Rigid cyanine dye nucleic acid labels†‡

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Cyanine dyes attached to DNA *via* a rigid linker show useful fluorescence and FRET properties without altering the stability of duplex DNA.

Due to their attractive fluorescence properties cyanine dyes are widely used for labelling DNA. The common method for covalently linking cyanine dyes to an oligonucleotide is *via* attachment to a DNA base. An incorporated nucleobase with a reactive amine is typically coupled with an activated ester of a cyanine dye to conjugate the dye *via* an amide (Fig. 1). There are a number of reports[1–3] describing the attachment of dyes to a DNA base. All these approaches use a flexible alkane linker to attach the dye to the DNA. An important point of consideration when labelling DNA is that cyanine dyes are known to interact with DNA through non-covalent interactions.[4] For example, NMR spectroscopy has shown that a Cy3 label attached *via* a flexible linker can stack on the end of a DNA duplex.[5] Interactions between the dye and the DNA may perturb the system being studied and can also alter the fluorescent properties of the dye.

Herein we report a strategy to introduce cyanine dyes *via* a rigid ethynyl linker whose structure should prevent the interaction of the dye with the DNA and hence reduce the influence of the label on the system. A range of groups and functionalities, such as pyrene, have been incorporated into oligonucleotides *via* an ethynyl linker for a variety of purposes that have included electron transfer systems.[6] Our approach involved the synthesis of cyanine dyes with terminal ethynyl functionalities suitable for coupling to a resin bound oligonucleotide (Scheme 1). As the C5 methyl group of thymidine is orientated into the major groove upon duplex formation, attaching a dye to this position *via* a rigid linker should ensure that the dye will be held away from the DNA, reducing the likelihood of interaction, providing that Watson–Crick base pairing of the associated base is not perturbed. This rigid linker restricts the mobility of the dye to rotation around the ethynyl linker, thus reducing the conformational space available to the dye. We have introduced both Cy3 and Cy5 labels with this rigid ethynyl linker and have studied their suitability to act as a Cy3–Cy5 FRET pair.

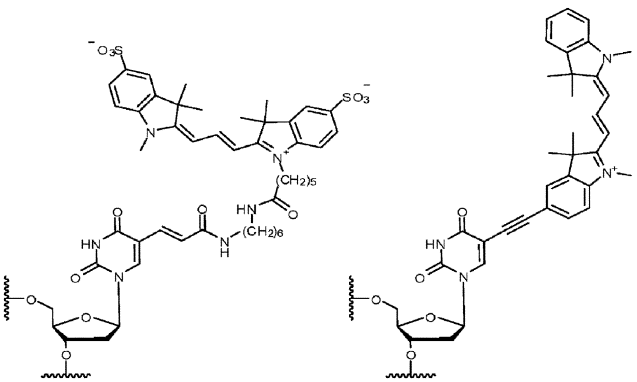
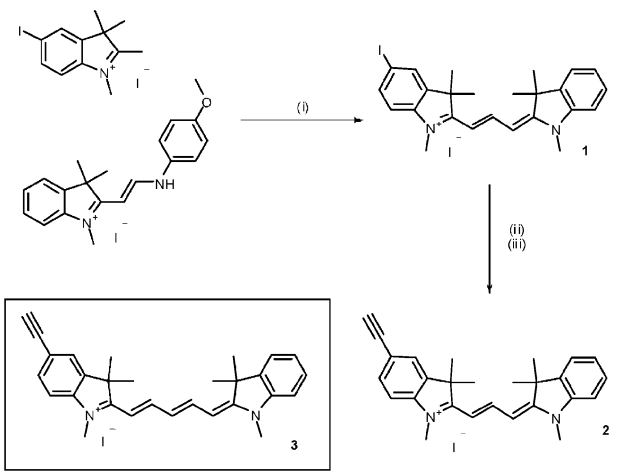


Fig. 1 Attachment of a Cy3 dye to DNA *via* a common flexible linker (left) and a rigid ethynyl linker (right).

An iodinated Cy3 dye **1** was synthesised by reaction of a hemicyanine[7] and an iodinated heterocycle (Scheme 1). The Sonogashira reaction of **1** with trimethylsilylacetylene using bis(triphenylphosphine)palladium(II) dichloride, Et_3N and copper iodide furnished the protected ethynyl dye. The silyl protecting group was removed using K_2CO_3 in MeOH–DCM at room temperature and purified to give the terminal ethynyl Cy3 dye **2**. An analogous procedure also furnished the Cy5 ethynyl dye **3** in good yields (see ESI‡). With the two dyes in hand we employed a solution phase coupling to furnish the free nucleoside–dye conjugates. Both nucleoside–dye conjugates were synthesised by cross-coupling of 5-iodo-2′-deoxyuridine with the relevant dye, using bis(triphenylphosphine)palladium(II) dichloride with Et_3N as base in the



Scheme 1 Synthesis of the ethynyl functionalised Cy3: (i) Ac_2O, pyridine, 50 °C, 5 h; (ii) trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, DMF, 50 °C, 1 h; (iii) K_2CO_3, MeOH, DCM, 20 °C, 2 h.

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† Dedicated to Professor Andrew B. Holmes on the occasion of his 65th birthday.

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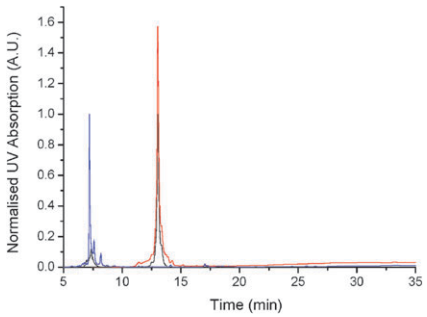

Fig. 2 HPLC trace showing overlay of HPLC traces at 260 nm for unreacted oligonucleotide (blue line) and Cy5 coupled reaction (black line). The UV absorbance at 650 nm is shown as the red line.

presence of copper(I) iodide. To introduce the ethynyl dyes into DNA a resin bound oligonucleotide containing a 5-iodo-2′-deoxyuridine at the 5′-terminus was obtained, d((IU)TA GCT CCT GAA GCG), where (IU) is 5-iodo-2′-deoxyuridine. It was produced with the 5′-O-DMTr protection on (ATDBio Ltd.). The on-resin Sonogashira cross-coupling[8] was performed following the procedure described for the incorporation of 1-ethynylpyrene.[6c] Upon cross-coupling of the ethynyl Cy5 dye **3**, the crude reaction mixture was analysed by HPLC giving two peaks: one at 7.5 min (corresponding to unreacted oligo) and one at 13 min (Fig. 2). The peak at 13 min absorbed at 650 nm as well as 260 nm, showing incorporation of a Cy5 dye. Mass spectrometry confirmed the mass for the Cy5 modified oligonucleotide, **Cy5-rigid** (ESI‡). The same procedure using **2** yielded the Cy3 modified oligonucleotide, **Cy3-rigid**. The efficiency of couplings was found to be up to 94%, as determined by the relative integration of the peaks at 260 nm for labelled and unreacted oligonucleotides for the coupling reactions, corrected for differing extinction coefficients. While this approach has used terminal modifications, an analogous chemistry[9] has been used to prepare internal and multiply labelled oligonucleotides.

To explore and compare the properties of these rigid dye DNA conjugates with more conventional labelled DNA constructs that employ flexible linkers, we acquired oligonucleotides **Cy3-flexible** and **Cy5-flexible** from a commercial supplier (IBA). The flexible linker for **Cy3-flexible** is shown in Fig. 1 (left) and the same linker was used for **Cy5-flexible**. The sequences of oligonucleotides **Cy3-rigid** and **Cy5-rigid** (prepared as described earlier) were identical to **Cy3-flexible** and **Cy5-flexible** (Table 1). The unmodified analogue of the fluorescent oligonucleotide (**Unlabelled**) was also obtained along with the complementary strand (**Comp**) (Table 1).

To study the effect of the attached dyes on the structure and stability of DNA, circular dichroism (CD) spectroscopy and UV–thermal melting analysis were carried out. The CD spectra for **Cy3-rigid:Comp** and **Cy5-rigid:Comp** duplexes (ESI‡) were found to be similar to that of the unmodified duplex, suggesting the attachment of the dyes to the nucleobases does not perturb the structure of the duplex. The thermal UV–melting temperatures, T_m, of these DNA duplexes can be determined by following UV absorbance at 260 nm, which exhibited hyperchromic melting transitions. These transitions were reversible as judged by their superimposable melting and annealing curves. The T_m of both the duplexes comprising rigid labels (57 ± 1 °C) was found to be the same as that of unlabelled duplex (56 ± 1 °C) (Table 2). This suggests that the base pairing ability of the nucleobase is not affected by the attachment of the dye with a rigid linker and furthermore that the overall stability of the duplex DNA system is not perturbed by the label. For the corresponding duplexes comprising DNA with a dye attached *via* a flexible linker, the T_m determined was 8 to 9 °C higher than that of the unlabelled duplex (Table 2), suggesting that a cyanine label with flexible linker stabilises the duplex.

Table 1 Sequences of DNA oligonucleotides

Name	Sequence (5′ to 3′)
Comp	CGC TTC AGG AGC TAA
Unlabelled	TTA GCT CCT GAA GCG
Cy3-rigid/flexible	T(**Cy3**)TA GCT CCT GAA GCG
Cy5-rigid/flexible	T(**Cy5**)TA GCT CCT GAA GCG

Table 2 T_m of unmodified and labelled duplexes[a]

Duplexes	T_m/°C
Unlabelled:Comp	56 ± 1
Cy3-rigid:Comp	57 ± 1
Cy5-rigid:Comp	57 ± 1
Cy3-flexible:Comp	64 ± 1
Cy5-flexible:Comp	65 ± 1

[a] Conditions: 2.5 μM DNA, 10 mM sodium phosphate, pH 7.0, 100 mM NaCl and 1 mM disodium EDTA.

The fluorescence emission and excitation spectra of the different dye conjugates were measured. The free dye–nucleobase conjugate and dye–DNA conjugate of rigid Cy3 dye show a 26 nm red shift in the emission maxima, whereas an 18 nm red shift was observed for the emission spectra of both the conjugates of the rigid Cy5 dye, as compared to their corresponding free iodinated dye. The excitation maxima for both rigid Cy3 and Cy5 dyes were red shifted by 14 nm for both the conjugates. The red shift may be due to the influence of geometry or electron conjugation of ethynyl linker on the cyanine moiety. In the case of labels with the flexible linker, no such red shift was observed.

We also explored the anisotropies (r) of the dye constructs. For **Cy3-rigid**, r = 0.29 ± 0.01, whereas for **Cy3-flexible**, r = 0.22 ± 0.01. For **Cy5-rigid**, r = 0.28 ± 0.02 whereas for **Cy5-flexible**, r = 0.20 ± 0.01. The lower anisotropy values for flexible constructs may reflect that the dye label is able to move freely during its fluorescence lifetime. Both the rigid dye conjugates show no change in the anisotropy upon hybridisation of a complementary strand (**Cy3-rigid:Comp**, r = 0.29 ± 0.01 and **Cy5-rigid:Comp**, r = 0.29 ± 0.01). In contrast,

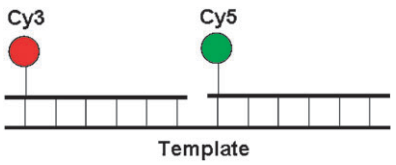

Fig. 3 Schematic of FRET system.

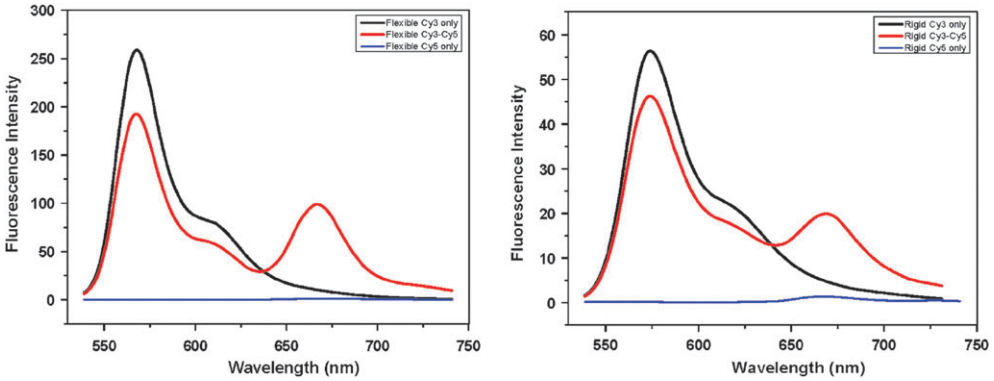

Fig. 4 Ensemble FRET spectra of (left) **Template:Cy3-flexible B:Cy5-flexible**, **Template:Cy3-flexible B**, **Template:Cy5-flexible** and (right) **Template:Cy3-rigid B:Cy5-rigid**, **Template:Cy3-rigid B**, **Template:Cy5-rigid** with excitation at 530 nm.

flexible dye conjugates show an increase in anisotropies after hybridisation of the complementary strand (**Cy3-flexible:-Comp**, $r = 0.25 \pm 0.01$ and **Cy5-flexible:Comp**, $r = 0.23 \pm 0.01$), signifying restricted dynamics of the fluorophore, suggesting the interaction of dye with DNA duplex stabilises the duplex as seen by their melting temperatures.

To assess the suitability of our new dye–DNA construct for FRET applications, we designed a system that employed a complementary 26 mer template strand (**Template**) to position the two fluorescently labelled oligonucleotides adjacent to each other (Fig. 3). The **Cy3-rigid:Cy5-rigid** system consisted of 1 : 1 : 1 equiv. of **Template:Cy3-rigid B:Cy5-rigid** (ESI‡). A system without the Cy5 label was also prepared. In the absence of the **Cy5-rigid** label (*i.e.* acceptor), excitation at 530 nm led to emission at 570 nm. In the presence of acceptor **Cy5-rigid**, reduction in the donor (**Cy3-rigid B**) emission and sensitized emission in acceptor (**Cy5-rigid**) at 670 nm were observed showing efficient FRET from the Cy3 to the Cy5 fluorophore (Fig. 4). This FRET was comparable in magnitude with the corresponding flexible dye–DNA construct (Fig. 4).

Our data suggest that rigid Cy3 and Cy5 labels in a DNA oligonucleotide do not perturb the stability or structure of a DNA duplex. In contrast, Cy3 and Cy5 linked *via* conventional flexible linkers cause the dyes to stabilise the formed duplex. The fluorescence and FRET properties of the cyanine dye labels attached to DNA *via* ethynyl linker suggest such constructs may be useful for fluorescence biophysics and assays in nucleic acid systems.

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