Nucleic Acid Structure and Sequence Probing Using Fluorescent Base Analogue tC^O

Karl Börjesson, Peter Sandin, L. Marcus Wilhelmsson[*]

Department of Chemical and Biological Engineering/Physical Chemistry, Chalmers University of Technology, SE-41296 Gothenburg, Sweden

email: marcus.wilhelmsson@chalmers.se

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[*] To whom correspondence should be addressed. Tel: +46 (0) 31 7723051; Fax: +46 (0) 31 7723858

Abstract

The fluorescent cytosine analog tC^O is on average the brightest probe of its kind and, moreover, it introduces minimal perturbations to the normal secondary structure of DNA. Here several ways of how tC^O, with an advantage, can be used as a local fluorescent probe in nucleic acid systems are presented. Most importantly, we show that tC^O is an excellent probe for the detection of individual melting processes of complex nucleic acid structures containing a large number of separate secondary structure motifs. Since conventional UV-melting investigations merely monitor the global melting process of the whole nucleic acid structure, *e.g.* multi-hairpin systems in RNA/DNA, and thus is incapable of estimating individual melting transitions of such systems, tC^O represents a new method of characterization. Furthermore, we find that tC^O may be used to detect bulges and loops in nucleic acids as well as to distinguish a matched base-pair from several of the mismatched.

Introduction

The most common and generally one of the first procedures used to chemically or physically characterize a nucleic acid structure is to investigate its melting transition(s). In addition to the actual melting temperature (T_m) of the structure, important parameters like the enthalpy and the entropy of the transition can be obtained from the measurements. The most frequently used method in these kind of investigations is to exploit the hyperchromic effect of the nucleobases when going from a double- to a single-stranded structure upon an increase in temperature, *i.e.* to measure the change in UV-absorption at the nucleobase peak (260 nm) at different temperatures. In this region of the spectrum all nucleobases absorb and thus the process studied here will be the melting transition of the whole structure. Since the investigated nucleic acid structure in many cases only contains a single duplex region, this is normally not a problem. However, in more complex nucleic acid structures, containing several separated regions of secondary structure or even tertiary folds, numerous melting transitions could overlap in temperature making comprehensive UV-monitored melting studies virtually impossible.

To study the melting of a particular region within a complex nucleic acid structure, such as a multi-hairpin-structure, some kind of local probe has to be introduced to the system. The primary modification in this respect is the introduction of fluorescent probes. Monitoring the change in a number of fluorescence properties, *e.g.* quenching of FRET donor, increase in anisotropy, difference in quantum yield, or variation in lifetimes, when altering the temperature can in such cases be exploited to study a single local melting transition.[1] However, the introduction of fluorescent probes to the structure could potentially perturb the system and thus affect its physical properties dramatically. For covalently attached fluorescent probes like the cyanine dyes and the family of rhodamines for example, there is a risk of both electrostatic and

hydrophobic interactions with the nucleic acid structure.[2,3] Moreover, these kinds of probes are bulky and may therefore be impossible to incorporate in close proximity to the position under investigation. In addition to the fluorophores mentioned above, monitoring local changes of nucleic acid structures has also been performed using fluorescent base analogues.[4-8] These probes are easy to position close to the site of investigation and normally perturb the overall geometry of the nucleic acid structure less. Common fluorescent base analogs such as 2-aminopurine (2AP) and 4-amino-6-methyl-8-(2'-deoxy-β-D-ribofuranosyl)-7(8*H*)-pteridone (6MAP) have both been successfully used as melting probes.[6,7] However, both these probes have a tendency to destabilize nucleic acid duplexes considerably and hence decrease the melting temperature significantly. This major disadvantage of for example 2-AP makes correct estimates of melting temperatures and detailed investigations of hybridisation processes unfeasible. There has also been a report using the fluorescent base, 2-amino-6-(2-thienyl) purine (s), as a promising probe for monitoring melting processes of 3D RNA molecules.[9] However, this fluorescent base does not pair with any of the natural nucleobases but instead with another unnatural base, pyrrole-2-carbaldehyd (Pa).[9]

Previously we have reported that tC^O (Figure 1) like its sulfur-analog tC has a negligible influence on DNA secondary structure.[10,11] Also, we have shown that tC^O on average has a slightly stabilizing effect on DNA duplexes but that, with an intelligent choice of nucleobases surrounding tC^O, the melting temperature can be virtually unaffected.[10] Like all other fluorescent base analogs except tC[12,13], tC^O has a quantum yield that is sensitive to hybridization ($0.17<\phi_f<0.41$)[10]. However, tC^O preserves significantly more of its high fluorescence quantum yield upon hybridization than any other of these base analogs. In summary these properties are very promising for a fluorescent probe of local nucleic acid structure.

In this paper we investigate the use of tC^O as a fluorescent probe in nucleic acid systems. Most importantly, we show that tC^O is an outstanding probe for monitoring specific melting transitions of multi-hairpin-structures. Also, we find that tC^O may be used to verify bulge- as well as loop-conformations in nucleic acid structures and that its fluorescence is sensitive to the nature of the nucleobase in the opposite strand (mismatch detection). Altogether, this shows that tC^O is an excellent non-perturbing fluorescent probe for nucleic acid local conformation and folding processes.

Materials and Methods

Chemicals

The buffer used in all experiments was a sodium phosphate buffer (10 mM) at pH 7.5 with Na^+ (NaCl) added to a total concentration of 20 or 200 mM for the hairpin and mismatch study, respectively. The tC^O nucleoside was synthesized following the procedure of Lin *et al.*[14] with the exception that toluoyl protection groups were used instead of acetyl. Preparation of the tC^O nucleoside for standard automated solid-phase methods using β-cyanoethyl phosphoramidite was carried out by dimethoxytritylation at the 5'-OH group and phosphitylation of the 3'-OH group to afford the dimethoxytrityl-protected phosphoramidite monomer suitable for oligonucleotide synthesis. Oligonucleotides containing tC^O were synthesized, purified and characterized in the same manner as for tC which is described elsewhere.[13,15] Unmodified, purified and characterized oligonucleotides, not containing tC^O, were purchased from ATDBio Ltd (Southampton, England).

Concentration Determination

The concentrations of oligonucleotides were determined by UV absorption measurements at 260 nm using a Varian Cary 4B spectrophotometer. The extinction coefficients for the modified oligonucleotides were calculated using a linear combination of the extinction coefficients of the natural nucleotides and the extinction coefficient of the tC^O nucleoside at 260 nm. To account for the base stacking interactions, this linear combination was multiplied by 0.9 to give a final estimate of the extinction coefficients for the oligonucleotides. The individual extinction coefficients at 260 nm used in the calculation were ε_T = 9300 M^{-1} cm^{-1}, ε_C = 7400 M^{-1} cm^{-1}, ε_G = 11800 M^{-1} cm^{-1}, ε_A = 15300 M^{-1} cm^{-1} [16] and ε_{tC^O} = 11000 M^{-1} cm^{-1} [10]. For sequences not containing tC^O, the nearest neighbour method was used to calculate the extinction coefficient of the oligonucleotide. Due to the ability of forming intramolecular secondary structures even at ambient temperature, the calculated extinction coefficients of the hairpin-sequences were merely used as rough estimates when determining the concentration of these sequences.

Absorption and Fluorescence Spectra Measurements

Absorption spectra of single stranded oligonucleotides as well as match-, mismatch-, and bulge-duplexes were recorded from 220 to 450 nm on a Varian Cary 4B spectrophotometer at approximately 5°C. Fluorescence spectra of the same samples were recorded between 375 and 700 nm using SPEX fluorolog 3 spectrofluorimeter at approximately 5°C. Samples were excited at 365 nm. The concentration of tC^O-strand in both the absorption and emission measurements was approximately 2 μM.

UV-Monitored Melting

The UV absorption melting studies were performed on a Varian Cary 4000 spectrophotometer equipped with a programmable multi-cell temperature block. The samples were heated from 5°C (25°C for the hairpin sequences) to 95°C at a maximum rate of 0.2°C min^{-1} whereupon they were cooled to 5°C (25°C for the hairpin sequences) at the same rate. The absorption at 260 nm was measured with a temperature interval of 1°C. Melting temperatures (T_m) were determined using the maximum of the derivatives of the unmodified melting curves. The concentration of hairpin-sequences was approximately 4 μM.

Emission-Monitored Melting

The tC^O-emission monitored melting studies were performed on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a programmable multi-cell temperature block. The samples were heated from 25°C (only hairpin sequences measured) to 94°C at a maximum rate of 0.2°C min^{-1}. The excitation and emission wavelengths were 365 and 455 nm, respectively, and data points were collected with an interval of 1°C. Melting temperatures (T_m) were determined using the maximum of the derivatives of the unmodified melting curves. The concentration of hairpin-sequences was approximately 4 μM.

Results and Discussion

Mismatch- and Bulge-Detection Using tC^O

To investigate the use of tC^O as a fluorescent probe for mismatches and bulges in nucleic acid systems a 10-mer oligonucleotide containing tC^O and a set of five standard strands were synthesized (Table 1). In the five standard oligonucleotides there are nine bases that are

complementary to the 10-mer tC^O-strand and the tenth base, the one opposite tC^O, is varied (complementary and A-, C-, or T-mismatch) or missing (tC^O as bulge).

As can be seen in Figure 2 the emission of tC^O decreases substantially when going from a single-stranded environment to a fully complementary duplex. Furthermore, as has been reported earlier, the emission becomes more structured upon hybridization to a complementary strand.[10] Figure 2 also shows that the emission intensity from tC^O forming a bulge in the duplex is virtually the same as for in the corresponding single strand. This suggests that tC^O as a bulging base experiences an environment similar to the one in the single strand.

Also the emission from tC^O in the C-mismatch sample is similar to the emission from the single strand, indicating that having a cytosine opposite to tC^O is very unfavourable and that either tC^O or cytosine in this case might be somewhat pushed out from the base stack. This conclusion is further evidenced by the considerable lowering in melting temperature of this mismatched duplex (T_m appr. 20°C) compared to the complementary duplex (T_m=46°C). Also the T-tC^O mismatch gives a considerable decrease (appr. 20°C) in melting temperature. However, in this case the emission intensity is instead similar to what is found for the fully complementary case (see Figure 2). The only difference between the two cases is the more structured emission for the complementary case, most likely an effect of the more fixed position of the tC^O in the matched duplex.

As can also be seen in Figure 2 the emission for the A-tC^O mismatch has a similar intensity to the complementary case. However, the emission as well as the absorption is highly structured compared to the other matched, mismatched, or bulged duplexes. Furthermore, the T_m of the A-tC^O mismatched duplex is considerably higher (T_m=34°C) than for the other mismatches. These observations implies a stronger interaction and a significantly firmer stacking

for tC^O in the duplex in the A-tC^O mismatch than in the other mismatches. It is interesting to note that we previously, in unpublished data, have found similar structured emission for the tC^O-monomer in a glass matrix at low temperature, in a nonpolar solvent as well as at high pH and, thus, it is difficult to determine whether it is a consequence of only firmer stacking, a different and more hydrophobic environment, an A+/C wobble base-pair or hydrogen tautomerisation induced by the adenine in the opposite strand or maybe a combination of all effects.

To summarize the findings from Figure 2 we have shown that tC^O, at least when its neighbouring bases are wisely chosen,[10] can be used as a fluorescent probe that can distinguish between guanine (complementary) and the group of cytosine, adenine, or a missing base (bulge) on the opposite strand. On the other hand, both the emission intensity and profile of tC^O when mismatched with a thymine are too similar to the fully complementary case to allow for unambiguous discrimination between a guanine and a thymine on the opposite strand. The highly structured emission that is only present for the A-mismatch, however, suggests that tC^O could be an interesting fluorescent probe to discriminate between adenines and any other base (or lack of base) on the opposite strand.

Melting Studies of Multi-Hairpin Systems Using tC^O

To explore the possibility for the future use of tC^O as a probe of local melting in for example multi-hairpin systems we designed and synthesized a set of eight oligonucleotides (Table 2). To demonstrate the potential of using tC^O in this kind of investigation we have selected two hairpins having well separated T_ms and a small probability to interact with each other. As we will return to below, these are by no means necessary criteria but were chosen in this study due to pedagogical reasons. Furthermore, in the design of sequences we have placed tC^O in sequence

surroundings that gives large differences in emission intensity upon going from single- to double-strand.[10] HP1tC^{O} is a sequence that can form a single *h*air*p*in structure with a stem region of up to nine base pairs, one of which containing a tC^{O}. HP1um is the corresponding *u*n*m*odified sequence. HP3tC^{O} contains the same hairpin sequence but instead has the reporter tC^{O} in the loop region. HP2tC^{O} is another sequence that can form a single hairpin structure with a stem region of six base pairs, one of which containing a tC^{O}. HP2um is the corresponding unmodified sequence. In addition to these single hairpin-forming oligonucleotides, three longer sequences that are all able to form two hairpins were synthesized. *D*ouble *h*air*p*in DHP12a_tC^{O} consists of the sequences HP1tC^{O} and HP2um separated by a stretch of nine thymines. DHP12b_tC^{O} instead consists of the sequences HP1um and HP2tC^{O} separated by the same stretch of thymines whereas DHP12um is the corresponding double hairpin structure lacking a reporter tC^{O}.

In Figure 3a two representative hairpin meltings monitored by the change in tC^{O} emission between single- and double-stranded systems are presented. As can be seen in the figure, the raw data of the melting of both HP1tC^{O} and HP2tC^{O} shows that tC^{O} is an excellent probe of single- to double-strand transitions. The large change in quantum yields between the two states in combination with the fact that the emission is high even for the duplex, gives melting curves with a high signal-to-noise ratio both at high and low temperatures as well as a distinctly monitored transition. Furthermore, the well-behaved constant decreases in the melting curves outside the transition regions simplify the conversion of raw data to extent of melting (α) and, consequently, allow for a detailed and quantitative analysis of the melting process. It should be noted that we have performed the melting investigations in this report both increasing and decreasing the temperature during the same experiments and that the curves were identical. This

proves that there is no photobleaching of the tC^O fluorophore during these kinds of melting experiments and that at the current rate of the experiment we have no hysteresis effects.

In Figure 3b normalized derivatives of melting curves of double hairpin structures DHP12um and DHP12b_tC^O are presented. As can easily be noticed the UV-monitored melting curves of DHP12um and DHP12b_tC^O are virtually identical. This is good evidence that the tC^O-modification present in DHP12b_tC^O has a negligible effect on the overall stability of this double hairpin structure. Furthermore, due to the large difference in stability of the two hairpins present in the structure it is easy to distinguish their individual melting processes. The tC^O-emission monitored melting of DHP12b_tC^O can also be seen in Figure 3b. Since the single reporter tC^O is positioned in hairpin 2 of the double hairpin structure only the melting of that particular hairpin is observed. When comparing the maxima of derivatives of the emission and the UV melting curves in Figure 3b, it can be seen that the T_m-value of hairpin 2 determined using these two methods is identical (see also Table 3). This proves that tC^O accurately monitors the process it was intended to follow and nothing else.

In Table 3 the melting temperatures of all the hairpin and double hairpin structures monitored using either UV or tC^O-emission are presented. Firstly, the melting temperatures that are measured both by UV and emission are almost identical ($|T_m^{UV}-T_m^{Em}|\leq 1°C$), confirming that tC^O correctly probes the melting processes. Secondly, the influence of tC^O-incorporation on T_ms should be examined. The difference in melting temperatures for the unmodified and corresponding tC^O-modified hairpin 1 and 2 is 3°C and 0°C, respectively. The same trend can be found when comparing the melting temperatures of the unmodified and corresponding tC^O-modified double hairpin structures (4°C and 0°C, respectively). Taking into consideration that tC^O in these cases is surrounded by the base combinations AG and AA, respectively, these

changes in melting temperatures are in very good agreement with what is expected from previous investigations on the influence of tC^O on duplex stability.[10] Consequently, with an intelligent choice of bases surrounding the reporter tC^O, natural melting temperatures will certainly be possible to estimate using the changes in tC^O-emission. Finally, the emission-monitored melting of HP3tC^O was investigated. For this hairpin the melting profile exhibited no transition but merely a monotonously decreasing curve (data not shown). This gives evidence that a tC^O situated in a loop-region behaves as if it were positioned in a normal single stranded region. Furthermore, the T_ms of this hairpin and the corresponding unmodified hairpin (HP1um) monitored using UV are exactly the same (see Table 3). This suggests that a tC^O situated in a loop region of a hairpin system will not interact with its surrounding nucleobases in a way to stabilize or destabilize the overall structure.

As an effect of our choice of having a large difference in stability of the hairpins above, the melting transition investigation of our double hairpin structures is easy to perform using normal UV monitored melting. However, when it comes to measuring melting of several overlapping T_ms in the same nucleic acid structure, conventional UV-melting studies give limited information. The data obtained will in such cases only give the overall melting of the structure and no details about individual transitions. A way to circumvent this is to cut the nucleic acid into several shorter sequences and to study the melting of each individual fragment. However, as the overall structure could influence local melting substantially, this method is prone to give errors in the estimate of T_m. Furthermore, introducing one or several bulky fluorophores (*e.g.* cyanine dyes or rhodamines) at specific positions in the system to probe local transitions might be impossible and will, as mentioned above, definitely influence the local stability and conformation of the nucleic acid structure. Also using the most common fluorescent base

analogues, like for example 2-AP, will introduce large errors in the investigation of melting temperatures of such systems since these probes drastically influence *e.g.* the stability of duplex regions and base pair dynamics.

Instead, we have here shown that tC^O with advantage can be used as a fluorescent probe for a detailed investigation of the melting of multi-hairpin structures. Incorporation of reporter tC^Os at several strategic positions in different batches of the nucleic acid structure under investigation will allow the investigation of one melting transition at the time. With an intelligent positioning between bases that give minimal changes to the secondary structure stability and large changes in emission upon the transition from double to single strands, tC^O will provide accurately estimated natural T_ms of these complex nucleic acid structures. The use of tC^O in this respect is obviously not limited to complex DNA-hairpins systems as has been used here. One can also envisage using it for studying other complicated folds or folding processes of DNA, DNA-aptamers, and in the expanding field of DNA-nanotechnology as well as perhaps more importantly for the corresponding ribonucleotide-systems. The results found here and in our previous studies suggest that tC^O will work very well in ribonucleotide-systems and efforts are currently made in this direction.

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Figure Legends.

Figure 1. Structure of a basepair of G and tC^O (1,3-diaza-2-oxophenoxazine).

Figure 2. Absorption and emission spectra of tC^O in different DNA environments: single strand (black), complementary duplex (red), mismatched with thymine (green), mismatched with cytosine (blue), mismatched with adenine (cyan), and hybridized to a strand that is lacking a base for tC^O to base-pair with, *i.e.* tC^O forms a bulge (magenta). The emission spectra are corrected for differences in absorption. Concentration of tC^O-strand was approximately 2 μM. Measurements performed at approximately 5°C in 10 mM phosphate buffer (pH 7.5, total Na^+ concentration of 200 mM).

Figure 3. a) Normalized raw data of melting of HP1tC^O (solid line) and HP2tC^O (dashed line) monitored by change in emission of tC^O. b) Derivative of melting curves of DHP12b_tC^O (solid line) and DHP12um (dotted line) monitored by UV as well as smoothed derivative of melting curve of DHP12b_tC^O (dashed line) monitored by change in tC^O emission. Concentration of hairpin-sequences was approximately 4 μM. Measurements performed in 10 mM phosphate buffer (pH 7.5, total Na^+ concentration of 20 mM).

Tables.

Table 1. *DNA Sequences Used in Mismatch and Bulge Detection Experiments as well as their UV-monitored Duplex Melting Temperatures.*

Name	DNA sequence	T_m^{UV} /°C
10-mer tC^O	5'-CGCAA**tC^O**GTCG-3'	
Complementary	5'-CGAC**G**TTGCG-3'	46
A-mismatch	5'-CGAC**A**TTGCG-3'	34
C-mismatch	5'-CGAC**C**TTGCG-3'	20
T-mismatch	5'-CGAC**T**TTGCG-3'	24
tC^O as bulge	5'-CGACTTGCG-3'	29

Table 2. *DNA Sequences Used in Hairpin Probing Studies.*

Hairpin name	DNA sequence*
HP1tC^O	5'-GCGAA**tC^O**GCAGCTTGCGTTCGC-3'
HP2tC^O	5'-CTA**tC^O**AATTTTTTGTAG-3'
DHP12a_tC^O †	5'-GCGAA**tC^O**GCAGCTTGCGTTCGCTTTTTTTTTCTACAATTTTTTGTAG-3'
DHP12b_tC^O †	5'-GCGAACGCAGCTTGCGTTCGCTTTTTTTTTCTA**tC^O**AATTTTTTGTAG-3'
HP3tC^O	5'-GCGAACGCAG**tC^O**TTGCGTTCGC-3'
HP1um‡	5'-GCGAACGCAGCTTGCGTTCGC-3'
HP2um‡	5'-CTACAATTTTTTGTAG-3'
DHP12um†,‡	5'-GCGAACGCAGCTTGCGTTCGCTTTTTTTTTCTACAATTTTTTGTAG-3'

[*] *Shaded regions of sequences are parts that have the ability to form stem regions in hairpins.*

[†] *Note that the two hairpin parts of the double hairpin sequences are made up by sequences HP1 and HP2.*

[‡] *Unmodified hairpins are assigned names containing um (unmodified) at the end.*

Table 3. *Hairpin and Double Hairpin Melting Temperatures Monitored Using UV-absorption from Natural Bases or Emission from* tC^O.

Hairpin	T_m^{UV} /°C		T_m^{Em} /°C
	Hairpin 2	Hairpin 1	
$HP1tC^O$	-	77	76
$HP2tC^O$	47	-	48
$DHP12a\_tC^O$	42	75	75
$DHP12b\_tC^O$	42	71	42
$HP3tC^O$	-	74	-[*]
HP1um	-	74	-
HP2um	47	-	-
DHP12um	42	71	-

[*] No melting transition profile observed.