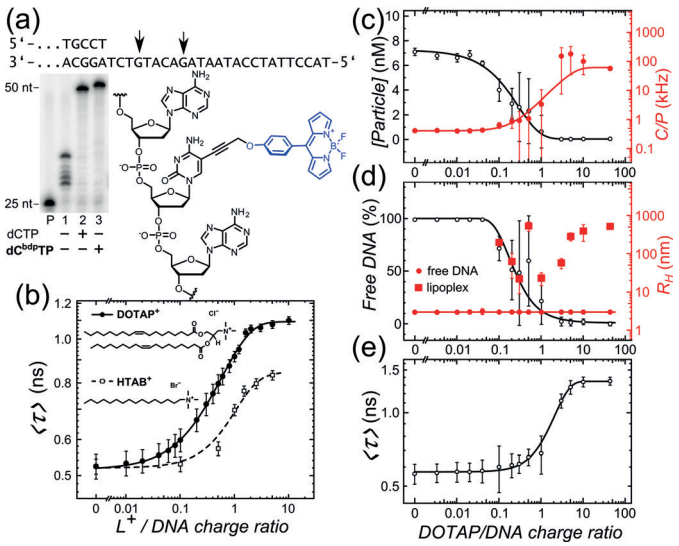

Figure 2. a) Top: Partial sequences of the template–primer complex used to prepare **DNA^{bdp}**, arrows indicate the positions for **dC^{bdp}** incorporation. Bottom left: PAGE analysis of **DNA^{bdp}** preparation; P = primer, lane 1 = reaction in the presence of dGTP, dATP, and dTTP, lane 2 = all four natural dNTPs; lane 3 = dGTP, dATP, dTTP and **dC^{bdp}TP**. Bottom right: fragment of the labeled DNA. b) Interaction of **DNA^{bdp}** with DOTAP and HTAB. Mean lifetime is plotted vs. lipid/DNA charge ratio. Results of FCS measurements: c) mean apparent particle concentration and count per particle (C/P); d) fraction of free **DNA^{bdp}** molecules, and apparent hydrodynamic radii of the particles (3D two-component model with fixed diffusion time of free **DNA^{bdp}**); e) mean fluorescence lifetime. Error bars show SE for $n=3$.

fluorescence decay of **DNA^{bdp}** in water was multiexponential, which was likely caused by a more complex anisotropic environment of the BODIPY rotor in comparison to **dC^{bdp}** in solution or a distribution of microenvironments. We also prepared a series of labeled dsDNA with different sequence and density of labeling. We found that the fluorescence decay of the FMR was best fitted with tri- or biexponential functions. All of the lifetimes components were sensitive to

the surrounding nucleoside context, multiplicity of the labeling, and to the temperature (Table S1, Figure S4). With these factors in mind, we investigated how the lifetime of a particular labelled DNA changes upon interactions with other molecules. For simplicity we further discuss only mean lifetimes.

Cationic lipids are common transfection agents in gene therapy.[20] Their tightly packed complexes with nucleic acids (lipoplexes) facilitate delivery of DNA and RNA into cells. We examined the sensitivity of **DNA^{bdp}** to its interaction with a cationic lipid (DOTAP) in the form of small liposomal vesicles. TCSPC measurements revealed a significant increase of fluorescence lifetime (from 0.5 to 1.1 ns) upon lipoplex formation (Figure 2b). The inflection point was observed at a lipid/DNA charge ratio of around 1. A similar but weaker effect was observed for cationic surfactant HTAB. The correlation between increased molecular packing and lipoplex formation was monitored by using TCSPC FCS (Figure 2c–e). A dramatic decrease in particle number with increased brightness of the particles (Figure 2c) indicated aggregation of **DNA^{bdp}** and DOTAP. The apparent hydrodynamic radius of the detected particles (Figure 2d, red) demonstrates the high variability of lipoplex sizes at the transition range (charge ratio 0.1–0.5), but stabilized at 520 ± 50 nm once lipoplex formation commenced. The final lipoplex size is in good agreement with 490 ± 50 nm obtained in FLIM experiments (see below). The increase of **DNA^{bdp}** lifetime simultaneously measured in TCSPC FCS experiments (Figure 2e) is similar to that measured in bulk TCSPC experiments (Figure 2b). The fluorescence lifetime curve (Figure 2e) was shifted to larger DOTAP/DNA charge ratios (inflection points at a charge ratio of ca. 1.1) in comparison with standard FCS parameters discussed above (Figure 2c–d, inflection points at 0.2–0.3). This behavior indicates further compaction of the lipoplexes, which was also observed by other authors at a lipid/DNA charge ratio of around 1.2,[20a] and was correlated with higher transfection efficiency. DNA should be well packed to be efficiently delivered; on the other hand, a large excess of positive charge is often toxic to cells.[21] Our results demonstrate advantages of the **dC^{bdp}** probe in combination with TCSPC/FCS for studies of lipoplex formation and precise characterization of particles used in DNA transfection and gene therapy.

We subsequently tested the impact of DNA-binding proteins on the lifetime of **DNA^{bdp}**. A previously described model system[22] comprising sequence-specific binding of the transcription factor p53 to DNA was used. An electrophoretic mobility shift assay (EMSA) showed comparable binding of the core-domain of GST-tagged p53 (**p53CD_GST**)[22c,23] to unmodified DNA and modified **DNA^{bdp}** (Figure 3A). Comparison of the lifetimes of **DNA^{bdp}** without and with **p53CD_GST** showed a dramatic increase from 0.8 to 2.1 ns (Figure 3B). Importantly, the effect was observed only in the presence of DNA-binding protein, whereas the negative control (BSA) showed only minor nonspecific binding (Figure 3C). We also observed the interactions between p53 and DNA by using the FCS setup. The apparent hydrodynamic radius of p53-bound **DNA^{bdp}** became significantly larger than the unbound **DNA^{bdp}** fraction (Figure 3D). With these

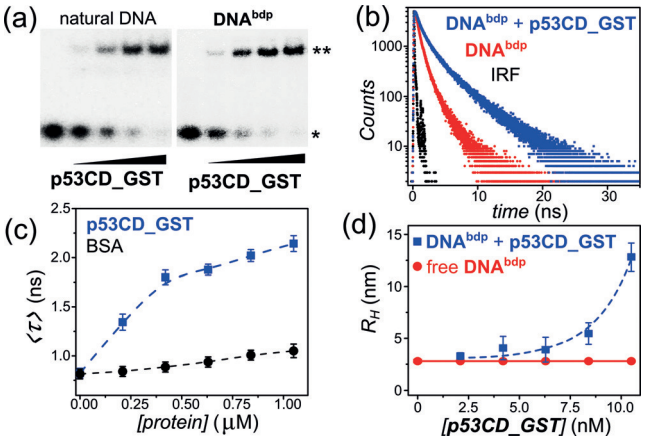

Figure 3. a) Binding of **p53CD_GST** to **DNA^{bdp}** (right) and natural control DNA (left) observed by EMSA; free (*) and p53-bound DNA (**) bands are indicated. b) Fluorescence decays of **DNA^{bdp}** before (red) and after (blue) binding to **p53CD_GST**; IRF: instrument response function. c) Changes of the mean lifetime of 0.18 μM **DNA^{bdp}** in the presence of **p53CD_GST** (blue) and BSA (black). d) FCS measurements: apparent hydrodynamic radii of the particles (3D two-component model with fixed diffusion time of free **DNA^{bdp}**). Error bars show SE for $n = 3$.

encouraging results showing the high proficiency of **dC^{bdp}** to probe interactions of DNA with lipids and proteins, we decided to examine its ability to probe the DNA microenvironment in cells.

Understanding of in vitro processes involved in forming lipoplexes helps the development of new compounds for DNA delivery into cells. Moreover, investigation of their fate in living cells provides valuable feedback for their further improvements for gene delivery and gene therapy.[20a,24] We therefore followed lipoplexes containing **DNA^{bdp}** in living human cells by using FLIM imaging. HeLa cells incubated with **DNA^{bdp}**–DOTAP lipoplexes for 30 min at 37 °C showed a wide range of lifetimes (Figure 4c, d, Figure S5), whereas no cytotoxicity was observed (Figure S6). The wider range of fluorescence lifetimes detected in cells compared to cell-free lipoplexes (Figure S5c) implies that exogenous DNA undergoes significant changes during the process of transfection.

Data acquired in living cells suggest that: 1) a relatively short lifetime is detected exclusively at the periphery of transfected cells; 2) this lifetime is shorter compared to lipoplexes that did not associate with cells (Figure 4a), thus suggesting unpacking of DNA from lipoplexes at rather early stage after association with the surface of the cell; 3) fluorescent molecules detected in the perinuclear region exhibit relatively longer lifetimes, suggesting tight molecular packing, probably because of protein binding to the probe. These data provide novel information on trafficking of exogenous DNA inside living cells emphasizing suitability of **dC^{bdp}** for in vivo studies.

To conclude, we used environment-sensitive fluorescent nucleoside **dC^{bdp}** as a lifetime probe for advanced fluorescence studies of DNA interactions in vitro and in cellulo. To the best of our knowledge, **dC^{bdp}** is the first probe for revealing the changes in the microenvironment of exogenous

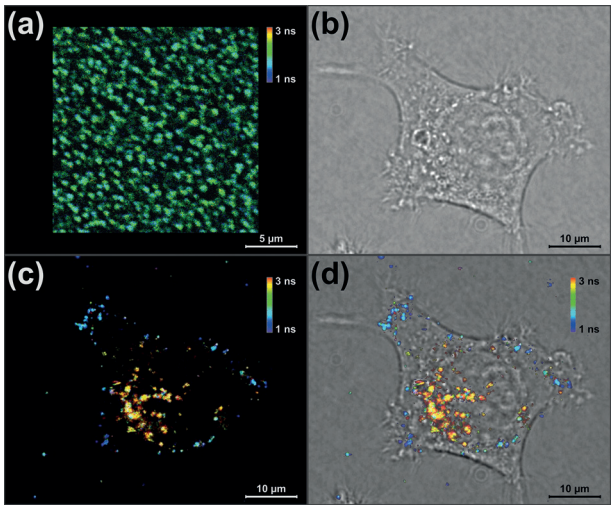

Figure 4. Transfection of HeLa cells with lipoplexes formed from 100 nM **DNA**^{bdp} and 10 µM DOTAP. a) Confocal FLIM image of the lipoplexes. Representative b) brightfield, c) confocal FLIM, and d) merged brightfield-FLIM image of a transfected cell ($n = 41$).

DNA in living cells. The provided data and detailed description of **dC**^{bdp} open new horizons of fluorescence analysis in life and material sciences. In particular, its tolerance by DNA polymerases enables metabolic labeling of endogenous DNA. This procedure would allow further investigation of local DNA microenvironment in healthy and transformed (e.g., cancer) cells. With the help of aptamers, the probe may be used for sensing local viscosity or molecular packing of potentially any cellular or synthetic structures. Finally, photostability, relatively long fluorescence lifetime, and respectable sensitivity to microenvironment, as well as its robustness in DNA synthesis make this probe a valuable tool for large-scale fluorescence applications.

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