Biophysical Chemistry 146 (2010) 1–6

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Conformation and thermostability of oligonucleotide d(GGTTGGTGTGGTTGG) containing thiophosphoryl internucleotide bonds at different positions

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ARTICLE INFO

Article history:
Received 3 June 2009
Received in revised form 11 September 2009
Accepted 22 September 2009
Available online 1 October 2009

Keywords:
G-quadruplex
Thrombin-binding aptamer
Phosphorothioate oligonucleotides
Circular dichroism
UV melting
MALDI TOF MS
Thrombin time
Biodegradation

ABSTRACT

The thrombin-binding aptamer d(GGTTGGTGTGGTTGG) (TBA) is an efficient tool for the inhibition of thrombin function. We have studied conformations and thermodynamic stability of a number of modified TBA oligonucleotides containing thiophosphoryl substitution at different internucleotide sites. Using circular dichroism such modifications were found not to disrupt the antiparallel intramolecular quadruplex specific for TBA. Nevertheless, the presence of a single thiophosphoryl bond between two G-quartet planes led to a significant decrease in the quadruplex thermostability. On the contrary, modifications in each of the loop regions either stabilized an aptamer structure or did not reduce its stability. According to the thrombin time test, the aptamer with thio-modifications in both TT loops (LL11) exhibits the same antithrombin efficiency as the original TBA. This aptamer shows better stability against DNA nuclease compared to that of TBA. We conclude that such thio-modification patterns are very promising for the design of anticoagulation agents.

1. Introduction

Inhibitory influence on thrombin activity was found for several short, single-stranded DNA sequences. It has been reported that all the sequences have a highly conserved 15-base region d(GGTTGGTGTGGTTGG) [1], called thrombin-binding aptamer (TBA). This sequence is able to fold in monomolecular antiparallel quadruplex (Fig. 1), as was shown by NMR, X-ray and circular dichroism spectrometry [2–5]. It was discovered that such structural motif is playing a major role in an aptamer's antithrombin activity [5]. However, practical applications of TBA are hampered by it's extremely short in vivo half-life, estimated to be ~100 s [6]. One way to overcome this problem is to use thiophosphoryl modification of TBA, which is a common approach to increase in vivo stability of synthetic oligonucleotides, since a sugar–phosphate backbone modification improves oligomers' resistance to various nucleases [7,8]. Nevertheless, these modifications can alter the spatial structure and target affinity of the oligonucleotides as well as potentially lead to system toxicity.

The completely thio-modified oligonucleotide (SATR) had been shown to maintain the G-quadruplex conformation [9]. However, SATR exhibits a high system toxicity, which could be decreased if a lesser number of phosphorothioate modifications would be introduced in the backbone. Our goal was to design TBA-derived oligonucleotides with a few numbers of phosphorothioates in such positions, that G-quadruplex structure and stability would be practically unaffected. With this in mind, we studied the influence of a number and localization of the backbone phosphorothioate modifications and its stability. Besides that, all synthesized oligonucleotides were preliminarily evaluated for their stability in blood serum and more importantly for their antithrombin properties.

2. Materials and methods

2.1. Phosphodiester and phosphorothioate oligonucleotides

In this study phosphodiester oligonucleotide TBA with sequence d(GGTTGGTGTGGTTGG) and 13 other oligonucleotides with different local phosphorothioate modifications were used (Fig. 1). The modifications were localized between guanines of G-quartets in P1011, P4, P1415, P111 and P511 oligonucleotides, or in the loops connecting G-quartets in L1, L11, L121, LL11 and LL (Fig. 1). Phosphorothioate modifications in oligonucleotides L343, L3 and SATR were localized as within G-quartets, as in the loops (Fig. 1). All phosphodiester and thiophosphoryl oligonucleotides were synthesized as in [10], purified by reverse-phase HPLC, and

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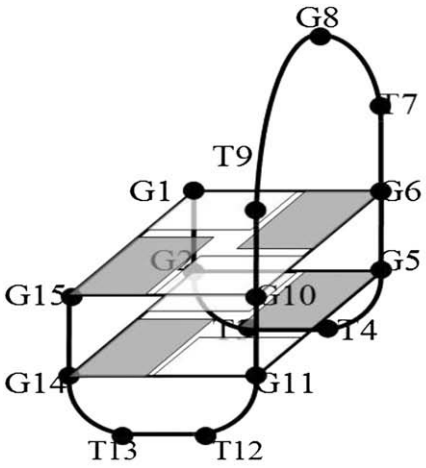
doi:10.1016/j.bpc.2009.09.011



TBA	d(GGTTGGTGTGGTTGG)
Modification in plane only:	
P1011	d(GGTTGGTGTGsGTTGG)
P4	d(GsGTTGsGTGTGsGTTGsG)
P1415	d(GGTTGGTGTGGTTGsG)
P111	d(GsGTTGGTGTGsGTTGG)
P511	d(GGTTGsGTGTGsGTTGG)
Modification in loop only:	
L1	d(GGTTGGTGTGGTsTGG)
L11	d(GGTsTGGTGTGGTsTGG)
L121	d(GGTsTGGTsGTGGTsTGG)
LL11	d(GGsTsTGGTGTGGsTsTGG)
LL	d(GGsTsTGGTsGTsGTGGsTsTGG)
Mixed modification:	
L343	d(GGTsTsGGTsGTsGGTsTsGG)
L3	d(GGTsTsGGTGTGGTTGG)
SATR	d(GsGsTsTGGTsGTsGGTsTsGG)

Fig. 1. Scheme of G-quadruplex structure of TBA. The oligonucleotide sequences with thiophosphoryl modifications. Mark "s" denotes the thiophosphoryl internucleotide bond.

desalted. The number of thiophosphoryl bonds in oligomers was verified by MALDI TOF MS (Matrix assisted laser desorption ionization time-of-flight mass-spectrometry).

The concentration of each oligonucleotide in water solution was determined at 90 °C using the calculated extinction coefficient 158,100 $M^{-1}cm^{-1}$ at 260 nm [11]. For all further experiments 5 μM oligonucleotide solutions in PBS (10 mM K-phosphate, pH 7.4, 0.1 M KCl) were utilized. The solutions were heated at 95 °C for 15 min and quickly cooled on ice.

2.2. Absorption spectra and melting curves

Absorption spectra and UV melting curves were recorded using Jasco V-550 spectrophotometer with thermostated cuvette holder. Melting curves were registered at λ = 295 nm in the 15–90 °C temperature range. Thermodynamic parameters were defined by fitting procedure using the two-state model for monomolecular melting [12].

2.3. Fluorescence polarization and lifetime of EtBr bound to TBA oligonucleotides

The fluorescence polarization (P) of ethidium bromide (EtBr) bound to TBA oligonucleotides was measured with Cary Eclipse spectrofluorimeter at the temperature 3 °C. Excitation wavelength was 540 nm and the fluorescence intensity was registered at 610 nm (I_{610}). The fluorescence polarization P was calculated using the equation [13]:

$$P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp}). \quad (1)$$

The vertical $I_{||}$ and horizontal $I_{\perp}$ components of bound EtBr fluorescence intensity were measured at excitation by the vertically polarized light. The free dye contribution was taken into account as described elsewhere [14]. Concentration of EtBr was 1 μM, concentration of TBA oligonucleotides — 5 μM.

The fluorescence lifetime (τ) of EtBr:TBA complexes was evaluated using Easy Life V. Fluorescence decay was registered through a RG610 long pass filter at excitation LED 525 nm.

2.4. Rotational relaxation times of EtBr:TBA complexes

Rotational relaxation time (ρ) for the EtBr:TBA complexes was estimated using the Perrin–Weber equation, valid for small particles having spherical or low-asymmetrical ellipsoidal shape [13]:

$$\rho = 3\tau(1/P_o - 1/3)/(1/P - 1/P_o). \quad (2)$$

P is the observed polarization, and $P_o = 41 \pm 1\%$ is its limiting value in the absence of rotational depolarization; τ is the fluorescence lifetime of adsorbed EtBr on TBA oligonucleotides, see also [14–16].

2.5. Circular dichroism

CD spectra of oligonucleotides were registered with a Jasco 715 spectropolarimeter using a thermostated cell. The CD values ($\Delta\varepsilon$) are given per moles of nucleotides. CD spectra were registered in 220–330 nm wavelengths and the 15–80 °C temperature range.

2.6. Impact of oligonucleotides on blood coagulation (thrombin time)

Thrombine time (TT) was measured according to the published procedure [17] and the protocols of an assay kit manufacturer ("Thrombin-TEST", Renam, Russia). Clotting times for the TT assay were initiated when 100 μl of citrate-stabilized serum was pipetted and incubated for 120 s. The last step – the addition of oligonucleotide to the final concentration of 0.1–1.5 pmol/μl – activated the final measurement with a coagulation analyzer (MiniLab-701, Unimed, Russia).

2.7. Oligonucleotide stability in blood serum

Citrate-stabilized serum specimens were obtained from healthy individuals, separated into 200 μl aliquots and stored at −20 °C until used. The hydrolysis was started by adding to the serum aliquot the mixture of the investigated oligonucleotide and TBA as an internal reference to the final concentration of 2 pmol/μl for each of them. Serum specimens were incubated at 37 °C for 120 s, followed by addition of the oligonucleotide–antidote A (5'dCCAACCACACCAACC) solution in PBS buffer (pH 7.4), which was added to the final concentration of 4–6 pmol/μl. Subsequently, after 30 s 1.5 ml of acetone

(Merck, Germany) was added and the mixture was vortexed intensively for several seconds. The obtained slurry was frozen and kept at −20 °C for 2 h followed by centrifugation at 14,000 rpm. After reconstitution of the precipitate in 200 µl of DI water the acetone (200 µl) was added and the mixture was intensively vortexed again. The resulting suspension was subjected to the second round of centrifugation (20 min, 14,000 rpm) and the supernatant was concentrated in vacuum (SpeepVac, Labconco) to the final volume of 40–50 µl, followed by MALDI mass-spectrometry analysis.

2.8. Mass-spectrometric analysis

MS analysis was performed on a MALDI mass spectrometer (Microflex, Bruker Daltonics, Billerica, MA) in linear mode. Accelerating voltage was +20 kV, IS2 = 18.65 kV, extraction delay = 400 ns. Recorded spectra were the average of 500 laser shots. Prior to analysis a sample was concentrated using SAX and C-18 Zip tips (Millipore, Billerica, MA), mixed upon elution with MALDI matrix (0.25 M solution of 3-hydroxypicolinic acid in DI water), spotted on to MALDI stainless steel target and air dried.

3. Results

3.1. Oligonucleotide conformation

To determine the influence of thiophosphoryl modification on oligonucleotide conformation we compared their CD spectra with that of phosphodiester oligonucleotide TBA. Fig. 2 displays CD spectra of the oligonucleotides at 15 °C. The positive band at 295 nm and the negative one around 268 nm reflect the formation of an antiparallel quadruplex by phosphodiester oligonucleotide TBA (Fig. 2, solid curve marked with filled circles) [18,19] and all other modified oligonucleotides. A positive CD band at 295 nm of oligonucleotides having thiophosphoryl modifications between guanines of G-quartets is reduced in comparison with that of TBA (Fig. 2A). Unlike this, modifications in the loops connecting G-quartets led to somewhat an increase in the CD at 295 nm (Fig. 2B). The "mixed" modifications placed as in the loops, as within G-quartets (L3, L343) resulted in CD spectra similar to that of phosphodiester TBA (Fig. 2C). The shape of the fully modified SATR oligonucleotide spectrum (Fig. 2C, solid line marked with triangles) is somewhat different from other spectra, but still has the characteristic bands.

Fig. 3 shows the dependence of the oligonucleotide CD spectra on temperature. CD spectra of phosphodiester TBA are shown in Fig. 3A, those of fully thio-modified SATR — in Fig. 3B, those of the modified in G-quartets region oligonucleotide P4 — in Fig. 3C, and CD spectra of L343 are given in Fig. 3D. The decrease in the characteristic band amplitudes reflects the denaturation of quadruplex structure. The CD spectra at 80 °C correspond to unordered oligonucleotide strands. The isodichroic point near 285 nm shows that the denaturation of quadruplex structure occurs by an "all-or-none" mechanism.

3.2. Intramolecular folding of the TBA oligonucleotides

TBA has been reported to form a monomolecular structure under similar experimental conditions [3,19]. In order to confirm the formation of intramolecular quadruplex structures by the new set of modified oligonucleotides, we measured the fluorescence polarization P and fluorescence lifetime τ of EtBr bounded to oligonucleotides (see Section 2). Fluorescence life time τ of EtBr:TBA complex was measured to be 14.0 ±0.5 ns. The fluorescence polarization P was examined for TBA, SATR and P4 oligonucleotides. The values of P were determined at 3 °C and were equal 0.13 ± 0.005 for all studied oligonucleotides. From Eq. (2) the rotational relaxation time was estimated to be $\rho = 17.6 \pm 2.3$ ns for TBA, SATR and P4 oligonucleotides. The computed ρ of the modified oligonucleotides were equal to

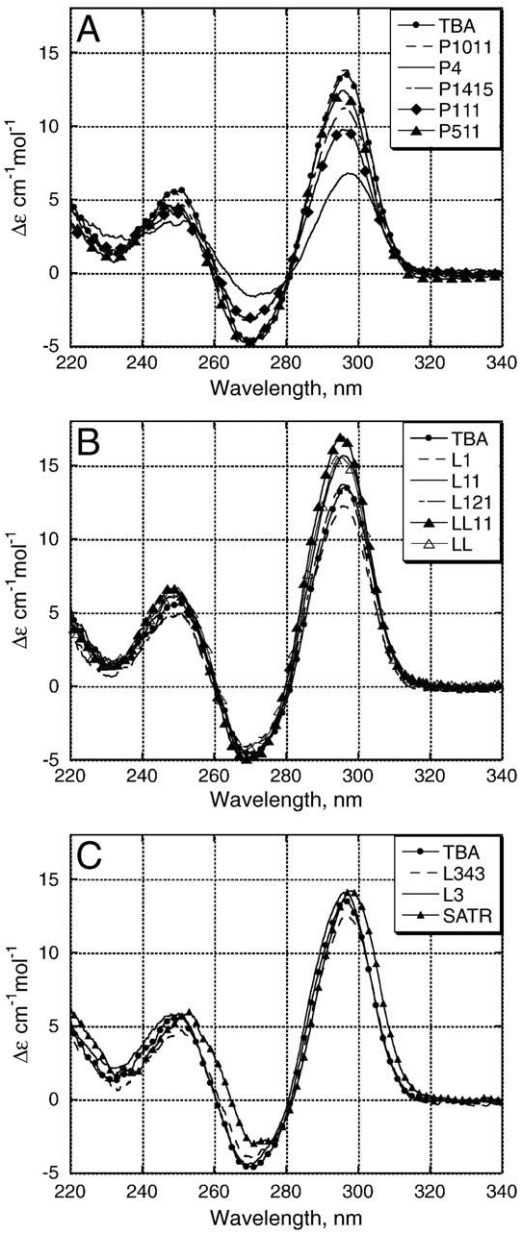


Fig. 2. CD spectra of TBA and thiophosphoryl oligonucleotides. TBA is marked with solid line with filled circles. Oligonucleotides (A) with modifications in "plane" between GG, (B) with modifications in "loop" regions, (C) with the both in "plane" and in "loop" modifications. Solution contained 10 mM K-phosphate, pH 7.4, 0.1 M KCl; temperature was 15 °C. Concentration of the oligonucleotides was 5 µM strands.

that for TBA. Our data corroborates the monomolecular fold of the studied modified oligonucleotides [3,14–16,20–25].

3.3. The stability of quadruplex structure formed by the oligonucleotides

The melting curve registered by absorption at 295 nm was shown to reflect a denaturation of the quadruplex structure [19]. In Fig. 4 the melting curves at 295 nm for the thio-modified TBA oligonucleotides are shown. Thermodynamic parameters, T_m, formation enthalpy and entropy for all studied oligonucleotides, were calculated using non-linear approximation with the two-state model (Table 1). The values of energetic characteristics for the melting of non-modified oligonucleotide TBA (Table 1, first row) were found to be in good agreement with literature data [19]. Fig. 5 represents a graphic presentation of the melting temperature (Fig. 5A) and the free energy dependence at 37 °C (Fig. 5B) on a number of thio-modification in oligonucleotides.

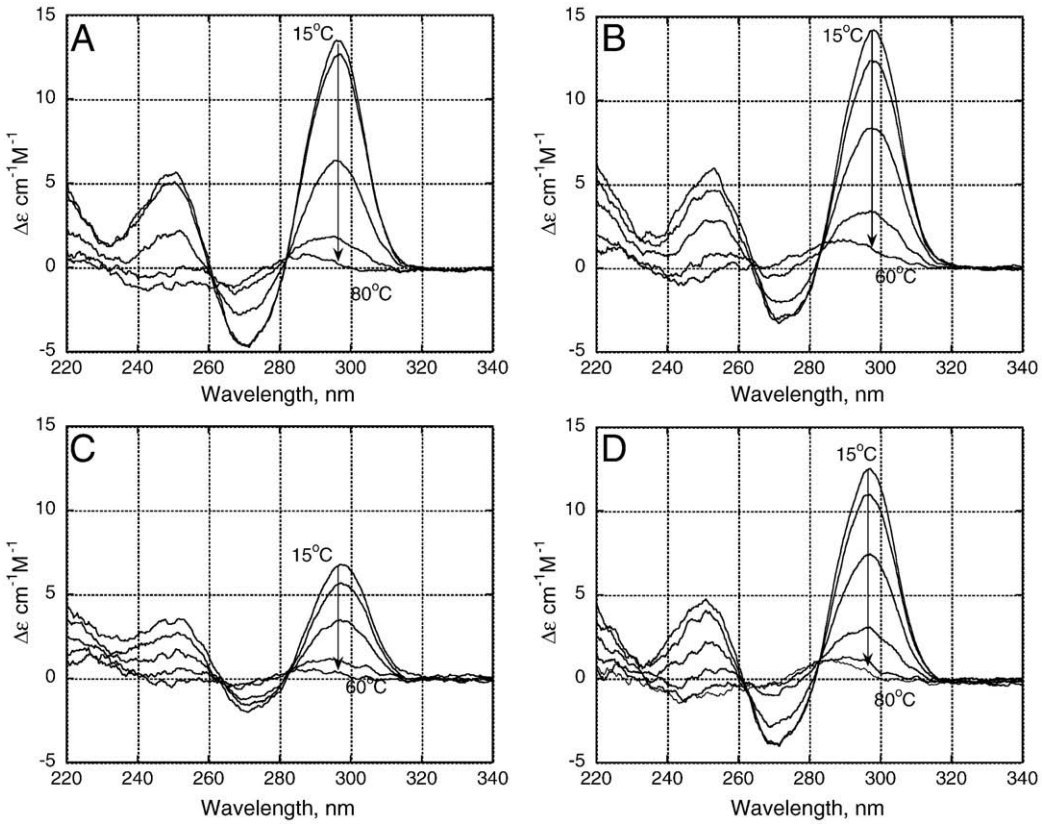


Fig. 3. Temperature dependence of CD spectra of TBA (A), SATR (B), P4 (C) and L343 (D). Temperature was 15, 30, 50, 60 and 80 °C for (A); 15, 30, 40, 50, 60 °C for (B) and (C); 15, 30, 40, 50, 60 and 80 °C for (D). Solution conditions were as in Fig. 2.

The number of modifications between two guanines in oligonucleotides named P1011, P111 and P4 exerts a cumulative effect on the quadruplex destabilization. On the other hand, modifications between T and G, G and T or T and T have an insignificant effect, and the mixed modifications give a more complex effect on oligonucleotide structural stability.

3.4. Anticoagulation properties of TBA analogs

The mechanism of anticoagulation activity of TBA is based on its ability to bind thrombin involved in blood coagulation pathway [26]. In order to compare the properties of TBA analogs, the standard medical test, thrombin time, was performed [17]. Concentration of the tested oligonucleotides in the serum was in the range of 0.1–0.8 nmol/ml, which is a typical level for TBA biological tests [26]. Interestingly, a fully thiolated analog (SATR) did not slow down clot formation, but accelerated it in comparison with the control serum sample. Some of the partially thiolated oligomers exhibit thrombin time close to that of TBA. In general, the activity of TBA analogs can be lined up in the following way: SATR ≪ P4 < L11 < LL11 ~ TBA.

3.5. Investigation of TBA analogs stability in blood serum

The stability of oligonucleotides in blood serum was investigated using MALDI MS method. Prior to isolation of TBA analogs after incubation with serum antidote — oligonucleotide "A" was added in order to free aptamers from thrombin complexes. The residual oligonucleotides were subjected to MALDI analysis after isolation and desalting step. MS spectrum of the mixture LL11 and TBA is presented in Fig. 6A. The oligonucleotides were isolated immediately after their addition to the serum sample without any incubation time. The starting ratio between intensities of TBA signal (4726 Da) and LL11 (4822) was 1.3 to 1. Upon 3 min of incubation (Fig. 6B) the ratio has been changed from 0.4 to 1, which suggests that LL11 degradation speed in serum is significantly (~3 times) slower than that of TBA.

4. Discussion

We have shown by means of CD spectroscopy that our set of oligonucleotides (Fig. 1) has the same structural motive as non-modified oligomer, intramolecular antiparallel G-quadruplex. We supported the monomolecular folding by non- and fully-modified oligonucleotide by measurements of bound EtBr fluorescence polarization. This result is in good agreement with other spectroscopic and structural data [3,20]. G-quadruplex folds formed by the oligonucleotides, differ in thermal stability. It was found that localization of the thiophosphoryl modification of internucleotide bonds plays the dominant role in the quadruplex structure stability. The thiophosphoryl modification of GG internucleotide bond decreased the structural stability (Fig. 5, Table 1). Presumably, substituting sulfur in place of oxygen in a phosphate group leads to changes in distance and angles between bonds. This may result in a loss of stacking interaction of two neighboring guanine quartets and a decrease the quadruplex structure stability. On the other hand, the entropic character of the destabilization (see ΔS values of SATR, P4, P111, P511, L343, Table 1) may denote an increased solvent organization. On the contrary, modifications of the internucleotide bonds in loop region exert minor effect on the structure stability. The loops govern the flexibility of the quadruplex fold, and the oligonucleotides with sulfur, in the loop region appear more stable than without modification. The oligonucleotides named L121 and LL have the same stability, which mean that the modification of the internucleotide bond between a loop region and planes does not affect the structural stability. Importantly, the concurrent "plane" and "loop" modification (Fig. 5, triangles) has a multiplicative effect on the TBA quadruplex stability. This is in good agreement with the data on SATR

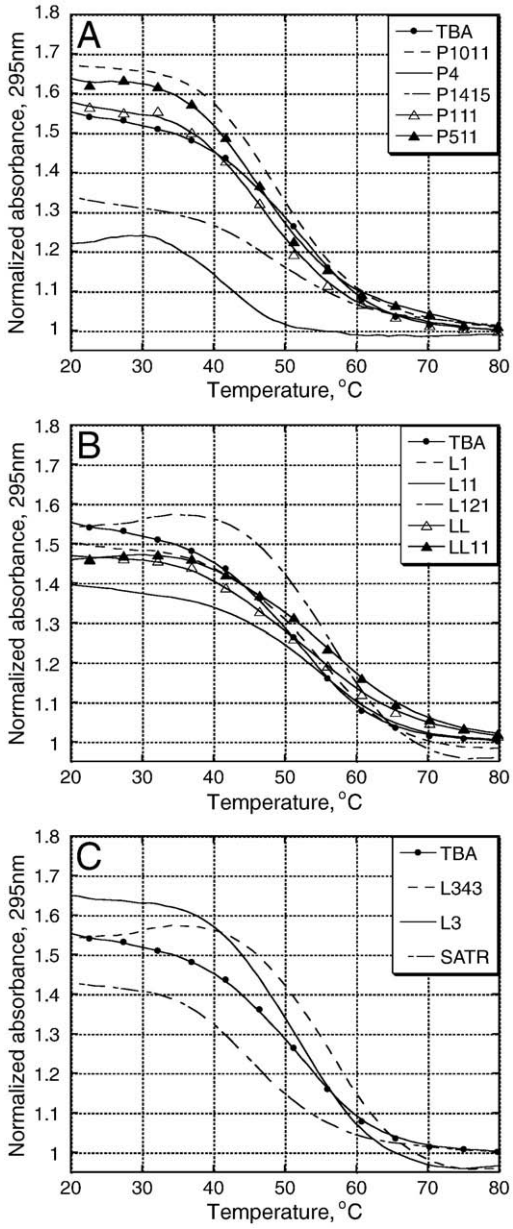


Fig. 4. UV melting curves of TBA and thiophosphoryl oligonucleotides detected with absorbance at 295 nm. TBA, solid line with filled circles; (A) the oligonucleotides with modifications in plane between GG; (B) the oligonucleotides with modifications in loop regions; (C) the oligonucleotides with the both plane and loop modifications. Solution conditions were as in Fig. 2.

reported earlier [9]. Interestingly, the effect of 2′-deoxy-2′-fluoro-D-arabinonucleic acid (2′F-ANA) modifications also depended on a localization of this modification in the thrombin-binding aptamer [21]. Namely, such modifications in the loop regions of TBA did not change the TBA structural features and increased its affinity to thrombin.

The found specific effects of the local thiophosphoryl modification on the TBA quadruplex conformation and stability may find their employment in attempts to stabilize other, quadruplex structures. Guanine-rich regions of DNA capable of forming G-quadruplex structures are found in a variety of chromosomal regions, including telomeres and promotor regions of DNA. Recently, it was demonstrated that a G-rich strand located within the proximal promoter region of oncogenes KRAS and C-MYC is able to form G-quadruplex structures that seem to be involved in the mechanisms of transcription regulation [22]. The study of natural as well as the design of

Table 1
Thermodynamic parameters determined with non-linear fitting of thiophosphoryl modified oligonucleotide UV melting curves.

Name	Number of modifications	ΔH, kJ/mol	T_m, °C	ΔS, J/mol	$\Delta G^{37\,°C}$, kJ/mol°K
TBA	0	−160	52	−492	−7,7
P1011	1	−166	49	−517	−6,3
P4	4	−200	40	−639	−2,1
P1415	1	−180	50	−558	−7,4
P111	2	−166	47	−520	−5,6
P511	2	−174	46	−545	−5,4
L1	1	−152	55	−464	−8,7
L11	2	−163	55	−497	−9,4
L121	4	−154	56	−467	−9,2
LL11	6	−104	54	−320	−5,6
LL	10	−163	55	−497	−9,4
L343	11	−130	46	−407	−3,7
L3	3	−154	52	−475	−7,5
SATR	14	−161	45	−506	−4,4

Solution contained 10 mM K-phosphate, pH 7.4, 0.1 M KCl. Standard deviation for ΔH and ΔS values was <5%, melting temperature were determined with 0.5 °C accuracy.

synthetic G-quadruplexes is useful for application in pharmacology as drug targets or mimics of protein ligands. Stabilization of a quadruplex structure is a pressing problem in these studies. There are several ways to increase G-quadruplex stability. At present, there are at least three separate groups of G-quadruplex-interactive compounds: anthraquinones, perylenes and porphyrins [23]. Another way is a special chemical design for G-quadruplex study. For example, it was discovered that LNA (Locked nucleic acid) backbone stabilizes G-quadruplex structure [24], but ribo-substitution for deoxy-

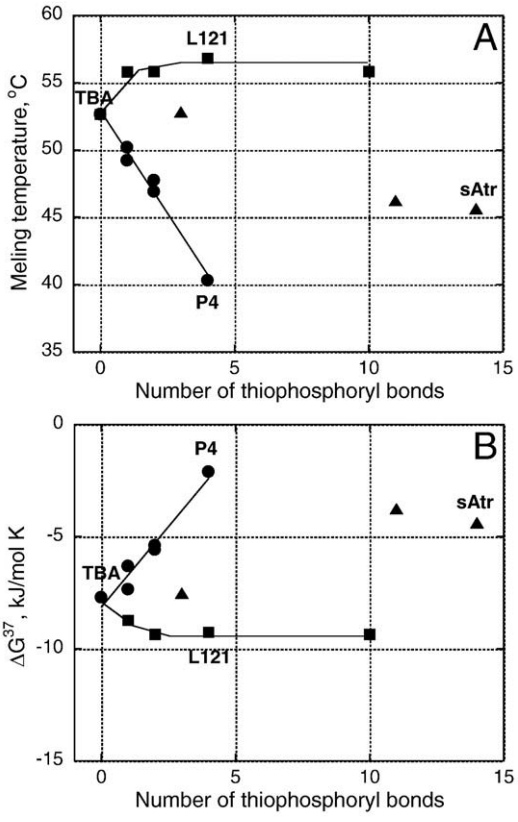


Fig. 5. Dependence of thermodynamic parameters of melting thiophosphoryl oligonucleotides on the number of the thio-modifications. The oligonucleotides modified only at GG bond are marked with filled circles, the modification only in loop regions are marked with filled squares, the oligonucleotides modified at both GG bonds and loop regions are marked with triangles. (A) melting temperature; (B) Gibbs free energy ΔG at 37 °C.

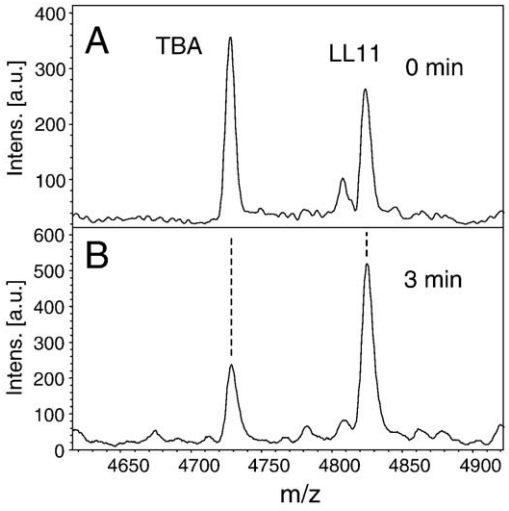


Fig. 6. MALDI MS spectra of TBA and LL11 in blood serum. Profile A — immediately after addition of both oligonucleotides to serum (0 min). Profile B — after 3 min of incubation at 37 °C.

backbone leads to changes in quadruplex structure [25]. The data reported here may contribute to a design of stable G-quartet-containing quadruplexes.

We investigated a number of thrombin-binding aptamer analogs with different thio- modifications of the sugar–phosphate backbone. All studied thio-modifications do not noticeably affect the oligonucleotide ability to fold in an antiparallel quadruplex. The effects of specific thio-modifications on aptamer stability were found. The substitutes placed between planes of G-quartets lead to a drop in formation free energy, and the stability decreases linearly with the number of these modifications. In contrast, the modifications in the loops connecting G4-quartets have no effect or even slightly increase quadruplex thermostability.

These data correlate with our biological tests. Oligonucleotides with a moderate number of modifications in both TT loops (such as LL11) did show very good thrombin time test results similar to TBA, while exhibiting better stability in blood serum compared to that of TBA. Although we have found that introduction of too many thio-groups led to the deterioration of antithrombin performance. A fully thiolated variant of TBA (SATR) had failed the thrombin test by causing immediate clogging, while an oligomer containing 10 thio-groups (LL) performed poorly. The plausible explanation is that aptamers containing a larger number of thio-groups (and thus are more hydrophobic) are prone to unspecific interactions with proteins, which trigger very fast blood clogging. It is worth noting that oligomers with a less stable conformation (such as P4, see Fig. 2) had demonstrated poor anticoagulation properties as well.

Thus, thiophosphoryl modifications of the sugar–phosphate backbone placed in the aptamer quadruplex loops allow constructing aptamer analogs with an intact stable quadruplex conformation, better stability in serum against nucleases (data not shown) and anticoagulation properties similar to TBA. Such TBA modifications can be valuable in medical applications.

Acknowledgments

This work was supported by Russian Foundation for Basic Research [07-04-00691a, 07-04-00645a and 09-04-12133-ofi_m]. The authors would like to thank M.A. Livshits for helpful discussion.

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