A circular dichroism study of the stability of guanine quadruplexes of thrombin DNA aptamers at presence of K^+ and Na^+ ions

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Abstract. The effect of K^+ and Na^+ on the properties of DNA aptamers that selective binds to fibrinogen (FIBRI) and heparin (HEPA) exosites of human α-thrombin was studied by circular dichroism (CD). The complexes of FIBRI-K^+ were slightly more stable than HEPA-K^+. However, lower stability was observed for HEPA-K^+ at presence of Na^+ in comparison with FIBRI-K^+. The analysis of CD melting curves suggests differences in thermal stability of both aptamers at presence of K^+. The melting temperatures (T_m) and changes in van't Hoff enthalpy for HEPA-K^+ complexes were lower in comparison with those for FIBRI-K^+. With increasing HEPA concentration the T_m value increased, but T_m did not change with increasing FIBRI concentration. This suggests formation of HEPA aggregates, while FIBRI aptamers are in monomeric form.

Key words: DNA aptamers — Circular dichroism — Guanine quadruplex — Potassium — Sodium

Introduction

DNA or RNA aptamers are synthetic, single-stranded oligonucleotides that fold up into unique three-dimensional structures, allowing them to bind specifically to other molecules, e.g. proteins, peptides (Nieuwlandt et al. 1995), amino acids (Geiger et al. 1996) or small molecules (Baker et al. 2006). They are prepared by iterative *in vitro* SELEX procedure (Ellington and Szostak 1990; Tuerk and Gold 1990). Aptamers are characterized with high affinity to their ligands that is comparable with those for antibodies. Increased interest to the aptamers is connected with their high potential for medical therapy and for biosensor development (Rimmele 2003; Centi et al. 2007; Hianik et al. 2007; Mairal et al. 2008). The DNA aptamer against thrombin was among the first developed (Bock et al. 1992). This aptamer is composed of 15 nucleotides of the sequence 5'-GGT TGG TGT GGT TGG-3' (FIBRI) and specifically binds thrombin at its fibrinogen-binding site (Wu et al. 1992). Later, Tasset et al. (1997) developed longer DNA aptamer against heparin-binding site of the thrombin of following sequence: 5'-A GTC CGT GGT AGG GCA GGT TGG GGT GAC T-3'. The underlined part (HEPA) corresponds to the binding motif of this aptamer that is also composed of 15 nucleotides. A unique peculiarity of these aptamers is formation of specific binding motif composed of guanine quadruplex (G-quadruplex). This is shown on Fig. 1 where the structures of FIBRI and HEPA are presented. It is seen that quadruplex consists of two guanine quartets that are stabilized by cyclic hydrogen bonds and connected by specific loops. In the case of FIBRI there are two TT loops and one TGT loop, while for HEPA T_4 is substituted by A_4 and GCA replaces TGT loop. So far the investigation of the structural peculiarities of DNA aptamers was mostly focused on FIBRI. It has been shown that this aptamer adopts an antiparallel chair-type G-quadruplex structure at presence of potassium ions (Macaya et al. 1993; Schultze et al. 1994; Marathias and Bolton 2000). Potassium is also required for inhibiting thrombin-clotting activity (Wang et al. 1993; Tsiang et al. 1995). The structure of this aptamer has been determined by NMR spectroscopy (Macaya et al. 1993; Wang et al. 1993; Schultze et al. 1994; Marathias and Bolton 2000) and those associated with thrombin by X-ray diffraction (Padmanabhan et al. 1993; Padmanabhan and Tulinsky 1996). Aptamer quadruplex conformation is stabilized also

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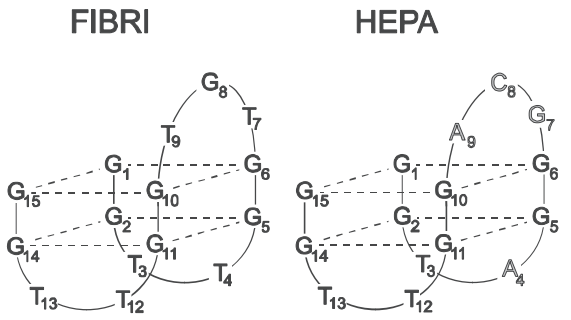

Figure 1. Structures of FIBRI and HEPA aptamer binding motifs.

at presence of other cations (Wang et al. 1995; Smirnov and Shafer 2000; Kankia and Marky 2001). Rather strong stabilizing effect on G-quadruplex was observed at presence of Sr^{2+}, Ba^{2+} and Pb^{2+} ions (Smirnov and Shafer 2000; Kankia and Marky 2001). However, sodium ions bind to the aptamer markedly weaker (Kankia and Marky 2001; Nagatoishi et al. 2007). This has been confirmed in particularly by circular dichroism (CD) spectroscopy. Molecular crowding (Nagatoishi et al. 2007) and decrease in melting temperature can support formation of G-quadruplex at deficiency of cations. In contrast with FIBRI, the properties of HEPA were studied in less extend. At the same time it has been pointed that above mentioned substitutions of nucleotides in the loops of HEPA should have unfavorable structural consequences on quadruplex. Since it is no longer allowed to form base pair between thymines at positions 4 and 13 in HEPA, some additional stabilizing factors are needed (Macaya et al. 1993). It has been therefore suggested that flanked sequences of spacers and duplex of 29-mer should be included in order to provide stability of quadruplex core (Tsiang et al. 1995; Tasset et al. 1997). However, this assumption has not been confirmed so far experimentally.

In this work we therefore performed comparative analysis of the properties of G-quadruplex in FIBRI and HEPA by means of CD spectroscopy at presence of potassium and sodium ions. We have shown that the effect of potassium cations on both aptamers is comparable and stabilize the G-quadruplex structure. However, in presence of sodium ions weaker binding of potassium to HEPA can be observed, whereas FIBRI-K^+ complex is only slightly affected. The thermodynamic analysis performed on the base of measurement of CD melting curves showed lower melting temperature (T_m), and van't Hoff enthalpy (ΔH_{vH}) for HEPA aptamer in comparison with those for FIBRI. Addition of Na^+ caused larger effect on the thermal stability of HEPA-K^+ complex in comparison with FIBRI-K^+. Moreover, with increasing HEPA concentration the T_m value increased, which suggests formation of aptamer aggregates that probably stabilize the G-quadruplex structure.

Materials and Methods

HPLC purified 15-mer oligonucleotides d(GGTTGGTGTGGTTGG) (FIBRI) and d(GGTAGGGCAGGTTGG) (HEPA) were purchased from Thermo Electron GmbH (Ulm, Germany). The concentration of oligonucleotide solutions was determined by measurement of absorbance at 260 nm and at 80°C using molar extinction coefficient of 146 l/(mmol·cm) for FIBRI and 158 l/(mmol·cm) for HEPA. These values were calculated by extrapolation of the tabulated values of the dimmers and monomer bases at 25°C to high temperatures (Cantor et al. 1970; Marky et al. 1983). Aptamers were suspended in 10 mmol/l or 20 mmol/l Tris, pH 7.4. KCl and NaCl (p.a. grade) were from Sigma (St. Louis, MO, USA). The absorbance was measured by Varian Cary 100 Bio UV-VIS spectrophotometer (USA). CD spectra were measured by JASCO J-810 spectropolarimeter (Japan) in a quartz cell with optical path length of 1 mm or 1 cm. The concentration of aptamer was 10 μmol/l in most experiments except thermal melting measurements as a function of aptamer concentration. The CD spectra were measured from 320 to 220 nm. The cell was thermostated by Peltier based electronic thermostat.

Binding isotherms

For analysis of binding of potassium ions to the aptamers the Langmuir isotherms were constructed and fitted using Origin7.5 according to the equation:

$$\Delta\theta = \frac{(\theta_{\max} - \theta_0)[K^+]}{K_D + [K^+]} \quad (1)$$

where $\Delta\theta = \theta - \theta_0$, θ is CD ellipticity at 292 nm and at corresponding equilibrium concentration of potassium ions, $[K^+]$, θ_0 is ellipticity of initial state, θ_{max} is CD ellipticity of final state of titration (saturation), and K_D is dissociation constant. Free energy of potassium binding was calculated using standard Gibbs equation $\Delta G = -RT \ln K_A$, where $K_A = 1/K_D$ is association constant.

CD melting

The melting curves were measured for each complex with ellipticity at 292 nm and the heating rate was 0.5–2°C/min. Thermal measurements were performed in a 10 mmol/l Tris-HCl buffer, pH = 7.4 and appropriate salt concentration of 50 mmol/l KCl and 140 mmol/l NaCl. The analysis of the melting curves yielded T_m and ΔH_{vH} values using two-state approximation (Marky and Breslauer 1987). Melting curves were fitted by equation:

$$\theta = \frac{\theta_F + \theta_U e^{(\Delta H_{vH}/R)(1/T - 1/T_m)}}{1 + e^{(\Delta H_{vH}/R)(1/T - 1/T_m)}} \quad (2)$$

where θ is ellipticity at 292 nm, θ_F and θ_U are ellipticities of folded and unfolded state, respectively. Transition temperature T_m is a midpoint temperature of the order-disorder transition and ΔH_{vH} is a van't Hoff enthalpy change.

Results and Discussion

As it has been shown previously, formation of G-quadruplexes can be clearly identified by CD spectroscopy (Lu et al. 1993). Two basic CD patterns that differ from those of double-stranded DNA are usually observed: quadruplex with antiparallel strand orientation exhibits positive Cotton effect around 295 nm and negative around 265 nm in contrast with parallel type, which exhibits positive band around 265 nm and negative around 240 nm (Lu et al. 1993).

In the first series of experiments we measured the CD spectra of the two aptamers at presence of various concentrations of potassium ions. Fig. 2a shows the dependence of CD spectra for FIBRI aptamer. In absence of potassium ions the peak amplitudes of the spectra are low, indicating small content of G-quadruplexes. With the increasing in K^+ concentration both positive peak around 292 nm and negative peak around 267 nm increases. This indicates formation of antiparallel strand orientation in G-quadruplexes (Lu et al. 1993). Two isoelliptic points at 257 nm and 278 nm indicate two-state nature of structural transitions upon binding of potassium ions.

Obtained results are consistent with previous CD studies of the properties of G-quadruplexes of FIBRI aptamer (Smirnov and Shafer 2000; Kankia and Marky 2001; Nagatoishi et al. 2007). Formation of G-quadruplex structure was also estimated in molecular crowding conditions at presence of polyethyleneglycol and at lower temperatures (Nagatoishi et al. 2007). The molecular crowding and low temperatures had no influence on the shape of CD profiles, but only on their magnitudes. This indicates that FIBRI aptamer is very stable in antiparallel strands conformation. The CD spectral characteristics for HEPA aptamer were not studied so far. In absence of potassium, CD spectra showed low band amplitudes as this is seen from Fig. 2b. However, with increasing K^+ concentration the amplitude of positive and negative bands increased. The sharp maximum at 292 nm and deep minimum around 267 nm indicates formation of antiparallel G-quadruplex. The presence of two isoelliptic points at 254 and 278 nm suggests two-state process of quadruplex folding. This observation shows that at presence of K^+ the quadruplex structure is cooperatively folded and that each addition of potassium caused complete folding of a certain fraction of quadruplexes, while the remaining molecules stay unfolded. This mechanism can be suggested for both thrombin-binding aptamers.

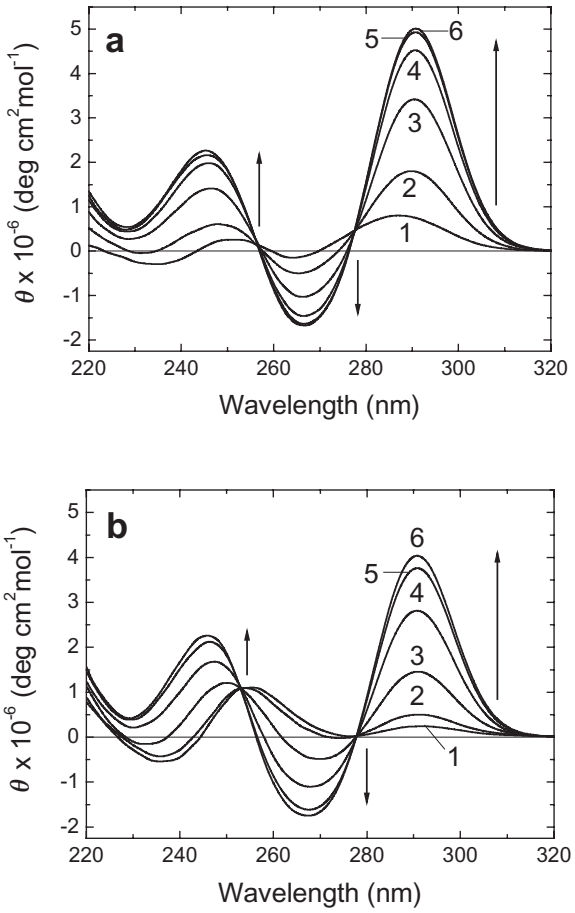

Figure 2. CD spectra of thrombin binding aptamers FIBRI (a) and HEPA (b) in the absence and at the presence of potassium ions at various concentrations (in mmol/l): 0 (line 1); 0.04 (line 2); 0.24 (line 3); 1 (line 4); 5.8 (line 5); 26 (line 6) in 10 mmol/l Tris-HCl buffer, pH7.4 at 25°C. The concentration of both aptamers was 10 μmol/l. The arrows indicate the direction of increase in K^+ concentration.

The comparison of CD spectra for FIBRI and HEPA aptamers suggests qualitatively similar interaction of quadruplexes with potassium ions. In order to estimate binding affinity of potassium to aptamer, the quantitative analysis was performed. The spectral data were analyzed at a wavelength 292 nm as a function of salt concentration and were used for construction of a binding curve, as it is presented on Fig. 3. It is seen that the binding curves for FIBRI and HEPA are similar. However, the broader curve for HEPA suggests weaker binding of potassium ions to the quadruplexes. Based on the analysis of these curves using Eq. (1) we estimated dissociation constants and determined changes in Gibbs energy (ΔG). These values are presented in Table 1. As it is seen from this table, at T = 25°C the dissociation constant for FIBRI is approx. four times lower in comparison with HEPA. This suggests higher stability of

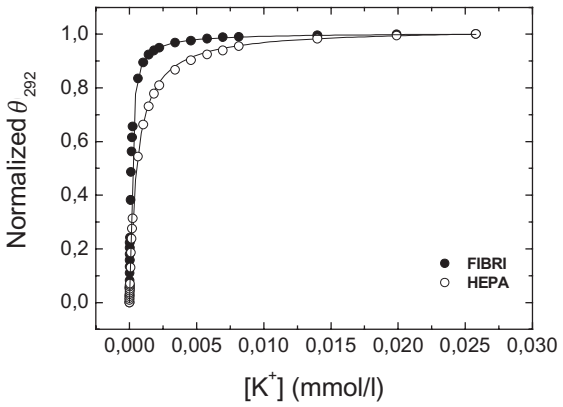

Figure 3. The Langmuir plot of normalized amplitude of CD spectra at 292 nm as a function of concentration of K^+ for FIBRI and HEPA aptamers. The symbols are experiments and the full lines represent fit according to Eq. (1). The concentration of aptamers was 10 μmol/l.

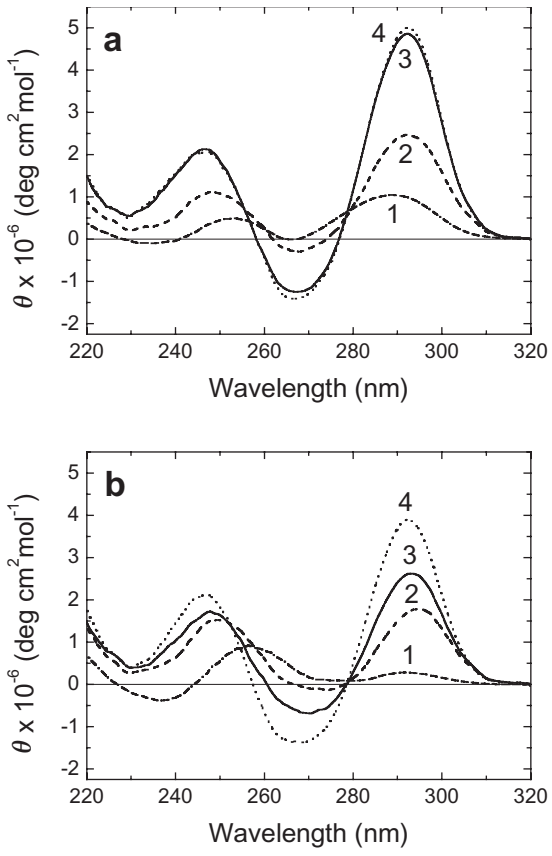

Figure 4. CD spectra of thrombin binding aptamers FIBRI (a) and HEPA (b) in 20 mmol/l Tris-HCl buffer, pH 7.4 at 15°C in absence of potassium ions and sodium ions (line 1) and at presence of 140 mmol/l NaCl and at various concentrations of potassium ions (in mmol/l): 0 (line 2); 2 (line 3); 20 (line 4). The concentration of aptamers was 10 μmol/l.

FIBRI-K^+ complexes in comparison with those for HEPA. Values of Δ*G* determined for FIBRI aptamer correspond to data published by Kankia et al. (2005) that used isothermal titration calorimetry to determine the thermodynamic parameters for FIBRI quadruplex folding. Reported value of Δ*G* for this system in 10 mmol/l Cs-Hepes buffer was –23.0 kJ/mol, which is in good agreement with our results of –22.1 kJ/mol (Table 1). Slight deviation might be caused by different buffer solutions used. For HEPA aptamer the Δ*G* value was estimated on –18.0 kJ/mol and reflects subtle hindered K^+-mediated formation of quadruplex in comparison with FIBRI. As we mentioned above, the structure of HEPA differs from that of FIBRI in positions 4, 7, and 9, where thymine residues were substituted by purines and in position 8, where guanine was substituted by cytosine (Fig. 1). Marathias and Bolton (1999) studied the effect of substitution of thymines by adenine residues in FIBRI aptamer by NMR spectroscopy. They studied the binding ability of the potassium ion to the FIBRI and those with certain nucleotide substitution. From one-dimensional NMR spectra obtained by means of potassium titrations of FIBRI with adenine on positions 3, 4, 7 or 9, which has the sequence similar to HEPA, they did not find any well-defined quadruplex structure. Therefore, it was

Table 1. The constant of dissociation (K_D) and changes of Gibbs energy (Δ*G*) connected with formation of G-quadruplexes of FIBRI and HEPA aptamers at presence of potassium and sodium ions determined from Langmuir isotherms (Fig. 3) according to Eq. (1) and Gibbs equation $\Delta G = -RT\ln K_A$, where $K_A = 1/K_D$. The pH of buffers was 7.4. Results represent mean ± S.D. obtained from 3 independent experiments in each series

Buffer composition	*T* (°C)	FIBRI		HEPA	
		K_D (μmol/l)	Δ*G* (kJ/mol)	K_D (μmol/l)	Δ*G* (kJ/mol)
10 mmol/l Tris-HCl	25	130 ± 1.0	–22.1 ± 0.2	540 ± 5.0	–18.0 ± 0.2
10 mmol/l Tris-HCl	15	30.0 ± 1.0	–24.9 ± 0.8	180 ± 10.0	–20.6 ± 1.1
20 mmol/l Tris-HCl + 140 mmol/l NaCl	15	35.0 ± 0.3	–24.5 ± 0.2	3350 ± 250	–13.6 ± 1.0

concluded that substitution of a purine for a thymine in any loop positions, other than position 8, would disrupt binding of potassium by this aptamer. Thus, based on these studies it can be expected that hindered quadruplex formation for HEPA upon potassium binding should take place. However, based on CD spectra of HEPA at presence of K^+ (Fig. 2b) one can see only by 15% lower band amplitudes in comparison with FIBRI. This suggests potassium-mediated G-quadruplex formation. Obtained results are different in comparison with conclusion based on NMR study mentioned above. The main reason of this discrepancy can be explained by different experimental conditions. NMR spectra were acquired at 15°C in 20 mmol/l Tris with 140 mmol/l sodium ions. At this temperature certain fraction of quadruplexes is folded (Baldrich et al. 2004; Nagatoishi et al. 2007). However, the presence of sodium ions in a concentration of 50 mmol/l affects the quadruplex folding (Kankia and Marky 2001). Similarly the CD spectra presented on Fig. 4, for FIBRI (a) and HEPA (b) at presence of 140 mmol/l NaCl indicate that about half of quadruplexes are folded in these conditions for both aptamers. After addition of 2 mmol/l KCl to FIBRI, folded fraction of this aptamer increased up to 97% and the structure was almost saturated. In the case of HEPA (Fig. 4b) the situation was different. In contrast with FIBRI, at 2 mmol/l KCl, the folded fraction of HEPA increased only by 35%. This result coincides with those obtained in NMR study of FIBRI with substituted thymines (Marathias and Bolton 1999). At 20 mmol/l KCl, CD spectra of HEPA reached the saturation. It is important to emphasize, that at each temperature or buffer composition used the CD spectra of both aptamers were nearly identical after saturation by potassium. In order to study the effect of lower temperature and the presence of sodium ions on binding of K^+ to aptamers we measured CD spectra also at 15°C in absence and presence of Na^+ and performed quantitative analysis of potassium binding based on Langmuir isotherms (Table 1). As it is seen from this table, low temperature has favorable effect on formation of the aptamer-potassium complexes as it is revealed by increase in ΔG absolute value for both aptamers. At the presence of sodium ions, changes in ΔG were more remarkable. The affinity of FIBRI aptamer to K^+ at 15°C was only slightly affected by Na^+. However, HEPA exhibited unfavorable changes at presence of Na^+ both on K_D and ΔG. It is seen from the Table 1, that sodium ions inhibit interaction with K^+, which result in increase of K_D and in decrease of absolute value of ΔG. This observation supports previous study that presence of the adenosines in telomeric sequence affects the quadruplex formation and its affinity to sodium and potassium ions (Guo et al. 1992).

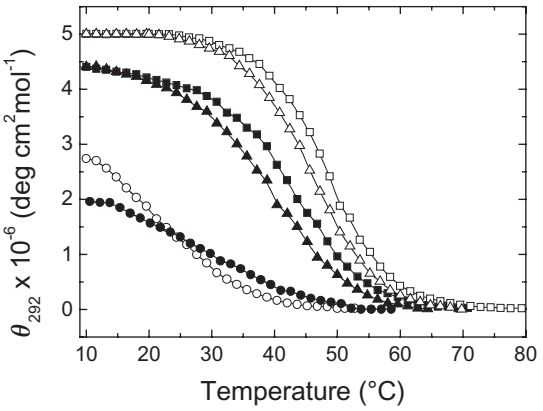

Figure 5. The plot of the ellipticity at wavelength 292 nm as a function of temperature for FIBRI (open symbols) and HEPA (closed symbols) in 10 mmol/l Tris-HCl buffer, pH 7.4 in presence of 140 mmol/l NaCl (circles), 50 mmol/l KCl (squares) and 140 mmol/l NaCl + 50 mmol/l KCl (triangles). The heating rate was 1°C/min. The concentration of both aptamers was 10 μmol/l.

For further comparison of both aptamers we investigated their thermal stability from CD melting profiles at presence of either 140 mmol/l NaCl or 50 mmol/l KCl or both 140 mmol/l NaCl + 50 mmol/l KCl. The plot of the amplitude of ellipticity at 292 nm as a function of temperature for both aptamers is presented on Fig. 5 where all melting curves have sigmoidal shape and with increasing of the temperature the CD magnitudes decrease. Using the data presented at Fig. 5 we estimated T_m and ΔH_{vH} of quadruplexes by means of fitting the experimental results using Eq. (2). Melting profiles in 50 mmol/l KCl for both species were independent on heating rate and no hysteresis was observed (results are not shown). T_m and ΔH_{vH} for FIBRI aptamer agrees with the data reported by Kankia and Marky (2001). At 50 mmol/l KCl HEPA aptamer showed lightly lower value of T_m and ΔH_{vH}, which indicate lower thermal stability of HEPA-K^+ complex. The results of thermal melting analysis are summarized in Table 2. As it is seen from this table, at presence of

Table 2. The transition temperature (T_m) and van't Hoff enthalpy changes (ΔH_{vH}) determined from melting curves recorded in 10 mmol/l Tris-HCl buffer, pH 7.4 containing 50 mmol/l KCl, or 50 mmol/l KCl + 140 mmol/l NaCl estimated by fitting the experimental results using Eq. (2)

Buffer composition	FIBRI		HEPA	
	T_m (°C)	ΔH_{vH} (kJ/mol)	T_m (°C)	ΔH_{vH} (kJ/mol)
50 mmol/l KCl	49.2 ± 0.1	168.9 ± 2.1	45.1 ± 0.3	146.2 ± 2.5
140 mmol/l NaCl + 50 mmol/l KCl	46.4 ± 0.5	157.5 ± 0.4	41.2 ± 0.1	127.4 ± 1.3

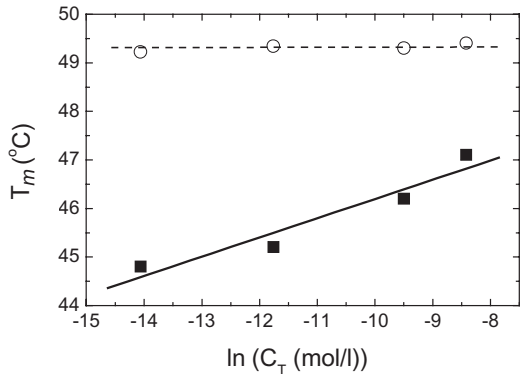

Figure 6. The plot of T_m as a function of the concentration of DNA aptamers (C_T) (in logarithmic scale) for FIBRI (○) and HEPA (■) in 10 mmol/l Tris-HCl buffer, pH 7.4 and 50 mmol/l KCl. The values of T_m were obtained from melting curves like those presented on Fig. 5 with a heating rate 1°C/min.

140 mmol/l NaCl, T_m decreased for both aptamers. However, thermodynamic values of HEPA were more affected in comparison with those for FIBRI. The T_m for FIBRI-K^+ complex at presence of Na^+ was 46.4°C, which is by 2.8°C less in comparison with those without Na^+. T_m value for HEPA-K^+ at presence of Na^+ has decreased by 3.9°C in comparison with those without Na^+. Presence of Na^+ also caused decrease in enthalpies of G-qudruplex melting by 11.4 kJ/mol and 18.8 kJ/mol for FIBRI and HEPA, respectively (see Table 2). This suggests larger influence of sodium ions on the stability of HEPA-K^+ complex in comparison with those for FIBRI-K^+. From Fig. 5 one can be seen that T_m values of aptamers at presence of Na^+ are markedly lower in comparison with those at presence of KCl. Therefore, decrease in thermal stability at presence of K^+ and Na^+ ions is probably caused by competition between these two cations.

Different thermodynamic properties of both aptamers follow also from analysis of their transition temperatures as a function of aptamer concentrations. The plots of the T_m as a function of the concentration of both FIBRI and HEPA are presented in Fig. 6. It is seen from this figure, that T_m for HEPA increases with increasing aptamer concentration while for FIBRI the T_m did not depend on the aptamer concentration. This phenomenon for FIBRI was reported earlier and suggests that this aptamer is in monomer form at wide concentration range (Kankia and Marky 2001; Tang and Shafer 2006). The increase in T_m with increasing HEPA concentration suggests formation of the aptamer aggregates (Marky and Breslauer 1987). Thus, HEPA probably form dimmers or higher aggregates that could stabilize the G-quadruplexes. We should mention, that in paper by Fialova et al. (2006) it has been suggested, that also FIBRI forms complexes. However, this has been showed for larger aptamer concentrations (0.01–1 mmol/l) in comparison with those used in our study (0.8–200 μmol/l).

Thus, our results confirm assumption on a less stability of HEPA aptamers in comparison with FIBRI especially at presence of sodium ions that is caused by substitution of certain nucleotides at loops of HEPA. However, at presence of potassium ions, the differences between stabilities of both aptamers are less expressed. Moreover, at presence of sufficient concentration of potassium ions (at least 2 mmol/l) substantial part of HEPA molecules forms stable G-quadruplexes. The sodium ions have disturbing effect on G-quadruplexes, especially for HEPA aptamers. However, potassium ions preserve the quadruplex conformation. On the other hand, our results show that the changes in oligonucleotide sequences in a loop of HEPA substantially affect the behavior of these aptamers, which results in their aggregation. The reason of this effect is not clear and requires further study. From practical point of view, the formation of stable quadruplexes of HEPA at certain ionic conditions need not supporting oligonucleotide sequences for stabilizing the aptamer conformation. Thus, for therapeutic or biosensing purposes the HEPA aptamers can be shorter and hence less expensive.

Acknowledgements. This work was supported by European Commission 6 FP Program STREP DVT-IMP (Contract FP6-2005-034256), by the Slovak Grant Agency (projects No. 2/6167/06 to M. A. and No. 1/4016/07 to T. H.) and by Comenius University (project G-08-228-00 to S. P.).

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Received: August 5, 2008
Final version accepted: August 15, 2008