ChemComm

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COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 5298

Received 22nd March 2013,
Accepted 23rd April 2013

DOI: 10.1039/c3cc42103a

www.rsc.org/chemcomm

J- *vs.* H-type assembly: pentamethine cyanine (Cy5) as a near-IR chiroptical reporter†

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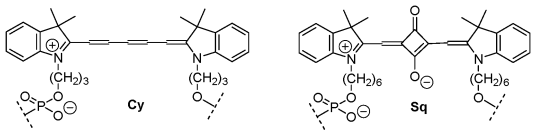
The DNA-enabled dimerization of pentamethine cyanine (Cy5) dyes was studied by optical methods. The value of cyanine as a chiroptical reporter using a monomer-to-dimer switch is demonstrated. The specific shape of the CD signal and its high intensity are a result of J-type assembly.

The use of chiroptical labels is usually not considered as a first line method for biological investigations because of their relatively low sensitivity compared to other optical techniques.[1] On the other hand, circular dichroism (CD) strongly depends on the three-dimensional arrangement of the interacting units and may, therefore, provide information not accessible by other methods. Chiroptical reporters are probes of structural information[2–4] with special value in applications of exciton-coupled CD for the characterization of complex materials, including the determination of absolute configuration.[2,5] Due to their excellent absorbance and fluorescence properties, cyanine dyes are a class of compounds that find use as sensitive labels for biology related applications, such as the study of DNA and proteins, including single molecule spectroscopy in live cells.[6–8] Herein, we demonstrate the value of cyanine dyes as chiroptical reporters in the visible to near-IR spectral range. Oligonucleotide–cyanine conjugates reveal a high susceptibility to monomer-to-dimer transitions that are observed by asymmetric exciton couplets due to J-aggregate formation.

We followed our general design of oligonucleotide conjugates with internally positioned artificial building blocks using the phosphoramidite approach (Scheme 1).[9–11] Due to the widespread interest in cyanine derivatives as labels and probes, the pentamethine cyanine (Cy5) phosphoramidite (abbreviated herein as **Cy**) is commercially available. Oligonucleotides **ON1**, **ON2** and unmodified references **ON3**, **ON4** were obtained from custom suppliers (ESI†). The recently reported squaraine (**Sq**) modified oligonucleotide **ON5**[12] served as a reference oligomer and also as a counter strand in mixed hybrid experiments.



ON1 5' AGC TCG GTC A**Cy**C GAG AGT GCA
ON2 3' TCG AGC CAG T**Cy**G CTC TCA CGT
ON3 5' AGC TCG GTC A**T**C GAG AGT GCA
ON4 3' TCG AGC CAG T**A**G CTC TCA CGT
ON5 3' TCG AGC CAG T**Sq**G CTC TCA CGT

Scheme 1 Cyanine- and squaraine-modified oligomers and reference oligonucleotides (**ON1–ON5**).

An overview of the absorbance and fluorescence data of cyanine modified oligonucleotides is presented in Table 1 and Fig. 1. Absorbance in the area up to 300 nm is mainly due to the nucleobases, whereas **Cy** and **Sq** absorption is manifested in the visible to near-IR area (500–700 nm). A key observation is the change in the shapes of the **Cy** and **Sq** spectra in the single strands (corresponding to the spectra of the monomer dyes) and those of the **Cy–Cy** and **Cy–Sq** assemblies in the hybrids. The development of a new, red-shifted absorption band of the **ON1*ON2** hybrid compared to the monomer (668 *vs.* 648 nm) is characteristic of J-aggregation of the **Cy** dyes,[13–15] a J-dimer in this case. In contrast, in the mixed assembly **Cy–Sq** (**ON1*ON5**) the major band is blue-shifted (591 nm) compared to the band of each monomer, revealing preferential H-type coupling.

Table 1 Spectroscopic properties of oligomers and hybrids (10 mM sodium phosphate buffer, pH = 7.4, 100 mM NaCl)

Sample	λ_{max}(Abs)/nm	fwhm[a]/cm^{-1}	λ_{max}(Em)/nm	Φ_F
ON1	255, 648	940	667	0.42[b]
ON2	260, 647	930	667	0.37[b]
ON5	259, 633	700	646	0.31[b]
ON1*ON2	257, 601, 668	—	668	0.039[c]
ON1*ON5	258, 591, 631	—	648	0.041[c]

[a] fwhm – full width at half maximum. [b] λ_{ex} = 600 nm. [c] λ_{ex} = 625 nm.

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† Electronic supplementary information (ESI) available: Synthetic and analytical details; additional spectra. See DOI: 10.1039/c3cc42103a

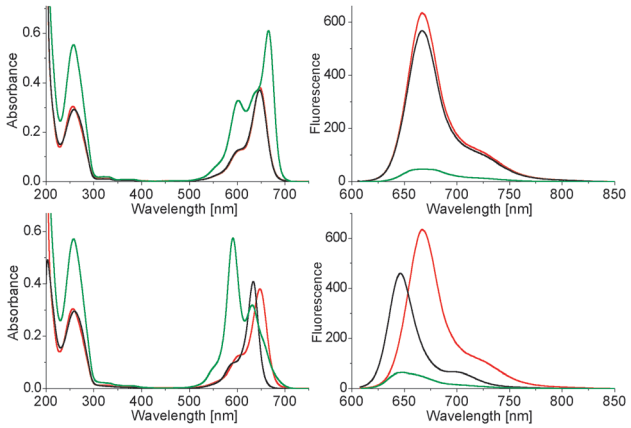

Fig. 1 Top: absorption (left) and fluorescence (right) spectra of single strands **ON1** (red) and **ON2** (black), and their hybrid **ON1*ON2** (green); bottom: absorption and fluorescence spectra of **ON1** (red), **ON5** (black) and hybrid **ON1*ON5** (green, 1.5 μM single strand conc., 10 mM phosphate, pH = 7.4, 100 mM NaCl).

Fluorescence data show that either form of assembly results in a substantial decrease in the **Cy** signal (>90%, Table 1 and Fig. 1). The excitation spectrum (ESI,† Fig. S4) has a maximum at 650 nm revealing that the remaining fluorescence signal of the **ON1*ON2** hybrid originates from a monomer type chromophore. Variable temperature absorption spectra of single strand **ON2** (ESI,† Fig. S5) disclose no specific interactions of the cyanine with other parts of the same oligomer (negligible deviations in absorbance). A decrease in fluorescence is observed with rising temperature (70% reduction, 20 → 80 °C), which is in agreement with the thermochromic behaviour described for cyanines.[16,17]

The higher tendency of cyanines to form J-type assemblies[15,17] compared to other organic molecules is well documented. H- and J-assembly of cyanines in crystals and extended aggregates on surfaces or in solution, including prolonged assemblies on DNA and other polymers as a template, has been reported.[18–22] The precise composition of J-assemblies is often ill-defined and the number of units participating in coherent J-coupling is uncertain.[15,23–25] The oligonucleotide-driven approach enables a molecularly defined constitution of the assembly. Spectroscopic changes distinctly show a transition between monomeric and dimeric forms of **Cy** molecules. The development of a J-band ($\Delta\lambda$ = 20 nm) upon formation of a **Cy**-dimer is evident upon titration of **ON1** to **ON2** (Fig. 2). More accurately, assembly of **Cy** within the **ON1*ON2** hybrid is accompanied by the development of two main bands consistent with Davydov exciton splitting[21,22] of the monomer band (M, λ_{max} = 648 nm) into two levels, at lower (λ_{max} = 668 nm, J-band) and higher (λ_{max} = 601 nm, H-band) energies, which reveals an "oblique" orientation of the chromophores.[3,26] This arrangement of the cyanines is crucial for the appearance of strong CD effects (see below).

The melting temperature (T_m) of the **ON1*ON2** hybrid (67 °C, Fig. 2) is 4 °C lower than that of the unmodified duplex **ON3*ON4** (71 °C). This is comparable to the effect of a nucleobase mismatch or an abasic site in a DNA duplex[27] and in contrast to the effects of other modifications of the same motif, such as pyrene,[28] PDI[29] or fluorene.[30] The latter modifications increase duplex stability due to stacking in a presumed face-to-face H-type fashion.

The most inspiring results came from circular dichroism (CD) experiments. The CD spectrum of the **ON1*ON2** hybrid is shown in Fig. 3 (left). A very strong intensity and a robust, specific signal shape are manifested. Several Cotton effects are evident: 668 nm ($\Delta\varepsilon$ + 250), 633 ($\Delta\varepsilon$ − 50) and 601 nm ($\Delta\varepsilon$ − 80). CD effects are absent or negligible in the corresponding single strands, which is attributed to the flexibility of the single strands. Signal shape and intensity are functions of dipolar strength of the electronic transitions and the mutual arrangement of the interacting transition dipoles, *i.e.* they depend on both, energetic and geometric factors. Usually, an exciton coupled CD of H-assembled chromophores is of symmetrical shape with positive and negative couplets of comparable intensities; it can be reasonably described by considering the main electronic transition only.[31,32] The complexity of the CD couplet in the **ON1*ON2** hybrid is due to the J-type dimer formation which leads to a substantial increase in the oscillator strength of the low energy band and its narrow character. The simulation of the observed spectrum has the best fit when the monomer band (648 nm) gives an asymmetric exciton couplet (+668 nm, −633 nm, with amplitude A = +300 and width W = 35 nm) and the other bands (601 and 556 nm) are (mono-directional) negative signals (see ESI,† S9). The CD spectrum is asymmetric in shape with a maximum that corresponds to the λ_{max} of the J-band, and a crossing point at 645 nm, which is close to the λ_{max} of the monomer absorbance. Notably, the overall integral intensity $\left(\int_{500-715nm}\right)$ of the positive (+) signal is significantly enhanced compared to the negative (−) one, $\int_{500-715(+)} \Big/ \int_{500-715(-)} = 1.4$. Non-equivalence in the intensities of exciton couplets of the main transition $\left(\int_{625-715nm}\right)$ is very pronounced and manifested in a 6-fold more intense positive couplet ($\int_{645-715(+)} \Big/ \int_{625-645(-)} = 6.2$; Fig. 3 and ESI,† S9). Quantitative characterization of signal asymmetry is possible using g-factor values,[2] which gives g_{668}/g_{633} = 4.2 and g_{668}/g_{601} = 2.6 (see ESI†).

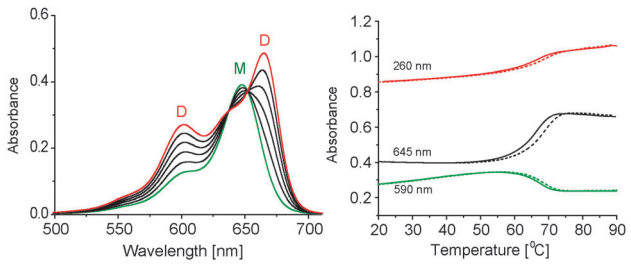

Fig. 2 Left: development of exciton splitting (J- and H-bands) upon **Cy–Cy** assembly; right: thermal denaturation of **ON1*ON2**; cooling (–), heating (---); (T_m = 67 °C; M: Cy monomer, D: Cy dimer); single strand conc. 1.5 μM, conditions: see Fig. 1.

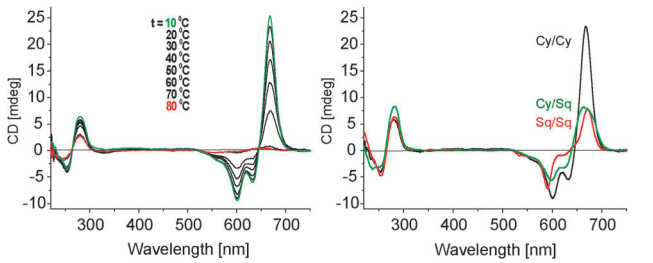

Fig. 3 Left: CD spectra of the **ON1*ON2** hybrid in the temperature range 10–80 °C; right: CD spectra of different assemblies (20 °C); single strand conc. 1.5 μM, conditions: see Fig. 1.

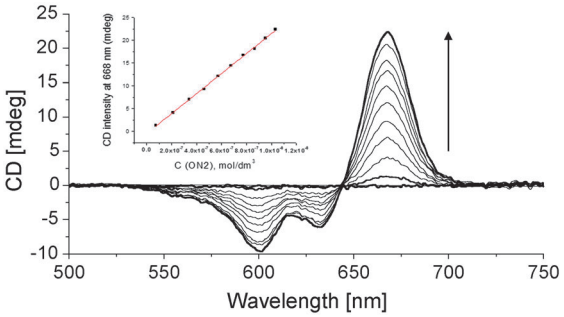

Fig. 4 Duplex formation by stepwise addition (arrow) of **ON2** to **ON1** (starting conc. 1.5 μM); first addition of **ON2**: 7.4×10^{-8} M. Inset: concentration dependence of signal intensity at 668 nm.

The **Sq** dye (Scheme 1) has similar optical properties to **Cy** (λ_{maxCy} = 645 nm, ε_{Cy} = 250 000 M^{-1} cm^{-1}; λ_{maxSq} = 635 nm, ε_{Sq} = 258 000 M^{-1} cm^{-1}) and was used as a reference compound and a counterpart in coupling experiments with non-identical chromophores. **Sq–Sq** excitonic interactions in the corresponding DNA hybrids[12] show a significantly more populated transition at higher energy (H-aggregation) and a symmetrical CD signal (Fig. 3, right). Non-degenerate interaction of **Cy–Sq** within **ON1*ON5** also results in a higher fraction of H-type coupling (λ_{max} = 591 nm, see absorption changes, Fig. 1) and gives a CD of complex shape with an overall broadening of bands. Most significantly, the CD signal intensity of the low energy band of **Cy–Sq** is at the level of that of **Sq–Sq** (H-type coupling,[12] Fig. 3, right), but is significantly lower than that observed for **Cy–Cy** (J-type) coupling. In absolute values, the signal intensity (I) of the **Cy–Cy** pair at 668 nm was 3.5 times higher than that of 20 natural oligonucleotide base pairs (bp) at 280 nm leading to a ratio $I_{CyCy}/I_{bp} \cong 70$. Semi-quantitative comparison of chiroptical labels in a DNA framework can be performed. Since the CD spectrum shows an averaged signal in the DNA region, the value per base pair should be approximately the same for a B-DNA of any given length, composition and base sequence. A value of $\Delta\varepsilon_{280}$ = 3–4 M^{-1} cm^{-1} per base-pair is commonly observed for a B-type DNA under physiological conditions.[33] Our data are consistent with this value: $\Delta\varepsilon_{280}$(B-DNA) ~ $\Delta\varepsilon_{280}$(**ON1*ON2**) ~ $\Delta\varepsilon_{280}$(**ON3*ON4**) ~ 3.5 M^{-1} cm^{-1} per base pair is found, which gives $\Delta\varepsilon_{668}$(**Cy–Cy**)/$\Delta\varepsilon_{280}$(bp) ≅ 70. This ratio can be used as a rough but useful value for a first approximation in comparison of CD data of modified oligonucleotides that are increasingly appearing,[34–44] and as a reference value for the design of DNA-based chiroptical reporters.[4,5] The remarkably high sensitivity of the **Cy** chiroptical label is further demonstrated by the titration experiment shown in Fig. 4: a 7.4×10^{-8} M oligonucleotide concentration is easily detected in a standard 1 cm cell (see ESI†).

In conclusion, using a derivative of the widely used chromophore Cy5, we have demonstrated the value of cyanine dyes as chiroptical reporters. **Cy–Cy** interaction leads to intense CD effects in a DNA duplex. The origin of the high signal intensity resides in the formation of a J-type dimeric assembly that exhibits an asymmetric exciton coupled CD signal with a significant enhancement of one of the couplets. This type of aggregation is well known for cyanine dyes,[16–20] however, to the best of our knowledge, was not applied so far in the design of chiroptical labels using a monomer-to-dimer switch.

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