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# G-Quadruplex Formation of Thrombin-Binding Aptamer Detected by Electrospray Ionization Mass Spectrometry

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The structure and properties of G-quadruplexes (Scheme 1) formed by oligodeoxynucleotides (ODNs)[1] are of interest because they exist in telomeric DNA and are stabilized by interactions with other molecules and ions, blocking telomeric activity and providing a basis for antitumor drug design.[2,3] The basic unit of a quadruplex is a quartet of guanine bases, stacked and held together by $\pi-\pi$ interactions. A recent crystal structure[4] shows that the loops holding together the G-quartets of telomere models are "propeller shaped", possibly explaining how they can be recognized by proteins.[4b] DNA aptamers also may contain G-quadruplex structures that allow them to bind to a desired target.[5] For example, the thrombin-binding, 15-mer aptamer d(GGTTGGTGTGGTTGG) specifically binds thrombin protein and inhibits thrombin-catalyzed fibrin-clot formation.[6]

Studies with circular dichroism, temperature-dependent UV spectroscopy, differential scanning calorimetry, isothermal titration calorimetry, and NMR[7−9] established the property of the 15-mer aptamer to form intramolecular G-quadruplex structures in the presence of various metal ions. There are only three reports, however, on mass spectrometric studies of G-quadruplexes in the gas phase: two on the observation of molecular ions of G-quadruplexes under electrospray ionization (ESI) conditions and one on their interaction with antitumor drugs.[10,11] Here we report for the first time evidence that ESI MS detects the formation of the G-quadruplex of the thrombin-binding aptamer in solution and establishes some of its properties in the gas phase, particularly its specific interaction with metal ions.

ESI of the thrombin-binding aptamer introduces into the gas phase $[M - nH]^{n-}$ ($n = 3-8$) ions.[12] In addition to the multiply deprotonated molecules, we expect ions in which $H^+$'s are replaced with alkali metal ions, if available. The key question is whether the metal-ion binding is specific. Many polar compounds undergo nonspecific, metal-ion binding upon desorption or spray ionization. Nonspecific addition would easily occur by the replacement of phosphate protons of the ODN. To determine if the metal-ion binding is specific, we selected d(AATTAATGTAATTAA), d(AGTTAGTGTAGTTAG), and d(GATTAGTGTGATTAG) as controls for which we replaced G bases with adenine. If the carbonyl groups of the guanine bases do coordinate to the metal ions to form a G-quadruplex, then those ODNs having that structure should form specific adducts to a greater extent than those that bind nonspecifically.

These control ODNs bind many $Li^+$, $Na^+$, $K^+$, and $Cs^+$ ions, as was seen by a cluster of ions in which addition of more than one metal ion occurs for lower charge states ($3^-$ and $4^-$) (data not shown), indicating that the binding is nonspecific. Addition of $Rb^+$ ion to the aptamer and to the control ODNs in various charge states is not significant.

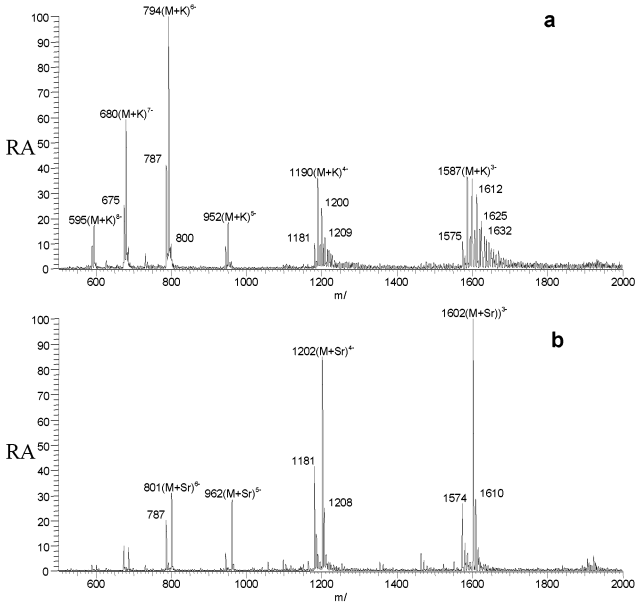
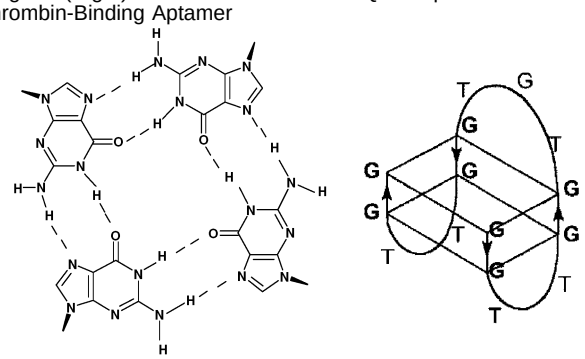



***Figure 1.*** Negative-ion electrospray mass spectra of the aptamer in the presence of KCl (a) and $SrCl_2$ (b). RA is relative abundance.

***Scheme 1.*** The Guanine Quadruplex (Left) and Schematic Diagram (Right) of the Intramolecular G-Quadruplex of the Thrombin-Binding Aptamer




In the case of $K^+$ (Figure 1a), however, we found evidence for enhanced adduct formation of the aptamer with one $K^+$; these adducts exist in the high charge states ($6^-$ to $8^-$). The control ODNs do not show such adduct formation. The addition of $Na^+$ to the aptamer may be partly specific, but the specificity is considerably lower than that exhibited by $K^+$ binding.

The divalent alkaline-earth metal ions $Mg^{2+}$ and $Ca^{2+}$ only interact weakly with the aptamer and the control ODNs. $Sr^{2+}$ (Figure 1b) and $Ba^{2+}$, however, clearly form abundant adducts with the aptamer, but not with the controls, pointing to stability and indicating specific interactions in this aptamer's binding (data not shown). The extent of adduct formation is similar for $Ba^{2+}$ and $Sr^{2+}$. Ion abundances show that adduct formation is more favorable

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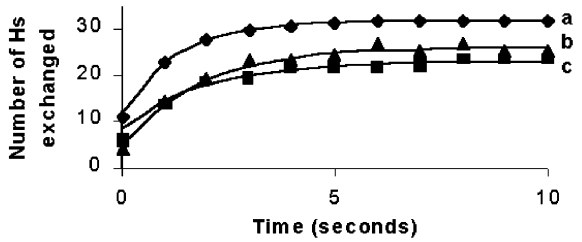


***Figure 2.*** The time-dependent number of H's exchanged with $CD_3OD$ in the gas phase for $(M - 7H)^{7-}$ (a), $(M - 8H + K)^{7-}$ (b), and $(M - 9H + Sr)^{7-}$ (c). The data are fit (solid curves) with two rel. rate constants of 1 and $10^5$; $P_{CD3OD}$ is unknown). The number of fast-exchanging H's is 12, 5, 8, whereas the slow-exchanging H's are 20, 21, 14 for (a), (b), (c), respectively. Only 50% of the experimental points are shown to avoid congestion.

for $Sr^{2+}$ and $Ba^{2+}$ ions than it is for $K^+$ (data not shown). These interpretations are consistent with those reached by using optical spectroscopy.[7]

Intrigued by these results, we studied in more detail the ability of the aptamer to bind $Sr^{2+}$. A 10-fold increase in the concentration of $Sr^{2+}$ (to 50 $\mu$M) with respect to that of the aptamer (5 $\mu$M) does not lead to any significant additional binding of $Sr^{2+}$. The abundance of the $(M + Sr)^{n-}$ adduct increases nearly linearly from 0 to 15% when the methanol concentration goes from 0 to 25% (by vol). We suggest that a decrease in the dielectric constant of the solution causes $Sr^{2+}$ to bind more effectively. We observe a similar effect when we used acetonitrile in place of methanol. The extent of adduct formation with $Sr^{2+}$ at different charge states does not vary significantly with respect to the changes in the concentration of methanol or acetonitrile.

Others reported that $Pb^{2+}$ and $Mn^{2+}$ ions also bind to this aptamer in its quadruplex form.[9,13] Our experiments with $PbCl_2$ and $MnCl_2$ show that $Pb^{2+}$ does form an adduct with the aptamer, although it is less abundant than those of $Ba^{2+}$ and $Sr^{2+}$. We draw this conclusion because, in a mixture containing equimolar amounts of $Ba^{2+}$, $Sr^{2+}$, and $Pb^{2+}$, the adduct with $Pb^{2+}$ is barely detectable. An adduct with $Mn^{2+}$ is also undetectable. The control ODNs do not form detectable adducts with $Pb^{2+}$ or $Mn^{2+}$. Others demonstrated, using NMR, that $Tl^+$ can replace $K^+$ in stabilizing the G-quadruplex structure, possibly because the ionic radii of the two ions are comparable.[14] We find that the binding with $Tl^+$, however, is insignificant.

Various measurements show that metal ions occupy the cavity between the two quadruplex structures formed by the eight guanine moieties and bind by coordination with the eight carbonyl groups of the guanines. Although the MS results presented thus far do not pinpoint the location of the metal ion, they do indicate that the interaction of the metal with the thrombin-binding aptamer is specific. The bonding in the gas phase is likely to be via the G-quadruplex form because $Sr^{2+}$, $Pb^{2+}$, $Ba^{2+}$, and $K^+$, which show enhanced binding, have similar ionic radii (1.26, 1.29, 1.42, and 1.51 Å, respectively).[15] When involved in eight-coordinate bonding, $Ba^{2+}$, $Sr^{2+}$, and $K^+$ do form quadruplex structures in solution.[7] The lack of a smooth correlation between the ionic radii and the extent of adduct formation for these four metal ions suggests that the size of the metal ions is not the only factor in stabilizing the intramolecular G-quadruplex structure. Nevertheless, ESI MS does offer another sensitive method for studying G quadruplex structures.

We then turned to a direct probe of the gas-phase structure, H/D exchange. When the thrombin-binding aptamer, with and without $K^+$ or $Sr^{2+}$, is stored in the ion trap as a function of time, the adducts undergo a time-dependent increase in H/D exchange with deuterated methanol (Figure 2). The $CD_3OD$ was admitted with the helium buffer gas and held at a constant, low partial pressure in the trap. The number of active H's in the $7^-$ state of the aptamer is 41, which are the maximum that can exchange. At the longest time (10 s), 32 exchanges occurred for the aptamer, but approximately 6 and 9 fewer exchanges occurred for the potassium and the strontium adducts, respectively. This additional protection is evidence that the interactions with these metal ions stabilize additional H's because of the specific binding of the G-quadruplex in the gas phase. Further work to establish the G-quadruplex structure in this and in related systems, using the H/D-exchange probe, is in progress.

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(12) All mass spectrometry experiments were conducted using a Finnigan LCQ quadrupole ion-trap mass spectrometer under negative-ion ESI conditions. The spray voltage was 2.2 kV, and the capillary was at 200 °C. The aptamer and other ODNs were from IDT, Coralville, IA, and were used without further purification. The 10 $\mu$M samples were prepared in deionized water. Metal chlorides were used as sources for the metal ions except for $Rb^+$ and $Cs^+$ where iodides were used. Metal-salt solutions of 10 $\mu$M concentration were prepared in water:methanol (1:1) and 1% $NH_3$. Equal volumes of the ODN and the metal-salt solutions were mixed and then introduced into the ESI source with a syringe pump at 5 $\mu$L/min. For H/D exchange, 2.5 $\mu$M samples were used, and the sample solutions were sprayed at 1 $\mu$L/min. Experiments were in the MS/MS mode using "zero" for the activation amplitude and changing the activation time from 0.03 to 10 000 ms. Five scans were averaged at each time point, the averaged spectrum was smoothed, and the centroids of the peak distribution, which represented D uptake, were used for the modeling.

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