Chapter 2

Thermal Melting Studies of Ligand DNA Interactions

Aurore Guédin, Laurent Lacroix, and Jean-Louis Mergny

Abstract

A simple thermal melting experiment may be used to demonstrate the stabilization of a given structure by a ligand (usually a small molecule, sometimes a peptide). Preparation of the sample is straightforward, and the experiment itself requires an inexpensive apparatus. Furthermore, reasonably low amounts of sample are required. A qualitative analysis of the data is simple: An increase in the melting temperature (T_m) indicates preferential binding to the folded form as compared to the unfolded form. However, it is perilous to derive an affinity constant from an increase in T_m as other factors play a role.

Key words: FRET, Telomeres, Telomerase inhibitor, G-quadruplex, G-Quartet, DNA ligands

1. Introduction

One of the possible methods to demonstrate an interaction between a compound and a nucleic acid is to perform a melting experiment: In the presence of the molecule, the melting temperature of the DNA or RNA should increase. This simple approach does not require any spectroscopic condition for the ligand – no fluorescence or absorbance special properties are required and has been applied for decades, initially on polynucleotides (see for example (1)), then on unusual DNA structures such as triplexes (2, 3) or quadruplexes. Quadruplexes can be formed by certain guanine-rich sequences in the presence of monovalent cations and are stabilized by G-quartets (Fig. 1). The theory behind these melting experiments, which is not fundamentally different when larger molecules such as polypeptides are considered can be complicated, especially for polynucleotides (4–6). Hence, it is not straightforward to translate an increase in melting temperature into affinity constants. Although the two phenomena are

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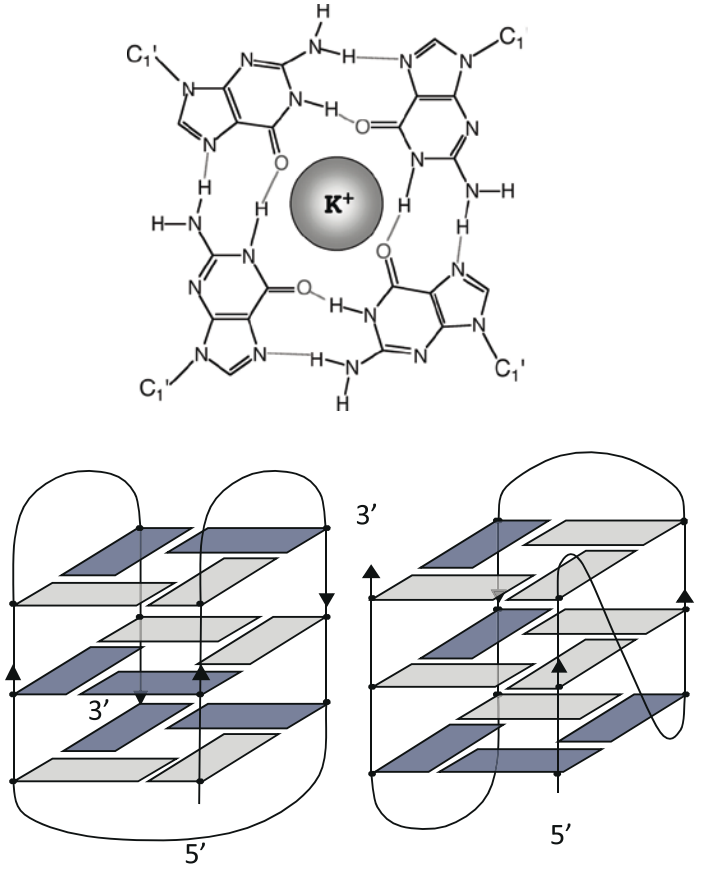

Fig. 1. G-quadruplexes. *Top*: A G-quartet, with four coplanar guanines and a central cation (here potassium; in reality the ion is sandwiched between the planes of two consecutive quartets). *Bottom*: Two possible folds of an oligonucleotide having 4 blocks of 3 guanines. These two conformations involve 3 quartets, but the loops are arranged in a different manner, and the syn (dark blue)/anti (light grey) orientations of the guanine sugar/phosphate backbone are different.

correlated (the strongest ligands tend to lead to the largest increase in T_m), other factors play a role, such as the number of binding sites, cooperativity of binding, and affinity of the ligand for the single strands. In other words, the increase in the melting temperature of a quadruplex induced by the presence of the ligand leads to a semi quantitative measurement of the interaction between a ligand and a nucleic acid.

An increase in the melting temperature of the nucleic acid may of course be followed by absorbance. Nucleic acids display a high absorbance around 260 nm, and it is known for decades that this signal increases when its secondary structure is denatured. For unusual structures, such as quadruplexes, one should select different wavelengths such as 240 or 295 nm (7). An alternative, although less used signal is provided by circular dichroism.

Provided that an appropriate wavelength is chosen, one can study the melting of a DNA structure in the presence of ligands by recording ellipticity as a function of temperature (8). But it is also possible and often desirable to label the nucleic acid with fluorescent groups and obtain fluorescence *vs.* temperature melting profile. One popular approach is to attach a donor and a quencher at both ends of an intramolecular structure and follow its thermal denaturation by a concomitant increase in donor emission (9–14) (Fig. 2). There are several important advantages of a fluorescence-based assay: (1) The difference between the emission levels of the folded and unfolded forms can be very significant; (2) The synthesis or screening of large chemical libraries of potential ligands needs an inexpensive, reproducible and fast assay to test nucleic acid binding: A fluorescent melting assay may easily be converted into 96- or 384-well format, allowing for the weekly screening of thousands of compounds; (3) Lower concentrations are required; (4) many ligands have a significant absorbance in the region where DNA absorbs light, which may interfere with the absorbance or ellipticity signal resulting from complex dissociation. On the other hand, only a handful of molecules were found to interfere with a fluorescence-based melting assay; (5) finally, as the targeted nucleic acid is labeled, one may add massive amounts of "unlabeled" nonspecific nucleic acid competitors without interfering with the fluorescent signal.

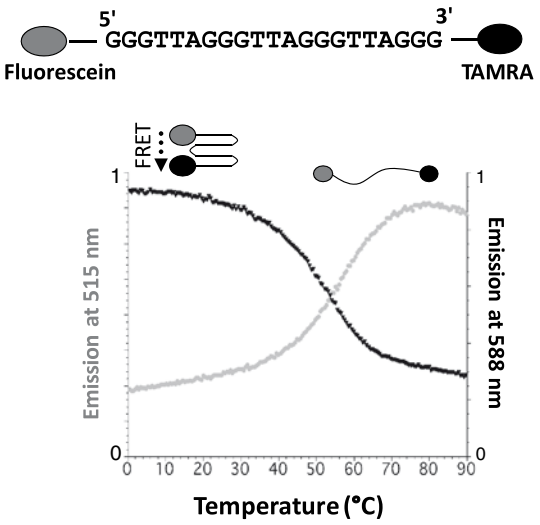

Fig. 2. Example of a FRET melting experiment for a quadruplex-forming oligonucleotide. *Top*: Sequence of the oligonucleotide used. *Bottom*: Fluorescence emission vs. temperature plot for the sequence above determined in a 10 mM sodium cacodylate pH 7.2 buffer with 0.1 M NaCl (total monocation concentration: 110 mM). Fluorescence intensities at both wavelengths have been normalized to 1. Unfolding of the quadruplex leads to a decrease in the emission at 515 nm (*donor, dark curve*) and a concomitant increase at 588 nm (*acceptor, grey curve*).

2. Materials

2.1. Nucleic Acids

2.1.1. Design and Synthesis

For absorbance, simple unlabeled oligonucleotides may be ordered. A synthesis on the 0.2 micromole scale is sufficient for most applications. Polynucleotides may also be tested.

For fluorescence studies, modified oligonucleotides must be synthesized. Fortunately, these compounds are commercially available. For quadruplex stabilization assay, we designed a doubly-labeled oligonucleotide that forms an intramolecular structure. Its sequence is 5′d-GGGTTAGGGTTAGGGTTAGGG3′. The FAM-TAMRA and FAM-Dabcyl dual-labeled oligomers are called "F21T" and "F21D", respectively. Other groups have chosen identical or different FRET pairs (15–19), such as for example fluorescein-Cy3, Cy3-Cy5, or pairs of chemically modified fluorescein, and rhodamine dyes (*i.e.*, Texas Red or ROX; for a recent review on FRET pairs, see (20)) (see Notes 1 and 2). Other quadruplex forming sequences may be tested. We recently analyzed oligonucleotides mimicking the Plasmodium falciparum degenerate telomeric motif $(GGGTTYA)_n$ (21). Nontelomeric repeats or RNA quadruplexes (22) may also be studied; they are reviewed in (23). Figure 3 summarizes results obtained with four different

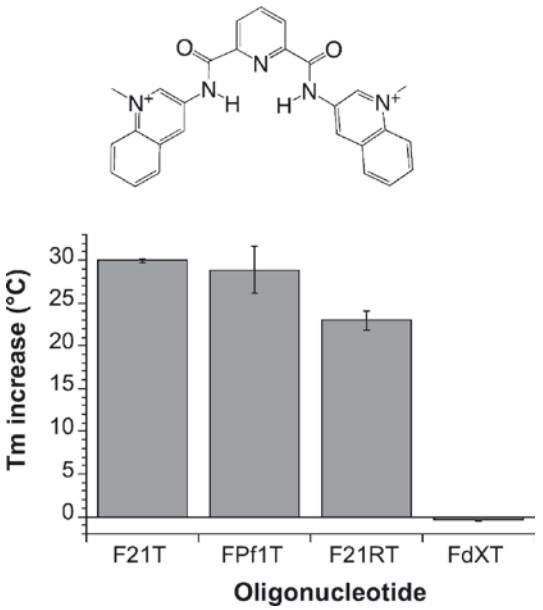

Fig. 3. T_m Stabilization by a quadruplex ligand. *Top*: Chemical formula of the G4 ligand (360A) (33, 34). *Bottom*: Stabilization obtained with the 360A ligand (22) for the human telomeric motif F21T $(GGGTTA)_3GGG$; the Plasmodium telomeric repeat $(GGGTTTA)_3GGG$ (FPf1T), the human telomeric RNA $(GGGUUA)_3GGG$ (F21RT); and hairpin duplex with a HEG loop $TATAGCTATAC_{18}TATAGCT$ ATA (FdXT) (*F* FAM, *T* TAMRA). All measurements were performed in a 10 mM lithium cacodylate pH 7.2 buffer with 10 mM KCl and 90 mM LiCl (total monocation concentration: ≈110 mM).

oligonucleotides (three quadruplex forming sequences and one hairpin duplex). We recommend checking the purity of these oligomers, using mass spectrometry, HPLC, or gel electrophoresis.

2.1.2. Determination of Concentration

Concentrations of all oligodeoxynucleotides were estimated using extinction coefficients provided by the manufacturer and calculated with a nearest neighbor model (24). The concentration should be checked even if the manufacturer provides an estimate of oligonucleotide amount.

2.2. Buffers

2.2.1. Choice of the Ionic Strength

A proper ionic environment is crucial as this parameter will influence the stability of the nucleic acid and modulate the interaction between a cationic ligand and a negatively charged DNA or RNA. The ionic strength and nature of the salt will also modulate the T_m. The cation will be condensed around nucleic acids, which are negatively-charged polyelectrolytes. There is a near-linear relationship between T_m and the logarithm of salt concentration, reaching a plateau around 1 M salt. A "reasonable" buffer contains 0.1–0.15 M salt (NaCl, LiCl or KCl). We suggest choosing buffer conditions that lead to a melting temperature between 40 and 50°C (*see* Note 3). For guanine quadruplexes, this parameter can also be tuned with a partial substitution of Na^+ or K^+ with Li^+ while keeping the total monocation concentration constant (14). For the human telomeric quadruplex, initial experiments were performed in a 0.1 M lithium chloride, 10 mM sodium cacodylate pH 7.2 buffer (25) (*i.e.* with 10 mM Na^+ ions). Later, we changed the reference conditions and compared stabilization values of this quadruplex in two different buffers. Both contain 10 mM cacodylic acid buffered to pH 7.2 with LiOH and either (1) 100 mM sodium chloride or (2) 10 mM potassium chloride + 90 mM lithium chloride. When studying extremely stable quadruplexes, such as c-myc DNA or some RNA G4 sequences, one can choose buffer conditions with an even lower potassium concentration (for example, 1 mM) supplemented with LiCl in order to keep total cation concentration around 0.1 M (26). An intermediate T_m value is required to follow the stabilization obtained in the presence of very « strong » ligands.

2.2.2. Storage and Preparation

2–10× buffer solutions may be prepared in advance, filtered and kept at 4°C for weeks.

2.3. Ligands

2.3.1. Storage and Preparation

Ligands should be solubilized in the mM concentration range using an adequate solvent (typically H_2O or DMSO). Stock solutions can be kept in the dark at –20°C for months. Fresh dilutions should be prepared before each melting experiment. High temperatures may lead to degradation of the ligand; in that case, one should reduce the duration of incubations at high temperatures.

2.3.2. Concentration Range

It is important to test the stabilization of the quadruplex at various ligand concentrations, as initially proposed by Neidle and coworkers (16). The final ligand concentration should be adjusted between two values: The lower corresponding to the oligonucleotide concentration as lower concentration may lead to complex melting profiles. On the other hand, the highest ligand concentration should not lead to a too-high stabilization (a T_m above 80°C is experimentally difficult to determine) and/or precipitation of the oligonucleotide (see Note 4). Comparison of the profiles obtained at different concentrations generates a ΔT_m *vs.* concentration curve (16, 27).

3. Methods

3.1. Absorbance

3.1.1. Instrumentation

Different models and trademarks of high-quality spectrophotometers are available on the market. We use Uvikon 940 and Secomam XL spectrophotometers. Their performance and stability over time in the far UV region (and at high temperature) should be guaranteed. The user should be able to select a temperature gradient over a wide range, and be able to monitor the sample temperature (with an external probe immerged in a control cuvet). Both heating and cooling profiles should be recorded. A multisample cell holder will allow a reasonable throughput, even with experiments that last 15 h. Besides 260 nm (or 295 nm for quadruplexes), it is sometimes appropriate to record absorbance around the ligand absorbance maximum and at a control wavelength. It is therefore useful to have the capability of recording absorbance at several wavelengths.

3.1.2. Preparation of the Samples

T_m of the (DNA (or RNA) + ligand) mixture should always be compared with the T_m of the DNA alone. A multisample cell changer allows performing this control in parallel. Make sure that the total absorbance stays in the linear range of the spectrophotometer – the ligand may significantly absorb light at 260 nm (see Note 5).

3.1.3. Absorbance vs. Temperature measurements

Data acquisition may be programmed for most spectrophotometers. The exact protocol depends on the instrument. Melting experiments are typically performed at a concentration of 2–5 μM per strand. Most transitions are reversible, as shown by superimposable heating and cooling profiles at a fixed rate of 0.1–0.5°C/min. This indicates that the denaturation curves correspond to a true equilibrium process, but prevent us from accessing kinetic parameters (28, 29). One should keep in mind that profiles may be superimposable for the oligonucleotide alone but not the drug-DNA (RNA) complex.

We recommend using the term $T_{1/2}$ (instead of T_m) for experiments in which no demonstration of reversibility is provided.

3.2. Circular Dichroism

The choice for a circular dichroism spectrophotometer is more limited. Nevertheless, different models from Aviv Biosciences, and Jasco are available. We use a Jasco J-810 instrument equipped with a Peltier controlled cell changer.

3.3. Fluorescence (FRET)

Melting measurements may either be performed on:

A traditional spectrofluorimeter (in our laboratory a Spex Fluoromax 3 instrument) with 600 µl of solution containing 0.2 µM of tagged oligonucleotide. An example of a FRET melting curve is provided in Fig. 2.

A real time PCR apparatus (MX3000P, Stratagene; or Roche LightCycler or DNA engine Opticon, MJ Research), allowing the simultaneous recording of up to 96 independent samples as first proposed by S. Neidle and coworkers. The typical concentration of fluorescent oligonucleotide is 0.2 µM (strand concentration), but acceptable curves may be obtained with concentrations in the 0.2–0.5 µM range depending on the volume (from 20 to 100 µl), the gain and the type of detection of the quantitative PCR apparatus used. Each condition is tested at least in duplicate, in a volume of 25 µL for each sample (see Fig. 4 for examples of FRET melting curves recorded on a real time PCR machine) (see Note 6).

3.4. T_m Determination

The melting temperature (T_m) corresponds to the equilibrium temperature at which half of the sample is folded, and half is unfolded. A time-consuming but precise method to determine T_m has been described in detail (30). In cases where equilibrium completion is unknown, $T_{1/2}$ should be used instead of T_m (see Note 7). A popular alternative to this baseline method is simply to determine the maximum of the first derivative of the absorbance or fluorescence signal (dA/dT or dF/dT). This is a simple and user-independent method, and many software programs provide an automatic "T_m" determination by this approach. However, one should keep in mind that this approach only gives an approximation of the true T_m. Nevertheless, this method is sufficient for most applications concerning nucleic acid ligands.

4. Notes

1. Some of the commonly used fluorescent labels have pH-sensitive emission properties. It is therefore recommended to check that the chosen pH does not quench the fluorescent properties of the dye.

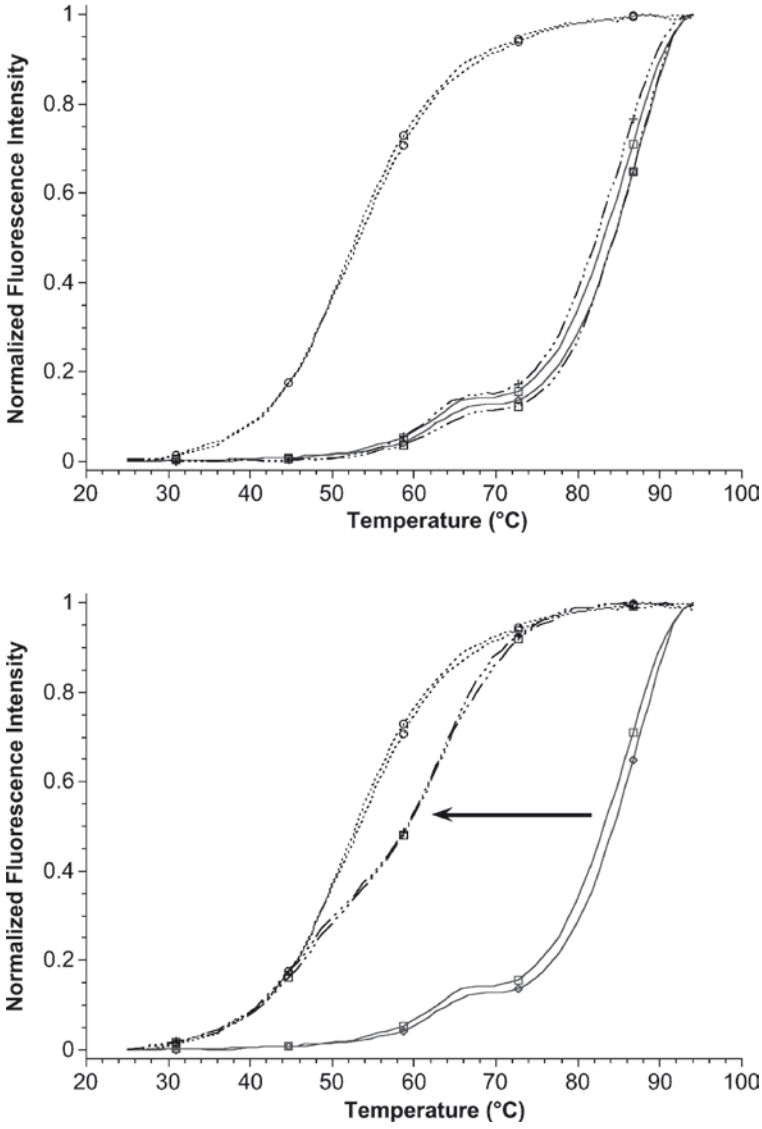

Fig. 4. Effect of competitors on $T_{½}$ increase. *Top*: Melting profiles for the human telomeric motif F21T $(GGGTTA)_3GGG$ alone (*circle*), in the presence of 1 µM 360A ligand (*full line*) and in the presence of a duplex competitor (ds26, 3 µM, hatched/dotted line). All curves are presented in duplicate. *Bottom*: Melting profiles for the human telomeric motif F21T $(GGGTTA)_3GGG$ alone (*circle*), in the presence of 1 µM 360A ligand (*full line*) and in the presence of an RNA quadruplex competitor (RhTR41 (26), 3 µM, hatched/dotted line). All curves are presented in duplicate. The duplex competitor does not lead to a significant decrease of the stabilization provided by the quadruplex ligand (*top*) while an excess of an RNA quadruplex nearly abolishes this effect: The curve reverts to the melting profile obtained in the absence of the ligand (*arrow*). All measurements were performed in a 10 mM lithium cacodylate pH 7.2 buffer with 10 mM KCl and 90 mM LiCl (total monocation concentration: ≈110 mM).

2. The stabilization value depends on the nature of the fluorescent tags, the incubation buffer and the method chosen for T_m calculation, complicating a direct comparison of the results obtained by different laboratories.
3. The buffer plays an important and often underestimated role. We strongly recommend, cacodylate (pKa: 6.14 at 25°C) and acetate (pKa: 4.62) for near neutral and slightly acidic conditions, respectively. These buffers do not absorb light in the far UV-region and their pKa is not too temperature-dependent. This is a key issue as some nucleic acid structures are extremely pH-dependent (31). The pKa of other buffers such as MES, BES, TES, Tricine, HEPES, MOPS or TAPS is extremely temperature-dependent (over 2 pH units between 0 and 100 °C) (32). These are therefore not appropriate for *Tm* experiments.
4. The ligand may interfere with the fluorescent labels rather than with the nucleic acid. In that case, an increase in melting temperature reflects an interaction with the fluorescent dye, not with the target structure, generating false positives. DNA/RNA interactions should therefore be confirmed by other methods.
5. A problem associated with heating is due to the reduced gas solubility at high temperature, which leads to the formation of air bubbles in the sample. These air bubbles will seriously alter absorbance, CD, or fluorescence measurements if they are in the optical pathway. Two simple methods solve this difficulty: It is possible to preheat the sample at 90°C for 5–15 min or to degas the sample under vacuum at room temperature for an equivalent amount of time. The latter solution should be preferred if the sample is extremely heat-sensitive. One may also preheat the buffer component before adding the nucleic acid.
6. For FRET-based fluorescent systems, we found that following the emission of the "donor" (in our case, fluorescein or FAM) gives more reproducible results than the sensitized emission of the acceptor (TAMRA).
7. Manual baseline determination, which is normally required for true *Tm* determination (30), is actually impractical when facing the amount of data generated by a 96-plate reader. Only two feasible automatable alternatives are possible: (1) $T_{1/2}$ determination and (2) first derivative analysis (14). Although both methods provide a less rigorous analysis than a full description of the melting process, they are much faster to implement, and values are obtained in a user-independent way. Although we tend to favour the first one (1), both methods are usually valid.

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