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Cy3 and Cy5 dyes attached to oligonucleotide terminus stabilize DNA duplexes: Predictive thermodynamic model

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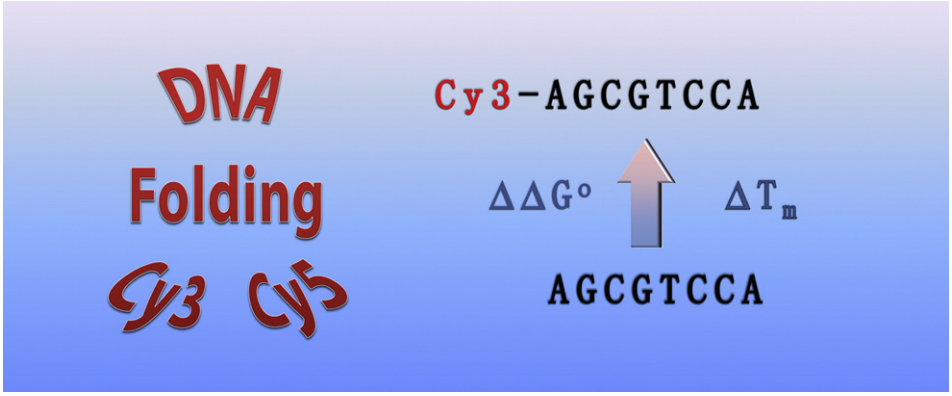
HIGHLIGHTS

- Cy3 and Cy5 dyes at termini stabilize DNA duplexes.
- Duplex stability is increased by 1.2 kcal/mol on average.
- The magnitude of stabilization depends on the base sequence.
- The dyes show larger thermodynamic effects than dangling nucleotides.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanine dyes are important chemical modifications of oligonucleotides exhibiting intensive and stable fluorescence at visible light wavelengths. When Cy3 or Cy5 dye is attached to 5′ end of a DNA duplex, the dye stacks on the terminal base pair and stabilizes the duplex. Using optical melting experiments, we have determined thermodynamic parameters that can predict the effects of the dyes on duplex stability quantitatively (ΔG°, T_m). Both Cy dyes enhance duplex formation by 1.2 kcal/mol on average, however, this Gibbs energy contribution is sequence-dependent. If the Cy5 is attached to a pyrimidine nucleotide of pyrimidine–purine base pair, the stabilization is larger compared to the attachment to a purine nucleotide. This is likely due to increased stacking interactions of the dye to the purine of the complementary strand. Dangling (unpaired) nucleotides at duplex terminus are also known to enhance duplex stability. Stabilization originated from the Cy dyes is significantly larger than the stabilization due to the presence of dangling nucleotides. If both the dangling base and Cy3 are present, their thermodynamic contributions are approximately additive. New thermodynamic parameters improve predictions of duplex folding, which will help design oligonucleotide sequences for biophysical, biological, engineering, and nanotechnology applications.

Abbreviations: C_t, total single strand concentration; Cy, Cy3 or Cy5 dye; ΔG°_{37}, transition Gibbs free energy at 37 °C; ΔH°, transition enthalpy; ΔS°, transition entropy; T_m, melting temperature, SVD, singular value decomposition

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1 . Introduction

Many biophysical and biological studies use oligonucleotides that contain additional chemical moieties attached to either 5′ or 3′ terminus. These moieties provide DNA oligomers with useful and unique properties. Cy dyes are one important class of such modifications. The dyes have sharp absorption bands, high extinction coefficients, excellent resistance to photobleaching and make DNA oligomers highly fluorescent, so that even single molecules can be observed [1,2]. Their chemical structure is built from two indole rings that are connected by a polymethine chain (Fig. 1). Cy3 dye (1,1′-bis(3-hydroxypropyl)-3,3,3′,3′-tetramethylindo carbocyanine) contains a connecting chain of *three* methine groups. The chain consists of *five* methine groups in Cy5 dye (1,1′-bis(3-hydroxypropyl)-3,3,3′,3′-tetramethylindodicarbocyanine).

In the case of oligonucleotides, the dyes are often linked to the ribose at 5′ terminus. In our experiments, the covalent linker consisted of 5′ phosphate group and the propyl chain that was connected to indole ring. Cyanine-modified oligonucleotides have been used in numerous studies of composition, structure and dynamics of nucleic acids including genotyping [3–5], real-time PCR [4,6,7], gene expression, microarray experiments [8,9], molecular beacons [10], *in situ* hybridizations [11], single molecule dynamics investigations [1,12,13], nanoparticles [8,14], optical switches for data storage [15], enzyme kinetics [16] and protein–nucleic acid interactions [17]. Typically, annealing of the probe to its target alters the fluorescence properties of the cyanine-labeled oligonucleotide. This could be accomplished by various schemes of dye quenching [4,18]. Another set of applications relies on Förster resonance energy transfer (FRET) between pair of dyes, allowing measurements of distances on nanometer scale [19]. For example, pairing of Cy3 with Cy5 is favored in single molecule studies [20]. Many aspects of Cy3 and Cy5 *fluorescent* properties have been investigated [2,21–23]. Levitus and Ranjit recently reviewed cyanine dye photophysics [24]. Our interest is focused on the energetics of molecular interactions.

Design of experiments and applications relies on predictions of melting temperatures (T_m) and Gibbs energies (ΔG°) of hybridization. The nearest-neighbor thermodynamic model has been very successful in predicting energetics and extent of hybridization for native DNA [25,26] and RNA duplexes [27,28]. Additional chemical groups can significantly affect duplex stability and melting temperature. We have observed that many dyes and quenchers attached to duplex terminus increase T_m values [29]. Cy dyes appear to stabilize DNA duplexes most from a group of seven common fluorescent dyes [29].

It has not been possible to model the dye effects accurately because sequence specificity of the stabilization has not been studied. We therefore measured and determined sequence-dependent thermodynamic parameters for Cy3 and Cy5 dyes attached to a terminal nucleotide. New parameters are compatible with the published nearest-neighbor model of DNA duplexes [26] and improve accuracy of T_m and ΔG° predictions. The results are important not only for oligonucleotide design, but also for interpretation of structure, dynamics and photophysics of Cy-modified nucleic acid molecules.

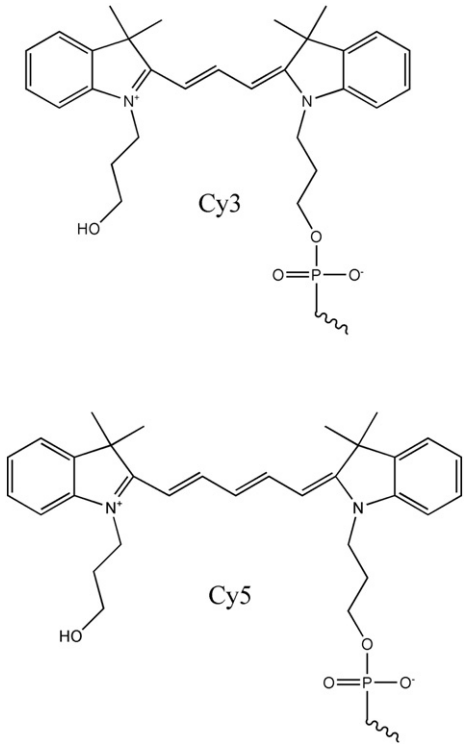

Fig. 1. Chemical structures of cyanine dyes and their attachment to 5′ terminus of oligonucleotides *via* phosphate group.

2. Materials and methods

2.1. Oligonucleotide synthesis

Oligodeoxynucleotides were chemically synthesized using phosphoramidites at Integrated DNA Technologies (Coralville, IA). DNA samples were purified with Transgenomic Wave HPLC system and/or 8 M urea denaturing polyacrylamide gel electrophoresis [29]. Attachment of dyes and purity of oligonucleotides were verified by electrospray-ionization liquid chromatography mass spectroscopy (ESI-LCMS). These tests were done on an Oligo HTCS system (Novatia, Princeton, NJ). Samples were discarded if the experimental molecular mass of oligonucleotide deviated more than 2 g/mol from the expected molecular mass. Oligonucleotide lots were also analyzed by capillary electrophoresis that was run using a Beckman PACE 5000 or Beckman MDQ systems [30]. Oligonucleotides that were less than 90% pure were purified again until they reached this level of purity. These assays confirmed quality and identity of oligonucleotides.

2.2 . UV melting experiments

We followed our published experimental protocol [30]. Oligonucleotides were rehydrated in 50 mM Na^+ buffer (50 mM NaCl, 10 mM phosphate, 1.0 mM Na_2EDTA, pH = 7.0) and their concentrations were determined from absorbance measurements at 260 nm using two different dilutions. Absorbance of self-complementary sequences was read at 85 °C to denature all base pairs. Extinction coefficients of oligomers were predicted from the nearest-neighbor model [31]. Extinction coefficients of Cy3 and Cy5 at 260 nm were added — they were assumed to be 4900 and 10,000 $L \cdot mol^{-1} \cdot cm^{-1}$, respectively [29]. Concentrated stocks of duplexes were prepared by mixing of strands in 1:1 molar ratio, heating the sample to 95 °C, and slowly cooling to ambient temperature.

Melting buffer consisted of 1 M NaCl, 10 mM sodium phosphate, 1 mM Na_2EDTA, adjusted with NaOH to pH 7.0. DNA stock samples were dialyzed against the melting buffer in 28-Well Microdialysis System (Gibco-BRL, Gaithersburg, MD) for at least 24 h. Samples for melting experiments were made by direct dilutions from the stock solution. UV melting profiles were measured using a Beckman DU 650 spectrophotometer with Micro T_m Analysis accessory. Absorbance values at 268 nm were recorded every 0.1 °C. Sample heating rate was 25 °C/h. Temperatures were obtained from an internal probe located inside of the Peltier holder and corrected based on our previous calibration measurements [30]. Custom-made 1 mm and 10 mm pathlength cuvettes (Helma GmbH, Müllheim, Germany) allowed us to measure total single strand concentrations (C_t) ranging from 1 to 175 μM. Absorbance of the upper baseline extrapolated to 25 °C was used to verify proper dilutions of samples and to estimate experimental C_t concentrations.