Combinatorial Synthesis of Thrombin-Binding Aptamers Containing iso-Guanine

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A library of all possible substitutions of guanine by iso-guanine (iG) in the thrombin aptamer was prepared by split and mix synthesis. A colorimetric assay was used to screen for functional oligomers in the library. Colorimetrically active oligonucleotides were selected and sequenced by the Maxam−Gilbert method. The sequenced oligonucleotides were individually resynthesized, and their affinities for thrombin were assayed by isothermal titration calorimetry. Three aptamer sequences containing iG were found to have enhanced binding activity to human α-thrombin compared to the parent aptamer.

In nature, DNA primarily functions as a genetic blueprint, transcribed into mRNA such that a particular gene may subsequently be expressed. However, oligonucleotides have demonstrated versatility well beyond their classical role as genetic information carriers, including in gene silencing,[1] antisense therapy,[2] RNA interference,[3] ribozymes and DNAzymes,[4] riboswitches,[5] and aptamers.[6] Many of these functions require control of tertiary structure. Analogous to protein-based enzymes, ribozymes and DNAzymes require a folded tertiary structure for proper function of their catalytic domain. Aptamers are oligonucleotides which, when properly folded, show binding specificity.

In 1992, Bock et al. selected a thrombin-binding aptamer from a pool of $\sim 10^{13}$ DNA 96-mers.[7] The consensus sequence was determined to be 5′-d(GGTTGGTGTGGTTGG)-3′ and was found to inhibit thrombin-catalyzed fibrin-clot formation with a K_i of approximately 120 nM.[8] The thrombin aptamer structure was determined by two groups through solution NMR studies as a quadruplex with two G-quartets as shown in Figure 1, panels A and B.[9,10] The X-ray crystal structure of the thrombin aptamer bound to α-thrombin has also been determined.[11,12]

iso-Guanosine is a naturally occurring nucleoside first reported by Cherbuliez and Bernhard in 1932[13] that has proven to be as capable of forming tetraplex structures as its isomer, guanosine (Figure 2, panel A and panel B).[14,15] In 1999, Chaput and Switzer demonstrated that DNA strands bearing iso-guanine also have the capacity to form pentaplexes (Figure 2, panel C).[16] Because of the versatility of iso-guanine (iG) in the formation of higher-order secondary structures, including an ability to form mixed tetraplexes with guanine,[15] we elected to conduct a combinatorial exploration of its properties in thrombin aptamers. Optimization of thrombin aptamer properties could help fulfill the need for alternative antithrombotic agents.[17] In addition, given that iso-guanine forms a nonstandard Watson−Crick base-pair with iso-cytosine, our study addresses the fitness of this pair with respect to a hypothetical RNA World by exploring the ability of one of its component bases to form functional macromolecules.

Results

Library synthesis was conducted on a PEGA support appended with a photocleavable linker based on the veratrole *o*-nitrobenzyl group, which upon irradiation liberates oligonucleotides as a 3′-phosphate. The linker as its carboxylate **1** was prepared as described by McMinn et al.[18] and coupled to PEGA with TBTU and *N*-ethylmorpholine in DMF as described by Will et al. (Figure 3).[19] Unreacted amino groups on the support after coupling to **1** were capped by overnight exposure to a 1:1 mixture of Cap-A (THF/Ac_2O, 9:1) and Cap-B (10% N-methylimidazole in THF) DNA synthesis reagents. Quantification of the support bound linker was performed spectrophotometrically by monitoring the extinction coefficient of the dimethoxytrityl cation after cleavage of a sample with 0.1 M *p*-toluenesulfonic acid in acetonitrile,[20] and determined to be 240 μmol/g.

A one bead−one sequence library was synthesized on the derivatized PEGA support **2** using an ABI 391 DNA synthesizer based on the consensus thrombin aptamer

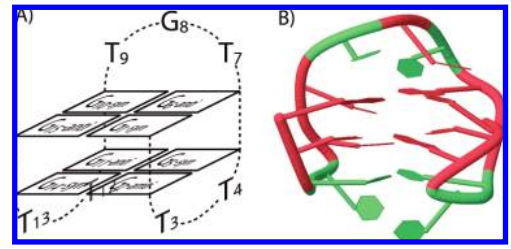

Figure 1. (A) Solution G-quadruplex structure adopted by the thrombin aptamer. (B) NMR structure (148D, Protein Data Bank (PDB)) of the thrombin aptamer.[9]

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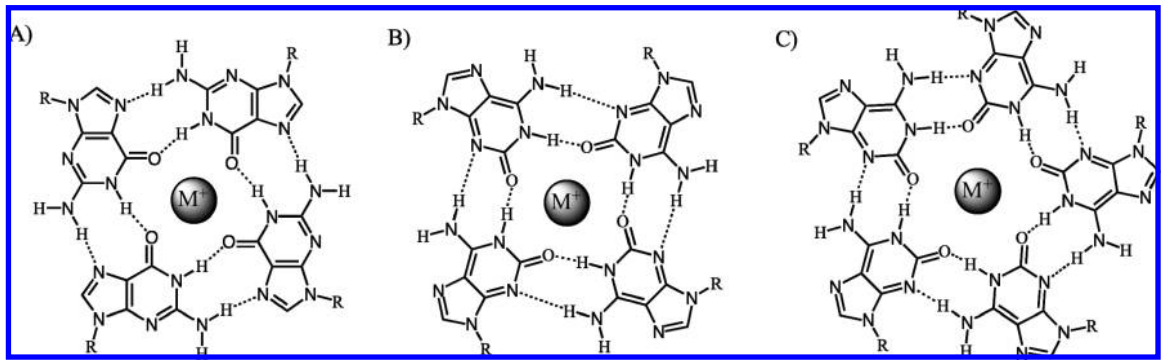

Figure 2. (A) Tetramer association of guanine, (B) tetramer association of iso-guanine, and (C) pentamer association of iso-guanine, in the presence of monovalent alkali cations.

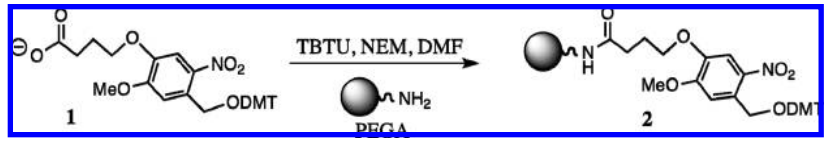

Figure 3. Attachment of the photocleavable linker **1** to PEGA to create the support used for library synthesis.

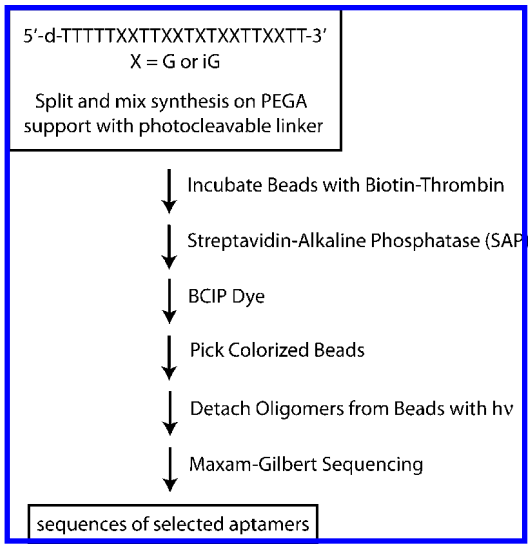

Figure 4. Steps used in selection of active aptamers from a one bead−one sequence library. BCIP = 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt.

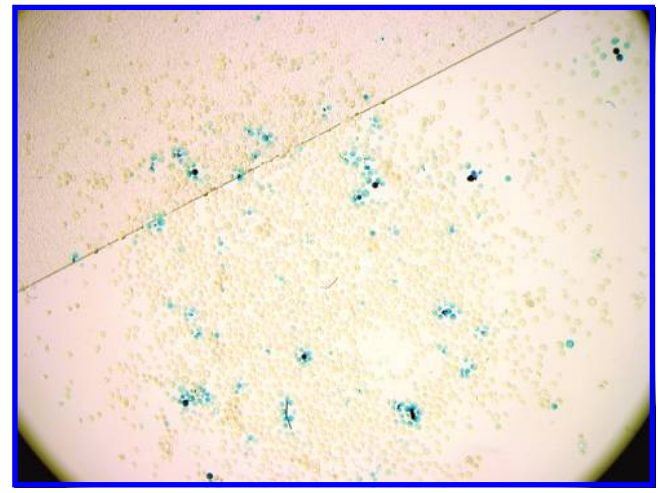

Figure 5. Photograph of a one bead−one sequence thrombin aptamer library after incubation with BCIP dye.

sequence and random substitution of 2′-deoxy-guanosine residues by 2′-deoxy-iso-guanosine. The library is expected to comprise an anticipated total of 512 different sequences. A summary of aptamer selection from the one bead−one sequence library is shown in Figure 4. "T-tails" were added to both terminii of the thrombin aptamer sequence during library synthesis to enable Maxam−Gilbert sequencing of selected beads. In practice, the addition of five T residues to the 5′-terminus and two residues to the 3′-terminus of the thrombin aptamer were sufficient to allow sequencing bands to be clearly distinguished for the G/iG variable regions.

The library was screened for human α-thrombin binding by adapting the protocols reported for screening a one bead−one compound combinatorial peptide library.[21] Thus, after library synthesis and oligonucleotide deprotection with NH_4OH, the library beads were incubated for 24 h with a 1−4.86 μM solution of α-thrombin conjugated with biotin at 4 °C. Following this incubation, the beads were washed thoroughly with buffer and were subsequently incubated with an 8.6 nM solution of streptavidin−alkaline phosphatase at 4 °C for 2 h. The beads were then washed with buffer once again, followed by exposure to BCIP dye. After 9 h of exposure to the dye solution, 26 colored beads were picked by hand, of which 9 beads were dark-, 7 were medium-, and 10 were light-blue in color. A representative photograph of a library is shown in Figure 5.

The selected beads were deproteinized by exposure to 8 M guanidinium hydrogen-chloride at 90 °C for 10 min. Photocleavage of aptamers from the support of individual picked beads was performed by irradiation for 45 min with a Rayonet reactor at 350 nm with a Pyrex filter. After photocleavage, aptamers were kinased to introduce a ^{32}P-label at the 5′-end, and then they were sequenced by an adaptation of the Maxam−Gilbert method. Despite the addition of five T residues to the 5′-terminus, it was still necessary to add myosin (ATPase) after the kinase reaction to achieve sufficient sensitivity with a phosphorimager for single-bead sequencing because otherwise the background contributed by unreacted gamma-^{32}P-ATP was found to obscure bands derived from the variable residues nearest the 5′-end. Maxam−Gilbert sequencing was conducted using

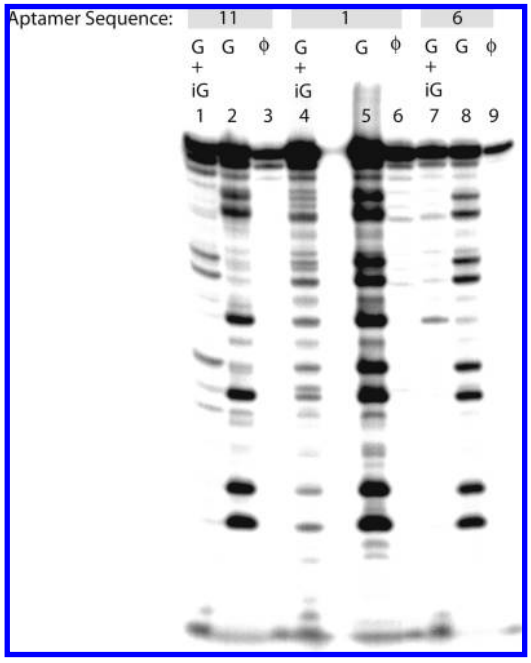

Figure 6. Maxam−Gilbert sequencing gel results for aptamer sequences **1**, **6**, and **11**. The lanes labeled "G + iG" correspond to "G + A" Maxam−Gilbert reaction conditions. The lanes labeled ϕ correspond to aptamer alone.

Table 1. Aptamer DNA Sequences and Data from ITC Studies

aptamer	sequence	K_d (nM)	n	ΔG°_{37} (kcal/mol)
1[a,b]	GGTTGGTGTGGTTGG	94.4 ± 26.6	1.13	−10.0
2[c]	GGTTGGT**T**TGGTTGG	76.4 ± 18.6	1.39	−10.1
3[c]	GGTTGGT**C**TGGTTGG	105.2 ± 20.8	1.37	−9.93
4[c]	GGTTGGT**A**TGGTTGG	72.4 ± 15.4	1.23	−10.2
5[b]	**iG**GTTGGTGTGGTTGG	84.4 ± 8.1	0.95	−10.0
6[a]	GGTTGGT**iG**TGGTTGG	55.0 ± 11.1	1.18	−10.3
7[b]	GGTTGGTGT**iG**GTTGG	65.8 ± 10.6	0.82	−10.2
8[c]	i**G**GTTGGTGT**iG**GTTGG			
9[d]	GGTTGGTGT**iG**GTT**iG**G			
10[d]	GGTTG**iG**TGTGGTT**iG**G			
11[a]	GGTTG**iG**TGT**iG**GTTGG			
12[d]	G**iG**TTGGTGTG**iG**TTGG			
13[d,e]	GGTTGGT**iG**TG**iG**GTTG**iG**			
14[d,e]	G**iG**TT**iG**GTGTGGTT**iG**G**iG**			
15[d]	GGTTG**iG**T**iG**TGGTT**iG**G**iG**			
16[d]	**iG**G**iG**TT**iG**G**iG**TGTG**iG**TTGG			
17[b]	**iG**G**iG**TTGGTGT**iG**G**iG**TT**iG**G**iG**			
18[b]	G**iG**TT**iG**G**iG**TGT**iG**G**iG**TTG**iG**			
19[d,e]	GGTT**iG**G**iG**T**iG**T**iG**G**iG**TTG**iG**			

[a] Dark-blue library bead. [b] Medium-blue library bead. [c] Not selected from the library. [d] Light-blue library bead. [e] ITC not investigated.

"G" and "G + A" reaction conditions on single aptamer sequences after bead picking and photocleavage. It was determined through control experiments with known sequence oligomers that iG reacts similar to A under Maxam−Gilbert conditions, and accordingly, these conditions have been dubbed the "G + iG" lane in the representative Maxam−Gilbert sequencing gel phosphorimage shown in Figure 6. All 26 picked library beads were sequenced in this way. The result was the 14 unique sequences as shown in Table 1 containing one to six 2′-deoxy-iso-guanosine residues. Sequence **1** in the Table corresponds to the thrombin aptamer consensus sequence, and sequences **2**, **3**, **4**, and **8** are controls that were synthesized for use in the binding assay (vide infra). As would be expected, several

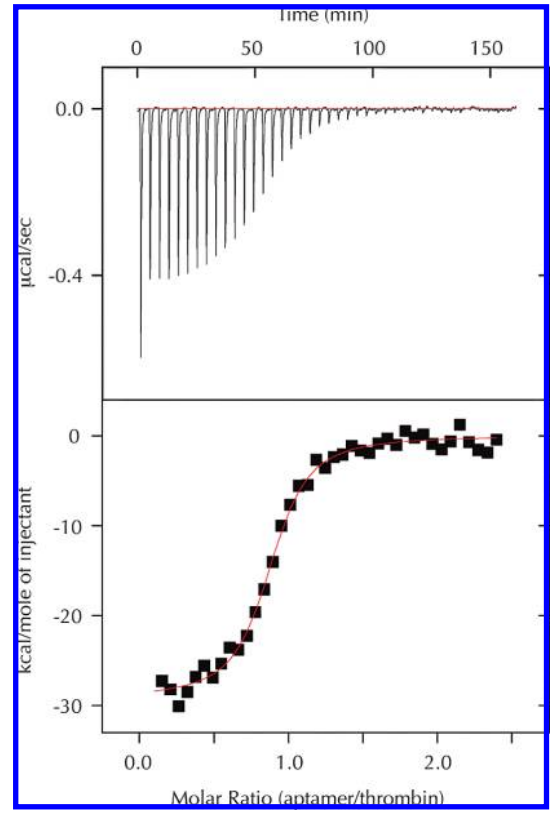

Figure 7. Isothermal titration calorimetry data for aptamer **7**/human α-thrombin binding.

of the sequences were found multiple times among the 26 picked beads.

The ability of the selected aptamer sequences to bind to human α-thrombin was determined by isothermal titration calorimetry (ITC). The K_d values that resulted from these ITC studies are shown in Table 1. Representative ITC data including a binding curve are shown in Figure 7. Only three of the eleven selected iG-aptamers tested for binding were found to have measurable K_d values: **5**, **6**, and **7**. These three oligomers correspond to the three sequences in Table 1 bearing single iG substitutions. The K_d values for **5**, **6**, and **7** are similar. Two of these K_d values, those for **6** and **7**, are lower than the K_d values determined for the consensus thrombin aptamer and its T, C, or A variants at position-8. These data indicate a favorable effect of iG substitution on aptamer binding affinity. The binding number, n, for all the aptamers was approximately 1, indicating 1:1 aptamer: thrombin stoichiometry.

Discussion

Of the eleven iG bearing sequences selected from screening the library and tested for thrombin binding activity, three showed binding in the ITC assay. There are several ways to interpret this result. The ITC assay is limited by the concentrations of the protein and aptamer used. For weaker associations a higher concentration of one of the components is required. We employed the same concentrations of aptamer and protein for all of the entries in Table 1. Further, the ITC assay is rather material intensive, and it was not practicable to optimize weaker affinity cases. Gel binding and filter binding assays were also investigated but led to inconsistent

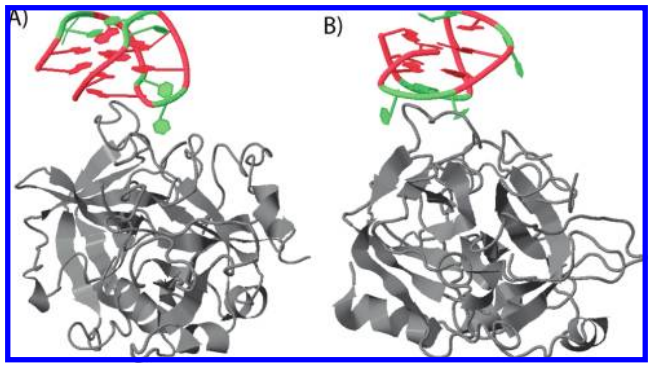

Figure 8. (A) The X-ray crystal structure of human α−thrombin (gray) bound to the "X-ray crystal structure-fold" of the thrombin aptamer at the fibrinogen-exosite (1HAP PDB) and (B) the X-ray crystal structure of the human α−thrombin (gray) bound to the "NMR-fold" of the aptamer (1HAO PDB) at the fibrinogen−exosite.

data. Thus, some of the sequences in Table 1 for which no K_d value was measureable are expected to bind thrombin, but with a much higher K_d (estimated as >10 μM) than for aptamers **5**−**7**. Another major contributor to the lack of activity for some of the selected aptamers is expected to be artifacts deriving from nonspecific binding during the selection assay to components other than the folded aptamers. One means to avoid false positives is to perform a negative selection after the initial positive selection. Specifically, a subsequent selection of noncolorized beads may be performed after a competition for thrombin binding between the aptamer and the known high affinity binder hirudin.[21] We avoided a hirudin negative selection as the number of positive selected beads was manageable with regard to manual sequencing, and the PEGA beads were sufficiently fragile that there were concerns about their surviving another selection intact. One last consideration vis-a-vis the 27% activity yield of selected sequences relates to the structure of the library relative to the structure of the parent aptamer; we have made the assumption that the 3′- and 5′-T appendages in the library are neutral with respect to their effect on aptamer structure and activity. We note that the K_d value observed in this work for the prototype aptamer **1** of 94.4 ± 26.6 nM is comparable to past reported values for this aptamer that range from 2.68 nM[22] to 75−100 nM[23] and, more recently, 333 nM.[24] The large difference in reported values appears to depend on the experimental method, as well as the conditions for obtaining the binding constant.

The initial crystal structure of the thrombin aptamer bound to thrombin shows the aptamer interacting with two positively charged regions of α-thrombin: the heparin binding-site (anion-binding exosite II) and the fibrinogen exosite (anion-binding exosite I) via TT-loops and TGT-loop of the aptamer, respectively.[11] Padmanabhan et al. deduced, in conjunction with their work[11] and the thrombin mutation studies by Wu et al.,[25] that it was the anion-binding exosite I through which the thrombin aptamer effected inhibition (Figure 8A). Interaction between the thrombin aptamer and anion-binding exosite II resulted as an apparent artifact of the crystal structure, where the aptamer was compensating for residual charge on an adjacent thrombin molecule. In a second structural report, Padmanabhan and Tulinsky[12] further

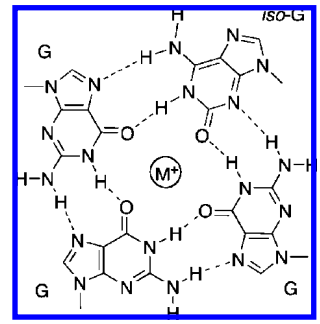

Figure 9. Mixed iG/G quartet of the type proposed to occur within aptamers **5** and **7**.

assessed the binding interaction between thrombin and the aptamer, as their earlier X-ray structure showed a reversed strand polarity of the aptamer in comparison to the NMR-solution structures.[26] In this work, it was shown that both the NMR fold and the prior determined X-ray fold of the aptamer refined to the same *R* values in aptamer−protein structures. The refined structures of the two aptamer folds bound to thrombin are compared in Figure 8, panels A and B. The aptamer interacts with thrombin differently in each of these two structures, via the TGT loop in one case, and the two TT loops in the other, respectively. Nevertheless, the NMR studies of Bolton suggest that the solution structure of the aptamer remains invariant upon binding thrombin.[27] The structures and activities of the selected aptamers are consistent with either of the aptamer/thrombin structures shown in Figure 8. However, the structure of the complex shown in Figure 8B would be expected to be more accommodating of the 5′ and 3′ T-tails used in the library for visualization of bands during Maxam−Gilbert sequencing.

Several inferences can be made about the structures of the iG-aptamers based on past structural work on the parent aptamer **1**. iG-aptamers can be split into two categories: quartet substitution (**5** and **7**) or loop substitution (**6**). A comparison of loop-substituted aptamer **6** against natural aptamers **1**−**4** shows iG substitution leads to more favorable binding than any of the four possibilities with the natural bases. The NMR structure of aptamer **1** suggests that substitution of iG for G at position 8 in the loop places its 6-amino group within range of hydrogen-bonding with the phosphate at position 5. In the case of the two quartet substituted aptamers, iG improves binding over the parent G containing aptamer **1** in both cases. In aptamer **1**, guanine bases alternate between syn and anti orientations (Figure 1). The sequence context of iso-guanine substitutions in aptamers **5** and **7** suggests a preference for syn-oriented guanines. Replacement of syn-oriented guanines is expected as such substitutions should lead to antioriented iso-guanines using an extension of Westhof's Rule[28] and a corresponding enthalpy gain.[29] We have previously observed[15] the formation of mixed iG and G quartets of the type proposed here, illustrated in Figure 9.

Since Bock et al. discovered the prototype aptamer **1**, other thrombin-inhibiting aptamers have also been selected with unique sequences. Latham et al.[30] and Kubik et al.[31] have described a 5-(1-pentynyl)-2‘-deoxyuridine-containing DNA aptamer and an RNA aptamer, respectively. Both novel aptamers contained putative hairpin structures instead of

quadruplex motifs. Macaya et al.[32] has reported an antithrombotic DNA aptamer that contains both quadruplex and duplex motifs. An additional quadruplex/duplex-containing DNA aptamer was selected against thrombin by Tasset et al.,[33] but their work indicated the aptamer was binding at the heparin-binding exosite in contrast to previous aptamers that bound to the fibrinogen-recognition exosite. Several modified variants of the original thrombin aptamer have been prepared, including aptamers with: formacetal linkages,[34] a 5′−5′ inversion of polarity site,[24,35] a G-LNA residue,[36] 2′-deoxy-2′-fluoro-arabino nucleic acids (2′F-ANA),[37] and an acyclic thymidine nucleoside.[38] Whereas the aptamer with formacetal linkages was found to be inactive, the remaining modified aptamers all showed antithrombin activity. In the case of 2′F-ANA modified aptamers, two were shown to give a 4-fold increase in thrombin binding affinity.

One property of the selected iG-aptamers **5**−**7** that awaits further study is their stability toward denaturation. While greater thrombin binding affinity was observed for aptamers **5**, **6**, and **7** in comparison to the prototypical aptamer **1** and could correspond to enhanced activities in vivo, differing thermodynamic stabilities of the quadruplexes may lead to attenuated serum half-lives. Thus, quartet stability in **5** and **7** could potentially allow the tuning of important therapeutic properties of these materials. In view of this, it has been our qualitative observation in past work that iG-tetraplexes tend to form and denature more easily than the corresponding structures bearing G.[15,16] A naïve prediction would be that the single-stranded state would be more accessible to aptamers **5** and **7** and that their serum half-lives would be shorter.

Conclusions

Methodology has been described for the preparation, selection, and sequencing of oligonucleotide libraries containing the nonstandard nucleoside 2′-deoxy-iso-guanosine. The viability of this methodology has been illustrated by the selection of three active thrombin aptamer variants, each incorporating a single iG residue. All three iG containing aptamer variants were found to bind human α-thrombin more tightly than the parent thrombin aptamer. In the most favorable case of iG aptamer **6**, an approximate binding enhancement of 2-fold is observed. Accordingly, the present work establishes an additional function for iG.[40]

Experimental Section

Library Synthesis. PEGA support **2**[18,19] (1.3 mg) was placed in two DNA synthesis columns and the sequence 5′-d-TTTTTXXTTXXTXTXXTTXXTT-3′ was synthesized using standard nucleoside phosphoramidites (Glen Research) on an ABI 391 synthesizer, where X corresponds to iG or G residues introduced by split and mix synthesis. iG phosphoramidite was prepared as previously described.[15] In practice, synthesis on the two columns began from the 3′-end and proceeded to the first X residue, at which point iG was incorporated via column 1 and G via column 2. The columns were then opened, the contents of the two columns combined, thoroughly mixed in the presence of acetonitrile, and then redistributed evenly back in the two columns, at which point the subsequent residue was coupled. This mixing procedure was repeated at all points after an X residue was encountered during synthesis. Upon completion of DNA synthesis, the library beads were deprotected by treatment with ammonium hydroxide at 55 °C for 18 h.

Aptamer Selection. The general procedure reported by Chen et al. for screening of a one bead−one compound peptide library was followed.[21] The library beads from above were washed successively with water and the following buffers: HSBB-T (4 × 400 μL), QHSBB-T (4 × 400 μL), and QHSBB-TG (2 × 400 μL). To the beads was added 100 μL of QHSBB-TG, and the mixture was agitated on a mechanical shaker for 1 h. The beads were then washed with 2 × 400 μL QHSBB-TG, and incubated with 1 μM of human α-thrombin (Enzyme Research Laboratories) conjugated with biotin for 24 h at 4 °C [successive selections were also conducted with 2 μM and 4.86 μM human α-thrombin]. The beads were washed with QHSBB-TG (2 × 400 μL) and then incubated with 8.6 nM SAP (Pierce) for 2 h at 4 °C. The beads were then washed with HSSB-T buffer, staining buffer, and then incubated with BCIP (Pierce). After incubation for 9 h at 4 °C, blue colorized beads were collected and deproteinized by treatment with 8 M guanidine hydrochloride for 10 min at 90 °C, followed by washing with DMF and then water.

Maxam−Gilbert Sequencing. Aptamers were cleaved from individual selected beads by irradiation for 45 min with a Rayonet reactor at 350 nm using a Pyrex filter. The aptamer was then kinased with T4 polynucleotide kinase (USB) in the presence of γ-^{32}P-ATP in a total reaction volume of 20 μL. After incubation for 30 min, 5 μL of ATPase (myosin; Sigma) was added, followed by an additional incubation at 25 °C for 5 min. Maxam−Gilbert sequencing was conducted on the kinased oligonucleotide as follows: G reaction,[39] 8 μL of the kinase reaction was combined with 1 μL of dimethylsulfate, 200 μL of 50 mM sodium cacodylate pH 8.0/10 mM $MgCl_2$/0.1 mM EDTA buffer, and incubated at 37 °C for 5 min, followed immediately by precipitation with ethanol; G + iG reaction, 4 μL of the kinase reaction was combined with 10 μL of 98% formic acid for 60 s at 25 °C, followed immediately by precipitation with ethanol. The precipitated products were treated with 100 μL of piperidine and heated at 90 °C for 30 min, lyophilized, and then analyzed by 20% denaturing PAGE.

Isothermal Titration Calorimetry. ITC experiments were performed in duplicate for aptamers **2**−**5** and **7**, triplicate for aptamer **6**, and quadruplicate for aptamer **1** on a VP-ITC MicroCalorimeter in a 1.4 mL cell at 25 °C. Either 41 or 61 injections of 7 or 4 μL of titrant (vide infra), respectively, were added to the cell solution every 240 s. ITC traces were fit to a single-site binding curve with the Origin 5 software package provided by the manufacturer. K_d values were reported as a range of the error from K as given by the curve-fit data.

Titrant Solution. Thirty-five nanomoles of aptamer and 20 μL of solution B were lyophilized to dryness and then taken up in 350 μL of solution A. The mixture was incubated at 90 °C for 10 min, kept at RT for 10 min, and then chilled at 4 °C overnight (100 μM aptamer concentration).

Cell Solution. One hundred fourteen microliters of solution C were lyophilized to dryness and then taken up in 2.0 mL of solution A (5.4 μM human α-thrombin concentration).

Reference Cell Solution. One hundred fourteen microliters of solution B were lyophilized to dryness and then taken up in 2.0 mL of solution A.

Solution A: 20 mM Tris acetate pH 7.3, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

Solution B: 50 mM sodium citrate pH 6.5, 200 mM NaCl, and 0.1% PEG-8000.

Solution C: 3.5 mg/mL human α-thrombin, 50 mM Sodium citrate pH 6.5, 200 mM NaCl, and 0.1% PEG-8000.

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