Polymerase synthesis of DNA labelled with benzylidene cyanoacetamide-based fluorescent molecular rotors: fluorescent light-up probes for DNA-binding proteins†

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Viscosity-sensitive fluorophores, fluorescent molecular rotors based on aminobenzylidene–cyanoacetamide moiety, were tethered to 2′-deoxycytidine triphosphate via a propargylamine linker and incorporated into DNA by polymerases in primer extension, nicking enzyme amplification or PCR. DNA probes incorporating modified nucleosides show a light-up response upon binding to a protein.

Fluorescent probes targeting DNA-binding proteins are of great value for cell biology to study signaling pathways, for medicinal chemistry to develop drug screening assays, and for clinical chemistry to detect biomarkers.1 Although fluorescence spectroscopy is a powerful method for studying biomolecular interactions, the existing methods of detection of DNA–protein binding in solutions using fluorescent reporting groups suffer several limitations. Most of the current DNA-based fluorescent probes are designed for the detection of proteins exhibiting enzymatic activity.1a Examples are nuclease2 and uracil DNA glycosylase analogues3 probes based on artificial emissive nucleoside analogues that are quenched by stacking interactions with the neighbouring nucleobases within the double helix. In contrast to enzymes which modify the chemical structure of DNA, many biologically important DNA-binding proteins, i.e. transcription factors or histones, do not alter the chemical composition of DNA and thus some more sophisticated approaches are required for their detection. Several methods based on molecular beacons4,5 and environmentally sensitive fluorescent nucleoside analogues6 have been developed for this purpose. These methods usually require de novo synthesis of the probe for each particular target via solid phase phosphoramidite synthesis, which makes them cost-consuming and can limit high-throughput applications. In this respect, polymerase construction7,8 of DNA-based probes using chemically modified deoxyribose triphosphates (dNTPs) would be advantageous. In our proof-of-the-principle studies we showed that dNTPs bearing suitable environmentally sensitive fluorescent reporter (either solvatochromic aminophthalimide7 or GFP-fluorophore as a molecular rotor9) can be incorporated into DNA by polymerases. The resulting probes showed a light-up response upon binding to proteins, although the increase of fluorescence was rather low (max. 2.5-fold).7,8 Therefore, development of more sensitive fluorophores is highly desirable.

Here we report improved fluorescent dNTPs based on molecular rotors, their enzymatic incorporation into DNA and use for sensing of DNA–protein interactions. Fluorescent molecular rotors (FMRs) are a class of fluorescent dyes sensitive to local viscosity. The most widely used are p-(N,N-dialkylamino)benzylidene-malononitriles DCVJ and CCVJ (1a, 2, Fig. 1a).8,10,14 These FMRs show increased fluorescence intensity in media of high viscosity (Fig. 1b). They found applications in life sciences as fluorescent viscosity probes for microheterogeneous systems, such as cytoplasm and cell membranes,9 but the possibility to probe biomolecular interactions by emission of FMRs is only poorly explored.10 In the field of DNA studies, FMRs were used to study pre-melting of DNA11 and probing of G-quadruplexes.12 Several emissive nucleoside analogues have been shown to be sensitive to the viscosity of the media,13 but none of them was used for the probing of interactions with proteins. In this study we hypothesized that a
nucleoside analogue bearing a CCVJ-type FMR attached to a nucleobase via a short linker will be a useful probe for protein binding. CCVJ is known to increase fluorescence upon binding with tight hydrophobic sites of proteins, such as alkaloids or antibodies. Although DNA binding proteins usually do not have hydrophobic pockets, the layer of water molecules next to the protein interface (the hydration shell) exhibits retarded molecular dynamics comparing to water in the bulk. This might provide a rational basis for protein-induced fluorescence enhancement (PIFE). 

Our design of FMR-labelled nucleosides is shown in Fig. 1c. We use a short propargylic linker to connect the p(N,N-dialkyldiamo)benzylidene-malononitrile to position 5 of cytosine. Two distinct N,N-dialkyldiamo-aryl groups were used to give a julolidine derivative (dCVY) and its less bulky N,N-dimethylamino analogue (dCVDP). The FMR-nucleosides were synthesized using a modular strategy based on the Sonogashira coupling of 5-iodo-dC with the corresponding fluorophore-linked acetylene (Scheme S1 in the ESI†).

Having in hands the nucleosides we compared their photophysical and viscosity-sensitive properties with reference compounds 1a,b,17 (Fig. 2, Table 1). The introduction of a nucleoside moiety blue-shifted the absorption spectra, whereas the positions of emission maxima were not affected. The sensitivity to viscosity was measured using the Forster–Hoffmann theory,9a,c,17 stating that the logarithm of fluorescence intensity (F) depends on the logarithm of the viscosity of the media (η) as follows:

\[
\log F = x \log \eta + C
\]

where x is the viscosity sensitivity which is an intrinsic characteristic of a molecular rotor. According to the theoretical predictions, the maximum value of x is 0.66, whereas, for the vast majority of FMRs, the x value ranges from 0.4 to 0.6.17 The sensitivity to viscosity was slightly higher for the nucleosides compared to the reference, whereas this improvement was more significant in the cases of 1b and 3b (Fig. 2). Notably, the values of x for nucleosides 3a and 3b (0.630 and 0.627, respectively) are pretty close to the theoretical limit (0.66) indicating that title compounds exhibit highly favourable sensing properties.

Then we proceeded to the synthesis of DNA. For the enzymatic incorporation, nucleosides 3a and 3b were phosphorylated at 5’ using the procedure of Ludwig18 to give corresponding triphosphates 4a and 4b (Fig. 1c; Scheme S1, ESI†). We examined the possibility of incorporating the modified dNTPs into DNA by enzymatic methods, i.e. primer extension (PEX), nicking enzyme amplification reaction (NEAR) and polymerase chain reaction (PCR).6,19

At first we tested the modified dCVTPs in combination with three natural dNTPs in PEX with KOD XL and Vent(exo−) DNA polymerases (Fig. 3a). PAGE analysis of the PEX reaction (Fig. 3b) shows that dCVDPTP was a good substrate for both enzymes tested (lanes 4 and 8) and gave full-length extended products, whereas the bulky dCVJTP was less efficient, giving a number of shorter products (lanes 3 and 7). Nucleotide dCVTP was nicely incorporated into DNA even at a high density (Fig. S1, ESI†). Single-stranded oligonucleotides (ssONs) were prepared by PEX using a biotinylated template and isolated by magnetoseparation with streptavidine-coated magnetic beads (Fig. 3c). MALDI-TOF analysis of the ssON containing modified nucleotides dCA confirmed the correct full-length products (Fig. S1 in the ESI†) and UV-vis spectroscopy confirmed the absence of non-specific binding of dCVJTP to DNA (Fig. S2, ESI†).

Then, we tested the modified triphosphates in the nicking enzyme amplification reaction (NEAR),18,b,19 a two-step linear isothermal amplification process for synthesis of short ssONs (Fig. 4a). The results of NEAR with different templates (12-mer ON, containing one or three dC modifications) are shown in Fig. 4b. The NEAR works well with both modified dCVTPs, but the yield was higher in the case of less dense

![Fig. 2](image-url) The improvement of viscosity-sensitive properties of FMR-nucleoside 3b compared to 1b. (a) Absorption (EG) and uncorrected fluorescence (EG–glycerol) spectra of 1b and 3b; the viscosity of samples was (from bottom to top): 74.0, 112.2, 170.2, 258.1 and 391.4 mPa s. (b) The Forster–Hoffmann plot for nucleosides 1b and 3b.

Table 1 Photophysical properties of fluorescent molecular rotors

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_{abs} ^a (nm)</th>
<th>λ_{em} ^b (nm)</th>
<th>x^c</th>
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<tbody>
<tr>
<td>1a</td>
<td>406</td>
<td>500</td>
<td>0.594 ± 0.028</td>
</tr>
<tr>
<td>1b</td>
<td>440</td>
<td>480</td>
<td>0.554 ± 0.020</td>
</tr>
<tr>
<td>3a (dCVY)</td>
<td>452</td>
<td>506</td>
<td>0.630 ± 0.021</td>
</tr>
<tr>
<td>3b (dCVDP)</td>
<td>423</td>
<td>488</td>
<td>0.627 ± 0.022</td>
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\(^a\) Position in nm of the maximum of the absorption in EG. \(^b\) Position in nm of the maximum of the emission in EG–glycerol. \(^c\) Viscosity sensitivity obtained from the Forster–Hoffmann equation.

![Fig. 3](image-url) (a) The scheme of primer extension (PEX) with FMR-modified dCVTPs; (b) PAGE analysis of PEX with template T1X and primer P1X performed by KOD XL (lanes 1–4) and Ventexo− (lanes 5–8) polymerases; and (c) preparation of labelled ssDNA by PEX followed by magnetic separation.

![Fig. 4](image-url) (a) Schematic representation of NEAR; (b) agarose gel analysis of NEAR products; and (c) agarose gel analysis of PCR products.
labelling. Correct incorporation of modified dNTPs was also confirmed by MALDI-TOF spectrometry. The NEAR on a semi-preparative scale was also performed using dC<sup>VDTP</sup>TP followed by HPLC purification, yielding the corresponding 12-mer modified oligonucleotide in 1.9 nmol yield (0.5 mL scale). To further explore the applicability of the FMR-modified dNTPs, we tested them in PCR. A series of optimization experiments have shown that dC<sup>VDTP</sup> (but not dC<sup>V</sup>) can be sufficiently incorporated by KOD XL DNA polymerase using dC<sup>VDTP</sup> as the substrate (Fig. 4c), although it requires higher concentration of dC<sup>VDTP</sup>, increased number of cycles and longer elongation time. Altogether, these results indicate that dC<sup>VDTP</sup> is a good substrate for enzymatic synthesis of DNA using PEX, NEAR and PCR.

Finally, we examined the usability of fluorescent molecular rotors attached to DNA as reporting groups for protein–DNA interactions. Binding studies in solution were performed using a 30-mer ssDNA probe ON1 (Table S2 in the ESI†) obtained by PEX with magnetoseparation (Fig. 3c) and single-strand binding protein from E. coli (SSB), exhibiting a non-specific sequence binding to ssDNA. We observed a significant 4-fold increase of fluorescence upon titration of DNA by SSB with a stoichiometry of interaction 2 : 1 (Fig. 5). Since at least 56 nt ssDNA is needed to wrap around the SSB tetramer (Fig. 5), we titrated the ssDNA probe with PBS–glycerol, which observed a significant 4-fold increase of fluorescence upon titration of DNA by SSB with a stoichiometry of interaction 2 : 1 (Fig. 5). Since at least 56 nt ssDNA is needed to wrap around the SSB tetramer (blue) and BSA (red); (b) relative fluorescence response upon binding to protein, which make siRNA prospect for probing DNA-protein interactions under homogenous conditions.

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Notes and references