

Construction, Stability, and Activity of Multivalent Circular Anticoagulant Aptamers*

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Here we describe the design and construction of multivalent circular DNA aptamers. Four aptameric binding motifs directed at blood-borne targets are used as a model set from which larger, multidomain aptamers are constructed in a straightforward manner. Intra- or intermolecular ligation of precursor oligonucleotides provides a stabilizing mechanism against degradation by the predominant exonuclease activity of blood products without the need for post-selection chemical modification. In many cases, circular DNA aptamer half-lives are extended beyond 10 h in serum and plasma, making such constructs viable for therapeutic and diagnostic applications. Duplexes and three-way junctions are used as scaffold architectures around which two, three, or four aptameric motifs can be arranged in a structurally defined manner, giving rise to improved binding characteristics through stability and avidity gains. Circular aptamers targeted against thrombin display improved anticoagulant potency with EC_{50} values 2–3-fold better than those of the canonical GS-522 thrombin DNA aptamer. Intrinsic duplex regions provide an opportunity to incorporate additional transcription factor binding motifs, whereas ancillary loops can be used to provide further functionality. These anticoagulant aptamers provide a starting point for merging the principles of DNA nanotechnology with aptameric functions.

Since the first reports on nucleic acid aptamers more than a decade ago (1–3), this class of molecules has developed steadily toward general therapeutic (4–6) and diagnostic (5, 7–9) applications. The relative ease of aptamer discovery has allowed for selection against a wide range of targets (10) by processes that have recently been automated (11). Simultaneous advances in signaling and detection strategies have improved the outlook for aptamer arrays in proteomic and biosensor applications. Whereas the high affinity and specificity of aptamers toward their molecular targets make them well suited for these tasks, the susceptibility of natural nucleic acids to nucleolytic degradation is a serious hurdle for uses in biological fluids. Strategies to improve the nuclease stability of aptamers, as for antisense nucleic acids, have included the use of chemical modifications such as phosphorothioate, locked nucleic acid, 2'-O-methyl- and 2'-fluoro-nucleotides (12), as well as mirror-image Spiegelmers (13). Of most relevance for potential therapeutic applications, some common nucleic acid stabilization strategies

have encountered problems associated with toxic degradation products (14). In addition, modification performed post-selection can alter the subtle binding interactions of the selected natural aptamer (15, 16).

Circularization of natural aptamers is an attractive alternative to chemical modification for improving aptamer stability. With the majority of nucleic acid degradation activity arising from plasma exonucleases (17), modification of exposed termini often achieves a sufficient improvement in stability for use *in vivo* (12), and circular constructs eliminate this primary source of degradation entirely. Moreover, circularization permits the use of natural nucleotides, which should avoid potential toxicity associated with chemical modification. In principle, circular aptamer constructs can be produced by either intra- or intermolecular ligation followed by exonucleolysis of unreacted products, a straightforward and efficient approach to their preparation (see Fig. 1A). With selected aptamers often forming a stem-loop structure, closing the stem into a duplex with a hairpin loop should not appreciably alter the binding loop structure and may confer added stability. Furthering this concept, two stem-loop aptamers with complementary overhangs can in principle be ligated to form a double-headed dumbbell aptamer linked by a duplex region. By analogy, higher order multiheaded aptamers could then be formed around single or double junction architectures (see Fig. 1B). With the ability to combine multiple aptamer activities into a single construct comes the notion of multitasking or pleiotropic aptamers. Functions are not confined to those associated with aptameric activity only, as transcription factor recognition or other sequences can be introduced into duplex regions and specific labels can be added as loop components (see Fig. 1C). In this way, modular pleiotropic constructs with a broad range of combinatorial functions could be produced from a smaller number of aptameric heads. The ability to incorporate multiple identical binding domains in a structurally determined manner may improve binding characteristics through avidity gains in a manner analogous to antibodies. Other benefits of multivalency may lie in the development of aptamer therapeutics for diseases that require simultaneous binding of two or more targets, such as disseminated intravascular coagulopathy, treated by combining anticoagulant and antiselectin therapy (18), or for cancers associated with coagulation enhancement (19).

Here we report the design and construction of multimeric DNA aptamers based largely around a blood coagulation model. The archetypal GS-522 DNA aptamer (3), which binds to the fibrinogen exosite I of thrombin, is used to compare the activities and stabilities of multivalent circular constructs also based around this aptamer domain.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Controls were GS-522 (thrombin exosite 1), GGT-TGGT-GAGGTTGG and 60–18(29) (thrombin exosite 2), AGTCCGTG-GTAGGGCAGGTTGGGGTGACT. Precursor monomers were as follows: pA-1, pAATTGTGCTAGTUATAGCAC; pT-1, pAATTCAGCACT-

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GGTTGGTGAGGTTGGGTGCTG; and pT-6, pTGACGTCATCGCTGTGGTTGGTGAGGTTGGCAGCGA, where p is 5'-phosphate and U is 5-aminocaproylpropargyl-dU.

Precursor dimers were as follows: pTT-1, pCTGGGTTGGTGAGGTTGGTCAGCACGGTTGGTGAGGTTGGTGTG; pTT-2, pCGTGTGGTTGGTGAGGTTGGCAGCGACTGGTTGGTGAGGTTGGGTGC; pTU-1, pGAGTCCGTGGTAGGGCAGGTTGGGGTGACTCGCTGTGGTTGGTGAGGTTGGCAGC; pTU-2, pGAGTCCGTGGTAGGGCAGGTTGGGGTGACTCGCTGTGGTTGGTGAGGTTGGACAGC; and pTU-3, pGAGTCCGTGGTAGGGCAGGTTGGGGTGACTCGCTGTGGTTGGTGAGGTTGGGCAGC.

Precursor dimers were as follows: pTT3-1, pAATTCTCAGGTTGCTGTGCTGGTTGGTGAGGTTGGCAGCAGACCGCTAGTGTGGTTGGTGAGGTTGGCACTACGCGCCTGAG; pTT3-2, pAATTCTGAGCTCAGTGTGTGGTTGGTGAGGTTGGCAGCACTGGACGATGCTGTGGTTGGTGAGGTTGGCAGCATCGCCGCTACG; pTU3-1, pAATTCTCAGGTTGCTGTGCTGGTTGGTGAGGTTGGCAGCAGACCGCTAGTGCAGGTAACCAAGTACAAGGTGCTAAACGTAATGGCTTCGCACTACGCGCCTGAG; and pTR3-1, pAATTCTCAGGTTGCTGTGCTGGTTGGTGAGGTTGGCAGCAGACCGCTAGTGCAGGTAACCAAGTACAAGGTGCTAAACGTAATGGCTTCGCTGTGGTTGGTGAGGTTGGCAGCAGACCGCTAGTGCAGTTCGCAACCGTTGCCAAGATTACGCGCCTGAG.

Dimeric aptamer formation was as follows: cTA-1 = pT-1 + pA-1 + ligation (L), cTT-1 = pTT-1 + L, cTT-2 = pTT-2 + L, cTU-1 = pTU-1 + L, cTU-2 = pTU-2 + L, cTU-3 = pTU-3 + L, cTT-6 = (2 × pT-6) + L.

Trimeric aptamer formation was as follows: cTTA-1 = pTT3-1 + pA-1 + L, cTTA-2 = pTT3-2 + pA-1 + L, cTUA-1 = pTU3-1 + pA-1 + L, cTSA-1 = pTS3-1 + pA-1 + L, cTRA-1 = pTR3-1 + pA-1 + L.

Tetrameric aptamer formation was as follows: cTTTT-1 = (2 × cTT3-1) + L and cTTTT-2 = (2 × cTT3-2) + L.

Aptamer Preparation—Oligonucleotides were purchased from Sigma Genosys, Oswel, Eurogentec, or Genset Pacific in desalted form with 5'-phosphate groups to allow for subsequent ligation. For constructs that required the ligation of multiple oligonucleotide sequences, each was added at an equimolar ratio typically at a concentration of 100 μ M. Prior to ligation, constructs were heated to 95 °C in T4 DNA ligase buffer (40 mM Tris-Cl, 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP, pH 7.8) and slow-cooled to room temperature. Typically, T4 DNA ligase was added at 10 units/ μ g oligonucleotide and incubated overnight at 15–37 °C. Following a further incubation at 75 °C for 10 min, products were exchanged into the appropriate buffer by spin column chromatography (Bio-Rad Micro Biospin-6), and any remaining uncircularized oligonucleotides were digested with either T4 DNA polymerase or exonuclease III. For the production of unsymmetrical or heteromeric constructs, EcoRI and MfeI restriction enzymes were included in the ligation reaction and/or added after ligation to ensure unwanted symmetrical or homomeric products were relinearized. Reaction mixtures were purified by phenol-chloroform extraction and ethanol precipitation prior to quantitation by UV spectrophotometry (Varian Cary100 Bio). Products were verified by mobility analysis following native and denaturing polyacrylamide gel electrophoresis (8–20%).

Thermal Melt Analysis—Melting profiles were obtained using a Varian Cary 100 Bio spectrophotometer and analyzed with Cary Thermal software. Aptamers at a concentration of 2 μ M were heated to 95 °C and slow-cooled in selection buffer (20 mM Tris acetate, pH 7.4, 100 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂) prior to obtaining melting profiles at a heating rate of 1 °C/min.

Physical Stability—Aptamer constructs at a concentration of 300 nM were incubated in blood products at 37 °C for periods up to 30 h. At each time point, 150 μ l of samples were removed and diluted into 350 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and 500 μ l of TE-saturated phenol-chloroform. The solutions were repeatedly heated to 50 °C and vortexed. An oligonucleotide standard was added prior to phenol-chloroform extraction and ethanol precipitation to allow for normalization of extracted samples. Purified samples were run on denaturing PAGE gels. Following staining with Sybr Green II (Sigma), the intact aptamer and oligonucleotide standard bands were quantitated with a Fluor-S imager (Bio-Rad).

Functional Stability—Aptamer constructs (100 nM) were incubated at 37 °C in 67 μ l of serum or plasma for periods up to 30 h. Following incubation, clotting in the incubated samples was initiated with the addition of thrombin (final concentration 12.3 nM) and fibrinogen (final concentration 2 mg/ml) (added only for serum assays) with selection buffer and 10 mM CaCl₂ up to a volume of 200 μ l. The clotting reaction was monitored at 410 nm in a microtitre plate reader. Clotting times were determined as the point of half-maximal absorbance change.

Anticoagulant Activity—Fibrinogen (2 mg/ml) and aptamer (0–1 μ M)

were preincubated in selection buffer plus 10 mM CaCl₂, 33% serum plus 10 mM CaCl₂, or 33% plasma at 37 °C. Coagulation was initiated with warmed thrombin (final concentration 12.3 nM) or thrombin plus CaCl₂ and monitored at 410 nm in a microtitre plate. Clotting times, which were typically standardized at 22 s, were determined as the point of half-maximal absorbance change. EC₅₀ values were determined as the aptamer concentration producing a doubling in clotting time.

RESULTS

Aptamer Design and Construction—To explore basic aspects of multivalent circular aptamer construction, four natural DNA aptamer domains that bind vascular protein targets were sourced from the literature. These domains, which were all compatible with hairpin loop formation, bound to thrombin exosite I (the “T” aptamer head) (3), thrombin exosite II (the “U” head) (20), L-selectin (“S”) (21) and a red blood cell marker (“R”) (22). A uniquely aminated (“A”) hairpin derived from a well characterized DNA loop (23) was included as an ancillary domain. All aptamer sequences were designed with additional flanking regions to form extended stem-loop structures. For the U, S, and R aptamer heads, whose minimal motifs already carried short stem regions, sequences were simply extended as necessary. For the T aptamer head, additional complementary sequence was added to both ends of the canonical 15-mer oligonucleotide to form the required stem. A non-pairing thymidine residue was generally included adjacent to the 5' end of the minimal motif to allow for a degree of structural compensation. In construct nomenclature, the prefix “p” denotes a 5'-phosphate group (indicating linearity) whereas “c” denotes a covalently circularized product, with suffix numerals denoting individual constructs. Thus, pTT-1 is a folded but unligated aptamer with two thrombin exosite I binding regions, whereas cTUA-2 is a circular construct with three heads: thrombin exosite I, thrombin exosite II, and an aminated hairpin. Combined with other construction principles described below, this set of building blocks permits the production of a wide diversity of aptamers with two, three or four functional heads (Fig. 1, A–C).

After annealing, the template oligonucleotide for the simplest circularization reaction forms a dumbbell structure with aptamers located at the extremities separated by a singly nicked duplex (Fig. 1A, *i*). This nick can be sealed by ligation using chemical methods or a DNA ligase to form a divalent circular aptamer (Fig. 1A, *ii*). Any unligated aptamer can be removed by exonucleolysis (Fig. 1A, *iii*) prior to final product cleanup. Good ligation efficiency is an essential requirement for efficient circular aptamer production. Although various constructs could be successfully ligated using chemical methods,¹ we here concentrate upon enzymatic ligation for compatibility with bioprocessing. Designing constructs with the shortest possible duplex region between functional aptamer domains may prove important for both functionality and production. Whereas pTT-1 containing a 6-bp internal duplex could not be circularized under any conditions attempted using T4 DNA ligase, pTT-2 containing an 8-bp duplex was ligated at a yield of ~40% under the reaction conditions tested (Fig. 2A). Because general rules for predicting ligation efficiency are not apparent, ligation of a set of three closely related pTU constructs differing only in a single duplex position adjacent to the T head was examined for comparison with pTT-2 (Fig. 2A). Addition of a single base pair to the duplex of pTU-1 (*i.e.* 9 bp in total) resulted in near quantitative ligation of the construct (Fig. 2A) consistent with the behavior of pTT-1 and pTT-2. For the pTU-2 construct, where the duplex length was increased to 10 bp by the addition of an adenine residue to pair with the non-bonding thymine adjacent to the T head, ligation efficiency

¹ S. M. Knox and G. C. King, unpublished data.

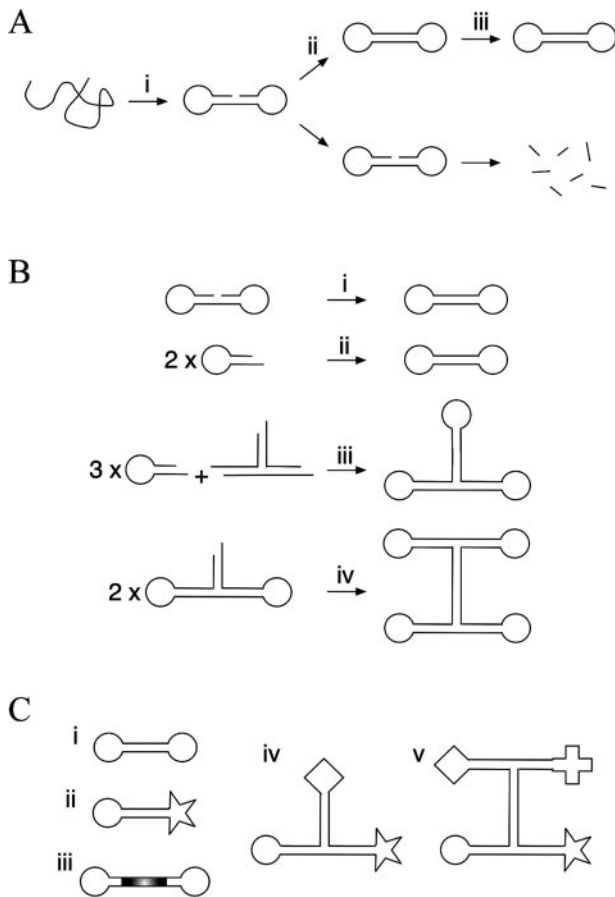


FIG. 1. Construction of multivalent circular aptamers. *A*, circular aptamer synthesis protocol. Oligonucleotide is (*i*) heated and slow-cooled to form aptamer heads and duplex region, followed by (*ii*) ligation, and (*iii*) exonucleolysis to degrade any uncircularized oligonucleotide, leaving only intact circular aptamer. *B*, approaches to multi-headed aptamers. Dimeric aptamers are formed by (*i*) intramolecular ligation of a single oligonucleotide containing two aptameric domains and a self-complementary central duplex region or (*ii*) intermolecular ligation of two aptamer-hairpin constructs; trimeric aptamers are based upon ligation of aptamer heads to a DNA three-way junction scaffold (*iii*), whereas tetrameric aptamers can be constructed by ligation of two three-way junctions each ligated to two aptamer heads (*iv*). *C*, heteromultivalent circular aptamers in homodimer (*i*), heterodimer (*ii*), dimer with DNA binding motif (*iii*), three-headed aptamer (*iv*), and four-headed aptamer (*v*) forms.

actually dropped slightly to 90%. Moreover, demonstrating the balance of influences upon ligation efficiency, replacement of this terminal A:T pair with a clamping G:C pair in the pTU-3 construct caused ligation efficiency to drop significantly to 60% yield (Fig. 2A).

To reduce the size and complexity of oligonucleotide precursors, single ligation of an aptamer (Fig. 1*B*, *i*) can be replaced by double ligation of two smaller precursors bearing complementary oligonucleotide overhangs (Fig. 1*B*, *ii-iv*), thereby introducing modularity through specific hybridization and ligation. To further generalize the concept of modularity, any desired binding activities should preferably be combined without the need to synthesize new precursor sequence(s) for every desired aptamer combination. The use of palindromic sequence overhangs (Fig. 2*B*, *i*) can facilitate this goal but introduces the complication of allowing hybridization between identical components, forming undesired homodimers at the same time as the desired heterodimer. For the production of pure heterodimers, this situation can be overcome by the use of a pair of related restriction enzymes that digest only homodimers while

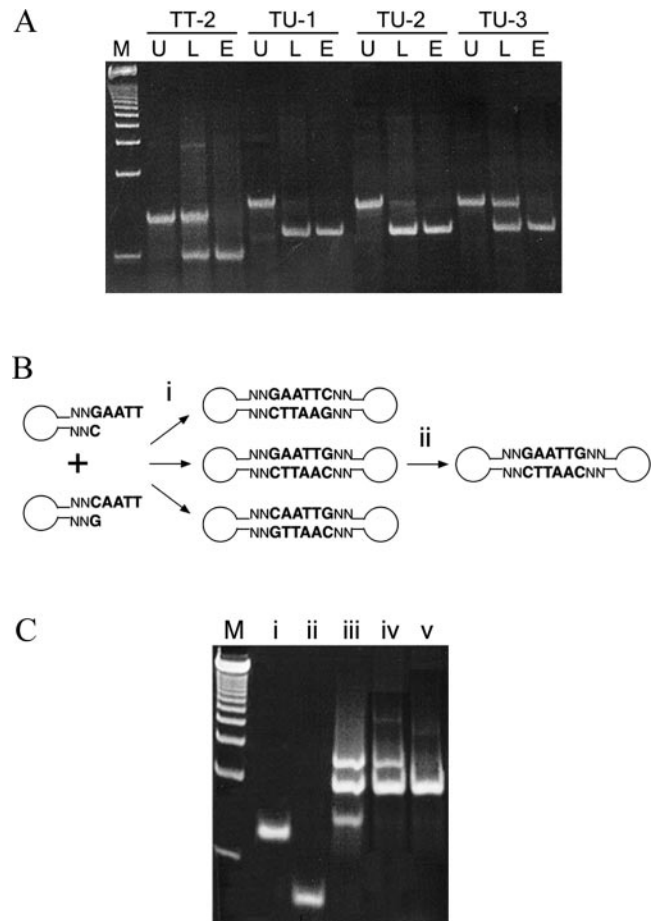


FIG. 2. Formation of heterovalent constructs. *A*, circular aptamer ligation efficiency. Denaturing polyacrylamide gel (20%) showing mobility shifts upon circularization of double-headed aptamer constructs TT-2, TU-1, TU-2, and TU-3. *M*, 25-bp DNA marker. Each triplet of lanes contains unligated (*U*), ligase-treated (*L*), and ligase followed by exonuclease-treated (*E*) aptamers. *B*, scheme for restriction enzyme-mediated formation of heterovalent constructs. Aptamer-hairpin constructs bearing complementary overhangs but differing flanking duplex nucleotides are ligated (*i*) to form a combination of hetero- and homodimers. Related restriction enzyme pairs can be used to digest homo- but not heterodimer products, resulting in pure heterodimer following subsequent exonucleolysis. *C*, use of the EcoR I/Mfe I restriction enzyme pair for heterodimer product resolution. A non-denaturing polyacrylamide gel (16%) shows: 25-bp marker ladder, (*i*) pT-1 standard, (*ii*) pA-1 standard, (*iii*) ligation of pT-1 and pA-1, showing the presence of both hetero and homodimers, (*iv*) ligation of pT-1 and pA-1 in the presence of EcoR I and Mfe I, driving the product toward majority heterodimer, (*v*) pure heterodimer where, following ligation, EcoR I and Mfe I were added along with exonuclease III.

leaving heterodimers intact (Fig. 2*B*, *ii*). The EcoR I/Mfe I restriction enzyme pairing works well in this context (Fig. 2*C*).

For higher order circular aptamers, specific architectures can be constructed depending upon the intended use and the desired orientations of the functional heads. Three-way junctions can form the underlying structural motif for triple-headed circular aptamers (Fig. 1*B*, *iii*), whereas four-headed constructs can be formed from a topologically distinct dimer of three-way junctions (Fig. 1*B*, *iv*) or from a single four-way (Holliday) junction (not shown). Three-way junction sequences can be specifically chosen to project functional heads in particular orientations either symmetrically or asymmetrically. Using rules previously developed for sequences defining three-way junction structure (24), two examples of asymmetrical junctions J3CC (25) and TWJ3 (26) were chosen for use here. The asymmetric orientation allows for the production of triple-headed circular aptamers that are topological analogues of

protein antibodies containing two (homo- or heteromeric) binding domains and an ancillary/signaling domain. Two structural isomers of the asymmetric junction can form with alternate arms stacked in a colinear manner (Fig. 3). The J3CC sequence produces a structure that strongly favors only one isomer. The TWJ3 sequence, although found primarily as the other isomer, is less stable in this form with more flexibility in the junction similar to the hinge region of antibodies. This design flexibility has potential use for tethered constructs where binding domains can be directed away from the surface, preventing unfavorable steric obstruction. The relative orientation of binding heads can also be manipulated by varying the length of intervening duplex. With approximately 10 nucleotides per turn of B-form DNA, the addition of five nucleotides to the linking duplex results in a 180° rotation of aptamer heads relative to each other.

In addition to heteromultivalency of the aptameric components (Fig. 1C), further functions can be incorporated into the duplex region(s) of circular aptamers. For DNA aptamers, a transcription factor decoy sequence (Fig. 1C, *iii*) can be employed to sequester a specific transcription factor to modulate gene expression. The result is a broad range of pleiotropic aptamer constructs produced from a relatively simple set of precursors. Quantitative stability and activity data for several multivalent anticoagulant aptamer constructs are summarized in Table I.

Stability—With enhancement of stability being a primary goal of circularization, thermal melting analysis was undertaken to examine the effect of circularization upon double-headed circular aptamers (Fig. 4A, Table I). For the pTT-2, pTU-1, pTU-2, and pTU-3 constructs, ligation to their covalent circular forms produced an average increase in melting tem-

perature of 24 ± 1 °C, a highly significant gain in thermal stability. The physical stability of circular constructs to nucleolytic degradation was then tested by incubation in blood products (Fig. 4B). The canonical GS-522 DNA aptamer displayed a physical half-life of 15 and 36 min in pooled human serum and plasma, respectively, consistent with expectations. Interestingly, even the unligated double-headed constructs showed a large improvement in serum and plasma stability compared to GS-522 with half-lives ranging between 3.6–5.6 h in serum and 7.6–16.9 h in plasma. Evidently, if termini of a linear sequence are held in a nicked duplex structure, exonuclease activity can be reduced dramatically in comparison to structures with exposed termini. When ligated, resistance to exonucleolytic degradation improved further with approximately an additional 2-fold gain in stability across the constructs tested, producing half-lives of 6.8–13.6 h and 15.4–32.1 h in serum and plasma, respectively (Fig. 4B, Table I). In serum, which lacks specific binding proteins for the cTT and cTU constructs, the somewhat higher nucleolytic stability of cTT-2 over comparable cTU constructs is indicative of the greater stability of the T aptamer head over the U head. This may be correlated to the more compact structure of the T head, restricting access of endonucleases to susceptible binding sites. Of interest for future exploration, a degree of variation in stability was observed in blood products obtained from individual donors compared with pooled serum and plasma (Fig. 4B).

In addition to the physical resistance of circular aptamers to degradation, measuring the ability of constructs to retain binding activity following extended incubation in serum or plasma will provide an indication of their functional stabilities. For constructs containing a T head, thrombin-induced fibrinogen clotting can be prolonged by aptamer binding and inhibition at thrombin exosite I, giving a measure of aptamer activity. Good exponential decay curves yielding well defined half-lives were observed in all cases examined (Fig. 4C, Table I). Measured functional half-lives of double-headed constructs ranged from 5.2–9.6 h in serum (Fig. 4C) and 10.2–25.3 h in plasma (Fig. 4D), in generally good agreement with the physical stability values. For cTT-2 and cTU-1 in serum, it appears that the functional half-lives in serum (18.2 and 19.0 h, respectively) may be substantially greater than the physical half-lives (13.6 and 7.3 h, respectively), suggesting the retention of significant activity by partially degraded aptamers. From these measure-

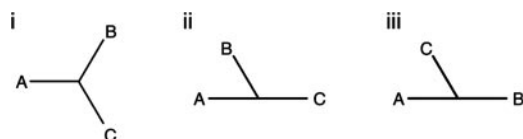


FIG. 3. Three-way junction geometrical configurations. Depending upon the identity of nucleotide sequences surrounding the junction, the presence or absence of an unpaired loop adjacent to the junction and the buffer conditions, a three-way junction may adopt (*i*) a symmetrical extended conformation, (*ii*) an asymmetrical conformation with two arms stacked coaxially, and (*iii*) an isomer of the asymmetric coaxially stacked conformation.

TABLE I
Stability and activity data for multivalent circular aptamers

Standard errors appear in parentheses.

Aptamer	Melting temp. °C	Activity EC ₅₀			Physical half-life		Functional half-life	
		Buffer	Serum	Plasma	Serum	Plasma	Serum	Plasma
			<i>nM</i>		<i>h</i>		<i>h</i>	
GS-522		19.3 (2.7)	107 (5)	123 (6)	0.25 (0.1)	0.6 (0.1)		
cTA-1		22.8 (1.2)	136 (9)	157 (12)				
pTU-1	54.9				3.9 (0.9)	12.4 (4.8)		
cTU-1	77.9	9.2 (1.5)	59.9 (2.9)	61.7 (5.8)	7.3 (2.2)	21.5 (4.2)	19.0 (0.8)	25.7 (2.2)
pTU-2	53.8				3.6 (0.4)	7.6 (2.1)		
cTU-2	77.9	11.8 (0.9)	67.7 (5.6)	77.2 (6.3)	6.8 (1.1)	15.4 (3.3)	8.4 (1.7)	10.9 (1.3)
pTU-3	57.0				5.5 (0.8)	8.5 (2.7)		
cTU-3	82.1	26.0 (2.7)	154 (14)	179 (5)	9.4 (0.8)	16.9 (2.9)	9.0 (1.6)	10.4 (1.2)
pTT-2	55.1				5.6 (0.6)	16.9 (5.4)		
cTT-2	79.8	7.0 (1.2)	40.6 (2.0)	45.0 (3.5)	13.6 (3.5)	32.1 (4.2)	18.2 (1.0)	34.5 (2.8)
cTT-6		27.7 (3.1)	158.2 (10)	182 (7)				
cTUA-2		19.9 (2.4)	125 (6)	130 (4)				
cTSA-2		29.4 (2.4)	174 (9)	201 (9)				
cTRA-2		29.6 (3.0)	169 (10)	190 (13)				
cTTA-1		11.1 (0.6)	63.9 (3.4)	66.6 (3.1)				
cTTA-2		12.9 (1.5)	76.9 (4.8)	85.0 (6.2)				
cTTTT-1		6.1 (1.5)	39.0 (1.6)	44.7 (3.0)				
cTTTT-2		9.2 (0.7)	55.4 (2.6)	59.5 (1.3)				

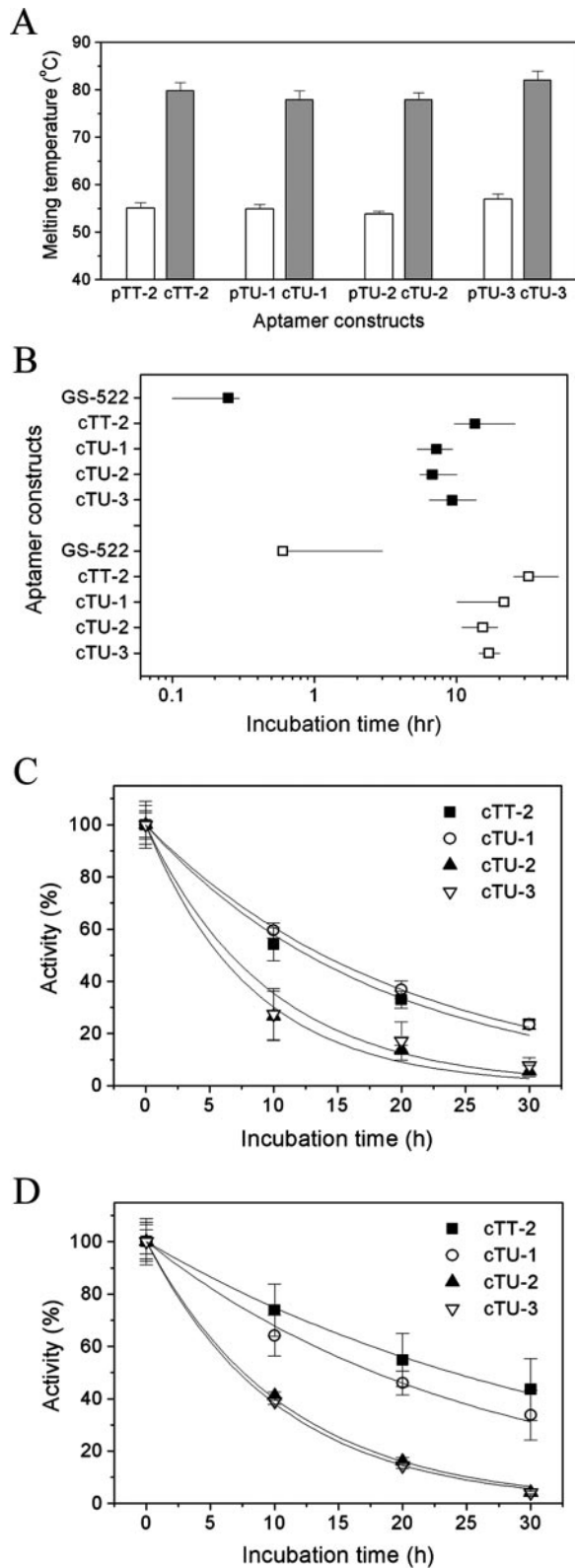


FIG. 4. Circular aptamer stability. A, thermal stability increases markedly upon circularization. The melting temperatures for pre-annealed unligated double-headed constructs (white bars) are compared with equivalent circularized constructs (gray bars). B, physical stability of aptamers in biological fluids. Half-lives of aptamer constructs incubated in pooled human serum (■) and pooled human plasma (□) are shown on a log scale. Thin lines represent the range of half-lives observed for individual (non-pooled) serum and plasma samples. C, functional stability of circular aptamer constructs determined from thrombin inhibitory activity following incubation in pooled human serum. D, functional stability following incubation in pooled human plasma.

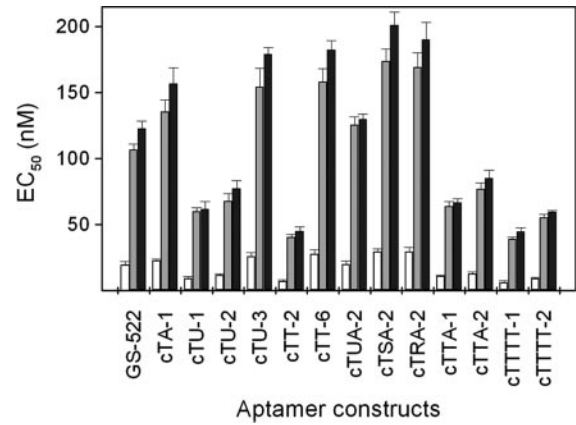


FIG. 5. Antithrombin activity of multiheaded aptamers. The anticoagulant activity of various multiheaded aptamer constructs in buffer (white bars), serum (gray bars), and plasma (black bars) is presented as EC_{50} values (the construct concentration required to double normal thrombin-mediated clotting time).

ments it is clear that circularization provides adequate protection for sustained aptamer activity in biological fluids.

Anticoagulant Activity—To examine the effect of the number and orientation of thrombin-binding domains upon the anticoagulant activity of related multivalent circular aptamers, one or more T heads were incorporated into di-, tri- and tetravalent constructs. Anticoagulant activities, expressed as the aptamer concentration required to double the fibrinogen assay clotting time (EC_{50}), were compared against the activity of the canonical GS-522 aptamer in buffer, serum, and plasma (Fig. 5, Table I). GS-522, which is closely related to the T head used here, displayed an EC_{50} of 19.3 ± 2.7 nM in buffer. In contrast, the thrombin exosite II-binding aptamer 60–18(29), from which the U head is derived, did not inhibit clotting at concentrations up to $1 \mu\text{M}$, some 2000 times the reported K_d value for thrombin binding (20) (data not shown). For all constructs EC_{50} values were ~5-fold higher in serum and plasma than in buffer alone, most likely because of nonspecific interactions with prevalent vascular proteins, reducing the effective aptamer concentration. Combination of T heads with other aptamer domains produced largely rationalizable results. Whereas the cTA-1 construct displayed EC_{50} values about the same as GS-522, consistent with its single inhibitory head, cTU-1 and cTU-2, which also possess only a single active inhibitory head, displayed EC_{50} values of 9.2 and 11.8 nM, respectively, in buffer (Fig. 5, Table I). On an active T head basis, these two constructs were the most potent of the set examined. This activity enhancement can be directly attributed to avidity gains caused by binding of the U domain to thrombin exosite II, although the U head is not explicitly inhibitory itself. Binding of the U domain is expected to increase the effective aptamer concentration in the vicinity of thrombin, resulting in an improved EC_{50} . Notably, the related cTU-3 construct did not display the activity advantage of its near siblings, highlighting the importance of local aptamer-stem structure upon activity.

Combination of two inhibitory T heads into the homodivalent cTT-2 construct caused EC_{50} to drop to 7 nM in buffer, almost a 3-fold improvement over GS-522. This improvement may arise from both an avidity gain and the greater structural integrity of the circular construct, producing additional activity beyond the simple doubling in aptamer functionality. Interestingly, cTT-6, which contains a longer internal duplex bearing a cAMP-response element transcription factor binding sequence, displayed an EC_{50} that was somewhat worse than GS-522 despite containing two inhibitory T heads.

To generate various tri- and tetravalent constructs, two dis-

tinct three-way junction architectures were employed for the production of these higher order species. Three-headed aptamers built around a three-way junction axis were constructed with an aminated hairpin loop, and either two T heads or a single T head with a single U, R, or S head (Fig. 5, Table I). Two alternative three-way junction architectures were used to examine whether arm orientation could significantly affect the anticoagulant activity of multivalent constructs. In comparing these alternative topologies for the cTTA-1 and cTTA-2 aptamers, only a small benefit was observed from employing the J3CC three-way junction sequence that favors orienting the two T heads in close proximity rather than at distal ends of the construct. However, the impact of the three-way junction sequence upon the inhibitory capacity of multiple T-containing aptamers is more pronounced for four-headed aptamers constructed from two three-way junctions. In this case, cTTTT-1, formed from two J3CC junctions, has an EC_{50} somewhat better than that of cTTTT-2, which is formed from two TWJ-3 junctions: 6.1 versus 9.2 nM in buffer, a trend that persists in serum and plasma. On an absolute concentration basis, cTTTT-1 was the most potent aptamer of the set examined although not on a per head basis.

DISCUSSION

Aptamers—The development of DNA as a molecule with utility beyond genetics has grown rapidly in the last decade. Research areas within both structural nanotechnology and biotechnology are strongly focused on deriving benefit from the unique recognition, folding, and organizational architecture properties of this molecule. The ability of usually linear DNA to form three- and four-way junctions, found in nature as replication forks and genetic exchange structures, can be harnessed to construct more intriguing two- and three-dimensional arrangements. Combined with the specificity of Watson-Crick base pairing and the inherent capacity for self-assembly, unusual topological constructs (27, 28) and regularly patterned lattices of DNA components can be formed (29). Separately, the development of *in vitro* selection methods (1, 2) has allowed nucleic acid aptamers to be derived that have the ability to bind ligands with a selectivity and specificity that can rival antibodies. A great advantage of this technology is that it can select sequences capable of binding to a variety of ligands, including small molecules, inorganics, proteins, and cellular targets (30). Good progress toward clinical applications of aptamers is being made (21, 31–35). At the same time, oligonucleotides containing G-quartets (36), CpG sequences (37, 38), and transcription factor decoys (39–41) have recently generated much interest for their ability to modulate disease states and the immune system.

Circular Aptamers—The circular constructs described here provide a framework upon which multiple aptamer, sequence, transcription factor binding, and ancillary motifs can be combined around a stable and structurally defined nano-architecture. This leads to possibilities for a wider range of improved binding functions with the ability to multitask by locating complementary activities within a single molecule. For practical applications in therapeutics or diagnostics, however, sufficient stability against environmental nucleases is a prerequisite. With natural oligonucleotides having half-lives of 5–15 min in blood products (42–44), significant increases in stability have been sought to make nucleic acids viable as therapeutic candidates. Other attempts to stabilize the canonical thrombin DNA aptamer for physiological uses have previously been described. One employed unnatural disulfide or triethylene glycol bridging of the 5' and 3' termini (45), yielding similar nucleolytic half-lives to those observed here. A second used 3'-biotin-streptavidin conjugates to increase both nuclease stability and

circulating half-life (46). Whereas these like most methods have concentrated on the use of unnatural chemistries to avoid recognition and cleavage by nucleases, we have employed circularization to prevent degradation by the more prevalent exonucleases. By extending circular construct half-lives up to 10–25 h in serum and plasma, this strategy can be seen to provide more than adequate protection for applications that are performed in biological fluids. The additional benefits of this method include low precursor cost and the absence of unnatural breakdown products, minimizing the risk of chemical toxicity.

The use of aptamer binding motifs in a circular format may have been expected to encounter problems associated with steric compatibility. The addition of non-native sequences to either end of a stem-loop aptamer could result in the specificity and affinity of the binding motif being attenuated by the structural and steric constraints imposed by the duplex arm. As shown here, this does not appear to be the case. The anti-thrombin aptamer GS-522 (3) makes a good test motif for judging how incorporation onto a duplex arm can affect activity. By comparing the activity of the double-headed cTU-1, -2, and -3 constructs it is apparent that imposing a G:C clamped duplex directly upon the thrombin aptamer motif (cTU-3) resulted in a significant loss of activity in comparison to a less tightly A:T clamped duplex (cTU-2) or the inclusion of a non-pairing thymine residue to loosely bridge the framework duplex with the binding motif (cTU-1). It is expected that most aptamer motifs could be incorporated into higher order circular structures without significant loss of activity by including a sufficient number of non-pairing residues, allowing increased flexibility for the binding motif at the cost of reducing construct thermal stability.

Activity measurements reveal that circular aptamer constructs can benefit from avidity gains in a manner analogous to antibodies. A 3-fold improvement in the potency of cTT-2 (Table I), which contains two exosite I aptamer heads, over GS-522 is indicative of this effect. Moreover, enhanced inhibition was observed for the cTU-1 and cTU-2 aptamer constructs containing two different binding domains directed at thrombin, even though only one of those domains was capable of inhibiting thrombin. Similar architectures incorporating multiple binding motifs for different epitopes on the same target molecule (*i.e.* simultaneous multiple binding) may also show promise in improving the specificity of aptamer constructs, a feature that could prove important in both therapeutic and diagnostic applications. This possibility is under investigation.

Enzymatic Ligation—Although the structure of T4 DNA ligase has not been solved, amino acid sequence similarity between many ligases is high (47), and the reported three-dimensional structures of ligases show highly conserved structural domains (48). Footprinting and modeling studies of T7 DNA ligase bound to nicked duplexes show contact of 7–9 nucleotides on the 5' side of the nick and 3–5 nucleotides on the 3' side (47). *Chlorella* ligase displays a slightly larger footprint, with 11–12 nucleotides on the 5' side and 8–9 nucleotides on the 3' side (49). With a minimum functional requirement shown here for four nucleotides on either side of the nick and assuming structural conservation between T4 and other ligases, it seems likely that the extended footprints for T7 and *Chlorella* ligases on a nicked duplex include inessential enzyme-duplex contacts. Alternatively, the aptamer domains on either end of the short duplex may be able to act as pseudo-duplex sequences participating in T4 DNA ligase recognition and binding. Comparing the current ligation efficiency (Fig. 2A) and thermal stability data (Table I), it appears that the presence of adequate conformational flexibility allowed by unpaired and more weakly

paired duplex nucleotides may be more important than overall duplex stability in determining ligation efficiency.

Conclusion—Here we have attempted to merge some of the principles of structural nanotechnology with aptamer functionality. By combining aptameric motifs in two-, three- and four-headed circular constructs, we have created architectures that by way of their structural framework have increased target binding affinities and much improved stability in biological fluids. The modular design and use of restriction enzymes allows for an extensible system in which a large number of binding motifs may be simply combined to form an even greater number of multivalent constructs. With improving selection methods and sophistication in structural nanotechnology, aptamer-based constructs should continue to demonstrate increasing value as tools for medicine and diagnostics.

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