Optical absorption assay for strand-exchange reactions in unlabeled nucleic acids

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ABSTRACT

The nucleic acid exchange reaction is a common feature for genetic recombination, DNA replication and transcription. Due to the fact that in the strand-exchange reactions the reactant and product molecules have similar or identical nucleotide sequences, the reaction is undetectable. As a rule, the nucleic acids with radioactive or fluorescence labels are used in such studies. Besides the fact that the labels can perturb the reaction and pose a health risk to the investigators, the assays usually involve extra experimental steps: quenching the reaction, separation, visualization and quantification of the products. Here, we describe a straightforward, direct and precise method to study strand-exchange reaction of unlabeled nucleic acids by real-time measurements of optical absorption. The method takes advantage of the property of some guanine-rich oligonucleotides to adopt monomolecular quadruplex conformation in the presence of certain cations. The conformation is characterized by significant absorption in long-wavelength range of the ultraviolet region where usually other secondary structures are transparent. The ‘signal’ oligonucleotide is incorporated into reactant duplex by annealing with target sequence. Adding the replacement sequence initiates the release of the ‘signal’ oligonucleotide into solution, which is accompanied by ultraviolet absorption in long-wavelength range.

INTRODUCTION

Displacement of a nucleic acid strand from a duplex by synchronized replacement with another equivalent or similar strand is a common feature for many reactions in vivo. For instance, the nucleic acid replacement occurs in genetic recombination, DNA replication and transcription. Since the displaced and replaced single strands, and consequently reactant and product duplexes have similar or identical sequences, the reaction is practically invisible. The replacement process becomes visible only after chemical modification of the nucleic acids; for instance, attaching fluorescence (1–3) or radioactive (4–10) labels. Obviously, this complicates the experiments: besides the costly labeling process, attached bulky fluorophores disturb the replacement reactions (1), and working with radiolabel materials is undesirable since they pose health risk and require special containment procedures. Furthermore, studies with labeled nucleic acids require additional experimental steps: quenching the replacement reaction at the appropriate time, separation, visualization and quantification of the products (1,4–10). It is obvious that there exists a need for rapid, sensitive and convenient method to study nucleic acid replacement reaction.

In this paper, we described a method to monitor the replacement kinetics of unmodified nucleic acids by simple measurements of optical absorption. The assay takes advantage of the unique optical characteristics of guanine-rich oligonucleotides folded into monomolecular quadruplex structure [strong circular dichroism (CD) and optical density (OD) signals in long-wavelength range of ultraviolet (UV) region]. These oligonucleotides can be incorporated into initial mismatched duplex by annealing with target sequence. Adding the replacement sequence induces the release of the ‘signal’ oligonucleotide into solution, which is accompanied by an increase in CD or OD at ~300 nm.

The validity of the assay is demonstrated on DNA oligonucleotide G2T2G2TGTG2T2G2, so-called thrombin aptamer (TBA) (11). In the presence of Sr2+, the oligonucleotide folds into monomolecular quadruplex (Figure 1A), which is

![Figure 1](https://academic.oup.com/nar/article-abstract/32/19/e154/1244814/29/March/2019)

Figure 1. (A) Sequence of the TBA and its schematic structure. (B) Scheme of the strand-exchange reaction. Mismatched base pairs are shown in bold face.

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characterized by strong positive band at ~300 nm in both CD and differential absorption spectra (12,13). TBA is preannealed with the complementary strand with two GA mismatches and used as a displacement strand (see Figure 1B). After adding the replacement strand, which is fully complementary to target strand, thermodynamically favorable but slow strand-exchange reaction takes place. The reaction products are fully base-paired duplex and released TBA (see Figure 1B). Released TBA binds Sr ions and folds into quadruplex structure with strong absorption at ~300 nm. Thus, monitoring of the CD or optical absorbance signal of the reaction allows study of strand-exchange kinetics.

MATERIALS AND METHODS

Oligonucleotides were supplied by Eppley Research Institute at UNMC (Nebraska) and were synthesized on solid support using standard phosphoramidite chemistry. Upon completion of synthesis, oligonucleotides were HPLC purified and desalted by dialysis at 4°C against water using Spectrum dialysis tubing with a molecular weight cutoff of 500 Da. The concentration of the oligonucleotide solutions was determined at 260 nm and 80°C using the following molar extinction coefficients, in mM⁻¹cm⁻¹ of strands: 145 for G₂T₂G₂TGTG₂T₂G₂, 141 for C₄A₅C₄ACAC₂A₃C₂, 140 for GT₂G₂TGTG₂T₂G and 149 for CA₂C₅ACAC₂A₅C. These values were calculated by extrapolation of the tabulated values of the dimers and monomer bases (14) at 25°C to high temperatures using procedures reported earlier (15).

UV absorption experiments were conducted on a GBC 918 spectrophotometer equipped with thermoelectrically controlled cell holder. Absorbance versus temperature profiles (melting curves) were measured at either 260 or 300 nm. The temperature was scanned at a heating rate of 0.5°C per minute. CD spectra were obtained with a JASCO J710 spectrophotometer equipped with a water-jacketed cell holder. Quartz cells with 10 mm path lengths were used in all studies. In a typical strand exchange experiment, the initial DNA duplex was incubated at the given temperature in the optical cell placed in the spectrophotometer cell holder. The exchange reaction was started when the displacement strand was added into the cell.

RESULTS

Spectral properties of the oligonucleotides

Here, we present detailed optical studies of the oligonucleotides involved in the reaction. In addition to UV absorption, we used CD technique because it is more sensitive to the sequence and secondary structure of the nucleic acids.

Figure 2 shows CD spectra of reactant (mismatched) and product (perfect) duplexes in 2 mM SrCl₂, 100 mM NaCl, 5 mM Na-HEPES, pH 7.5. CD spectra of the final (perfect) duplex (straight line) and initial (mismatched) duplex (dashed line) in 100 mM NaCl, 2 mM SrCl₂, 5 mM Na-HEPES, pH 7.5. Concentration per duplex: 5 μM (data not shown). Thus, the duplexes on both sides of the reaction (Figure 1) contribute to UV absorbance identically.

Figure 3 shows circular dichroism (CD), absorbance (A) and absorbance difference (ΔA) spectra of displacement (left panel) and replacement (right panel) strands in 2 mM SrCl₂, 100 mM NaCl, 5 mM Na-HEPES, pH 7.5 at 20°C (solid lines) and in the same buffer without Sr ions (dashed lines). In the absence of Sr ions, both strands show similar CD and absorbance spectra (dashed lines). Adding of Sr ions into displacement strand or TBA solution reveals dramatic effects (left panel), while the spectra of replacement strand stays essentially the same (right panel). CD spectrum of TBA in the presence of Sr ions is characterized by strong positive bands at ~250 and ~300 nm, and negative band ~275 nm, which is in excellent agreement with earlier measurements under similar conditions (12). Similarly, adding of Sr⁺ induces significant effects in absorbance spectrum of the displacement strand.

Figure 3 visualizes two important aspects. First, importance of Sr ions in quadruplex formation, and subsequently in the strand-exchange reaction. As one can see from Figure 3, in the absence of Sr ions, reactant and product oligonucleotides have similar optical properties, and the strand-exchange reaction, shown in Figure 1B, is invisible. Second, two single mutations, exchange of guanines for thymines at positions 2 and 14 (see Figure 1), is completely enough to inhibit Sr²⁺-induced G-quartet formation.

UV difference spectrum of displacement strand in the presence of Sr⁺ shows three peaks: ~240, 275 and 300 nm. It is clear that the best optical window for the tracking of the strand-exchange reaction is at ~300 nm: (i) the effect is the largest; and (ii) this optical window is almost transparent for all other reagents.

Thermodynamic properties

In order to choose the reaction temperatures properly, we have studied unfolding of all reactant and product molecules. Figure 4A shows UV melting curves of the duplexes and displacement single-strand, TBA, in 100 mM NaCl, 2 mM SrCl₂, 5 mM Na-HEPES at pH 7.5 and at ~4 μM strand.
concentration. All melting curves are sigmoidal indicating a typical two-state transition. In the case of TBA, the process is hypochromic, which is opposite to the hyperchromic effects of the duplexes, and typical for quadruplex melting. Due to the fact that our duplexes are bimolecular, their melting temperature, \( T_m \), depends on the strand concentration. Therefore the melting curves of the duplexes were recorded at different strand concentrations and the results are shown on Figure 4B. We used van’t Hoff analysis to determine the unfolding parameters from the dependence of \( T_m \) on strand concentration (Figure 4B). The data are collected in Table 1 and are in excellent agreement with the parameters calculated from perfect (18) and mismatched (19) DNA nearest-neighbor analysis. The \( T_m \) value of TBA is \( 57 \pm 1 \) °C, which is similar to earlier measurements in 10 mM SrCl\(_2\), 10 mM Cs-HEPES, pH 7.5 (12). The TBA quadruplex is monomolecular and therefore its \( T_m \) does not depend on strand concentration (12).

Figure 3. Circular dichroism (CD), absorbance (A) and absorbance difference spectra (\( \Delta A \)) of displacement (left column) and the replacement (right column) without (dashed lines) and with 2 mM Sr ions (straight lines). Spectra are recorded in 100 mM NaCl, 5 mM Na-HEPES, pH 7.5 at 20 °C. Concentration per strand: 5 μM.

Table 1. Thermodynamic parameters for duplex formation*

<table>
<thead>
<tr>
<th>Duplex</th>
<th>( T_m ) (^{b} ) (°C)</th>
<th>(-AG_{20} ) (kcal/mol)</th>
<th>(-AH ) (kcal/mol)</th>
<th>(-AH_{pred} ) (kcal/mol)</th>
<th>(-AS ) (su)</th>
<th>(-AS_{pred} ) (su)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfect (product)</td>
<td>55.1</td>
<td>20.3</td>
<td>108.7</td>
<td>115.0</td>
<td>301.6</td>
<td>314.6</td>
</tr>
<tr>
<td>Mismatched (reactant)</td>
<td>47.0</td>
<td>14.8</td>
<td>77.9</td>
<td>80.1</td>
<td>215.4</td>
<td>219.1</td>
</tr>
</tbody>
</table>

*aThe parameters determined in 100 mM NaCl, 2 mM SrCl\(_2\), 5 mM Na-HEPES, pH 7.5.

*bThe \( T_m \) values refer to a total strand concentration of 3.5 μM.
Strand-exchange reactions

Figure 5 demonstrates kinetics of strand-exchange reactions measured at 4 \( \mu \text{M} \) of the reactant molecules and at temperatures between 28 and 37\(^\circ\)C. The experimental temperatures are significantly lower than \( T_m \) of the molecules involved in the reaction (see Figure 4). The rate constant, \( k \), was determined by fitting the experimental data to a first-order reaction equation:

\[
(A_t - A_0) = (A_\infty - A_0)(1 - \exp(-kt))
\]

where \( t \) is time, and \( A_t, A_0 \) and \( A_\infty \) are the optical absorbance at time \( t \), zero time and infinite time, respectively.

The rate constants are collected in Table 2. The rate constant increase with increasing the temperature, which is in good agreement with earlier measurements of strand-displacement rates on perfect duplexes using electrophoresis assay of labeled oligonucleotides (1). As it was expected, the present rate constants, obtained for mismatched reactant duplexes, are significantly higher than the parameters reported for perfect duplexes (1).

The strand-exchange kinetic is not affected by folding of the displaced strand

We remind that in the present assay, the role of ‘signal’ molecule is played by displacement strand, which upon release folds into the monomolecular quadruplex structure. It is known that monomolecular quadruplexes are formed readily (12). However, it is important to see that our strand-exchange kinetic is not affected by kinetics of the quadruplex formation. We performed additional experiments in which the unfolded TBA molecule was titrated into the buffer (100 mM NaCl, 2 mM SrCl\(_2\), 5 mM Na-HEPES, pH 7.5).
2 mM SrCl₂, 5 mM Na-HEPES, pH 7.5) at different temperatures. In all cases, we observed immediate increase of absorbance signal at 300 nm, corresponding to folding of the strand. Waiting periods of ~15 min did not reveal any further changes in the measured parameter.

**DISCUSSION**

The main point of the study is that the process of the nucleic acid strand-exchange can be studied by the real-time measurements of optical absorbance without any modification of the strands. The foundation of the assay is formed by following two facts. First, in the presence of Sr ions single-stranded TBA strands. The foundation of the assay is formed by following two facts. First, in the presence of Sr ions single-stranded TBA folds into intramolecular quadruplex structure, which strongly absorbs light at ~300 nm where all other secondary structures are transparent, including TBA incorporated into the duplex. Second, exchange of a few guanines for thymidines in TBA molecule completely inhibits quadruplex formation. Thus, both reactants (left side of Figure 1B), mismatched duplex with TBA and mutated version of TBA, GT₃TGTG₂T₃G do not absorb the light at ~300 nm. Upon their mixing, TBA is replaced in the duplex by perfectly base-pairing GT₃TGTG₂T₃G. The release of TBA into solution is accompanied by immediate quadruplex formation and increase of optical absorbance at 300 nm.

It is obvious that the reaction described here can be used as a model system for fundamental studies on the mechanism of the nucleic acid strand-exchange reactions. In addition, the assay can be used to study the role of non-specific binding proteins in the strand-exchange reactions, such as HIV-1 nucleocapsid protein (2,3).

The assay is not limited only to TBA molecule. There are other sequences forming monomolecular quadruplexes with light absorbance in near-UV region: e.g. human telomeric repeat (G₃TTA)₃G₃ ([17], B.I.Kankia, unpublished data) and oxytria telomeric repeat (G₄T₄)₂G₄ (17). It is also known that exchange of the nucleotides in loop regions does not inhibit quadruplex structure ([17,20]; B.I. Kankia, unpublished data). Keeping in mind that we also need to introduce some mutations in G-track regions to inhibit quadruplex formation of displacement strand, it became clear that one can design almost any nucleotide sequence in fully base-paired product duplex (for instance, a protein-binding site). In addition, one can link the signal oligonucleotide with the desired sequence.

The assay is easily adaptable to desired reaction rate and experimental conditions. In addition to the ionic strength, temperature and the duplex length, one can vary the type of cation used for quadruplex formation. It was shown earlier that the stability of TBA is strongly dependent on the type and size of cations (12,21). For instance, Tₘ of TBA in the presence of Sr²⁺ is ~60°C, while in the case of Rb⁺ it drops to ~33°C (12). Thus, to slow down the reaction kinetics of an unstable reactant duplex, one can use a small amount of Rb ions in combination with lower temperature. Another factor strongly influencing the reaction rate is type and number of mismatches in the reactant duplex. There are eight possible mismatches or mispairs and depending on their neighboring nucleotides all of them have different stabilities (19,22). For instance, by choosing the most stable mismatches, i.e. G₃A, one can increase the number of the mismatches in the reactant duplex, or shift the reaction into slower rates.

In the present assay, the replaced strand forms thermodynamically stable Sr²⁺-quadruplex which facilitates initial strand-exchange reaction and as a result complicates the reverse reaction. While beneficial in some cases (for instance, to accelerate very slow reaction), this can prevent multiple rounds of the experiment. Since (i) TBA quadruplex easily integrates into duplex upon addition of the complementary structure (B.I.Kankia, unpublished results) and (ii) quadruplex stability is sensitive to the type of cations used (12,21), one can choose the experimental conditions favorable for reversible reactions. For instance, one can perform the reactions in the presence of cations which form unstable quadruplexes (i.e. Rb⁺ or NH₄⁺) (12), and by selecting proper temperature and ionic strength to make the strand-exchange reaction reversible.

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**REFERENCES**