3′ Poly(dA)-Tailed Thrombin DNA Aptamer to Increase DNase-Resistance and Clotting Inhibitory Activity

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Aptamers are an attractive candidate for a molecular-targeted drug since they can show ability to precisely recognize proteins with a large affinity similar to antibodies. A drawback of aptamers is that they can be easily degraded under biological conditions by DNase (or RNase). In this study, we attached poly(dA) tail to the 3′ end of thrombin DNA aptamer and found that its survival time in human plasma was prolonged by about threefold compared to the original aptamers (i.e., without the tail). We found that the attachment of a poly(dA) tail at the 3′ end caused an increase of the clotting inhibitory activity. A preliminary in vivo assay using BALB/c mice confirmed a considerably elongated clotting time for the tailed aptamer. When the poly(dA) tail was attached to three other DNA aptamers, DNase resistance was increased, indicating that the present finding can be universally applied to increase stability of DNA aptamers.

DNA aptamers are synthetic oligonucleotides with a specific sequence optimized for the ability to bind to target proteins using a so called "in vitro selection (or evolution) technique" (SELEX).[1,2] Because of this selection formula, aptamers have excellent molecular recognition ability: the dissociation constant is in the high pico-molar or low nano-molar range and the specificity constant is 10^3 or more.[3] These abilities are comparable to antibodies. Aptamers offer advantages over antibodies since they can be produced with simple chemical synthesis and show little immunogenicity. One of the major issues in their clinical application is to provide stability in biological fluids since native DNA degrade rapidly under biological conditions and thus they are not suited for use as practical drugs. DNA analogues, including phosphothioate, 2′-*O*-methyl, and 2′-*O*-fluoro modifications, can provide good resistance against enzymatic hydrolysis, while toxicity due to dosing these non-natural compounds becomes a serious problem.[4,5] Conjugation of poly(ethylene glycol) (PEG) helps to stabilize the aptamer and also facilitates clinical delivery. However, PEG reduces binding affinity between the PEG/aptamers and target molecules.[6] Therefore, an alternative way to provide both stability and high affinity to native polynucleotides has been sought for long time. This is an aim of the present paper.

The DNA aptamer with the highest affinity for thrombin was found to have a sequence of d(GGTTGGTGTGGTTGG) (denoted TBA hereinafter)[7] by SELEX and this DNA aptamer disrupts thrombin-mediated clotting and does not compete with other inhibitors for thrombin.[8] Structural studies with NMR and X-ray revealed that TBA adopts a highly compacted symmetrical structure which consists of two tetrads of guanosine base pairs and three loops, as presented in Figure 1. The TBA binding site on thrombin was determined by Paborsky et al.[9] and called exocite 1 and is mainly comprised of positively charged residues such as Arg, Lys, and His. Recently,

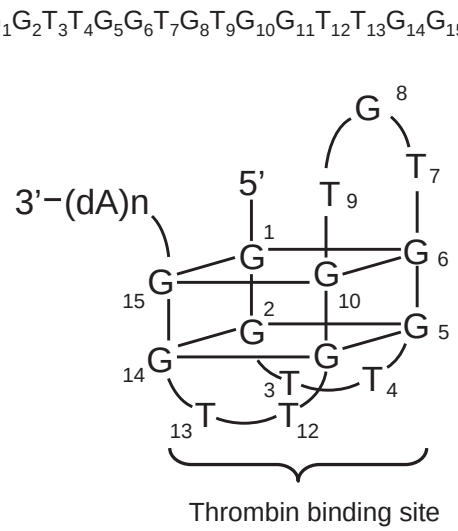

Figure 1. Three-dimensional structure of thrombin aptamer and the position of the 3′ end attached poly(dA) tail.

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Padmanabhan et al.[10] showed that the portions $G_2T_3T_4G_5$ and $G_{11}T_{12}T_{13}G_{14}$ (see Figure 1) are responsible for binding with exocite 1. The driving force of this interaction is thought to be a combination of electrostatic interaction and molecular pattern recognition.[10] The biological and chemical properties of TBA are well understood and thus we decided to use TBA as a model aptamer in this work.

There have been many studies carried out to improve binding affinity of thrombin aptamers as well as increasing their stability. This is because the thrombin aptamers can be an excellent anticoagulant that offers controlled onset and offset of action, reduced bleeding complications, and low risk of heparin-induced thrombocytopenia.[11] Among others, Ikebukuro et al.[12] have improved the in vitro selection technique and reported that when they added a specific combination of 8 mer oligonucleotides to both 5′ and 3′ ends of TBA, the aptamer showed a higher thrombin inhibitory activity than that of the original TBA. Recently, one TBA analogue has been in pre-clinical trials for acute cardiovascular procedures and this analogue utilizes both chemical modification and PEG conjugation.[3]

When we carried out other work that focused on a novel complex made from β-1,3-glucan and polynucleotides, we found that the polynucleotide stability in vitro and vivo unexpectedly increased when poly(dA) tails were attached.[13] In this paper, we extend this poly(dA) tail attachment to TBA and explore its stability and inhibitory activity.

Experimental

Materials. We attached various homo-oligonucleotide tails at either the 3′ or 5′ of TBA and the sequences and sample codes are summarized in Table 1. All oligonucleotides were obtained from Hokkaido System Science. Each oligonucleotide was dissolved in 1.0×10^{-2} M Tris (pH 7.4, 1.0×10^{-1} M KCl) and its concentration was determined from the absorbance at 260 nm at 80 °C. Before measurement, the samples were annealed to 90 °C and cooled slowly to room temperature for 12 h. Human thrombin and fibrinogen were purchased from CALBIOCHEM (product numbers are 605195 and 341576, respectively). Human plasma was purchased from COSMO BIO (12181201). For all solutions, super purified water (MilliQ) was used.

Spectroscopy. Circular dichroism (CD) spectra were measured at 37 °C on a JASCO J-720WI spectrometer with a 1 cm path length cuvete. UV–visible spectra were measured on a Jasco V-570 UV spectrophotometer with a 1 cm cell to determine the turbidity (or transmittance).

Clotting Inhibitory Assay. In order to determine the inhibitory effect of the aptamers, the time course of clotting was measured as transmittance change over time. 0.1 NIH unit of thrombin was added to a 2.0 mL of fibrinogen solution (3.0×10^{-6} M) and the transmittance was measured at intervals. The aptamer solution with a final concentration of 3.7×10^{-8} M was added at the same time as thrombin. Before and after mixing, all solutions were kept at 37 °C.

Table 1. Sample Codes and Sequences[a)]

Sample codes	Sequences
TBA	GGTTGGTGTGGTTGG
TBAA20	GGTTGGTGTGGTTGG (dA)20
A20TBA	(dA)20GGTTGGTGTGGTTGG
TBANx (in general)	GGTTGGTGTGGTTGG(dN)x
TBA31	CACTGGTAGGTTGGTGTGGTG GCCAGTG

a) N: base, x: number.

Polyacrylamide Gel Electrophoresis. The stability of aptamers in human plasma was examined with gel electrophoresis. A typical procedure is as follows: 8.5 μg of oligonucleotide was added to 500 μL of human plasma and the mixture was incubated at 37 °C for an appropriate interval. After incubation, the oligonucleotide was extracted with phenol and examined by gel electrophoresis at room temperature. The migrated oligonucleotide in the gel was stained with a SYBR Gold nucleic acid gel stain kit (Invitrogen). The molecular weight (MW) of the aptamer fragment was determined from the migration length in polyacrylamide/urea gels (15% acrylamide/7 M urea). The dissociation constant (K_d) between the aptamers and thrombin was approximately evaluated with the electrophoresis mobility shift assay.[14] A thrombin solution at various concentrations (from 0.25×10^{-6} to 3.0×10^{-6} M) was mixed with an aptamer solution (fixed at 3×10^{-6} M) and the mixture was examined by gel electrophoresis at 2 °C. The bound to unbound aptamer molar ratio was determined from the band brightness for each mixing ratio.

Clotting Assay. TBA (9 nmole) or TBAA (3 or 9 nmole) was injected through the tail vein of ether-anesthetized BALB/c 8 to 10 weeks old female mice. Immediately after the injection, blood was directly taken from the heart, and the clotting time was measured with an Amelung coagulometer (Amelung, Lemgo, Germany) according to the method indicated by the manufacturer.

Results and Discussion

Prolonged Degradation Due to Poly(dA) Tail Attachment. Figure 2 shows two sets of electrophoresis photographs: (a) & (c) and (b) & (d), comparing the time course of hydrolysis of TBA (a) and TBAA20 (b) when they were in human plasma, and their thrombin binding assay (c) & (d). For TBA, the migration length did not change for all samples and no smeared bands were observed, while the brightness of the band decreased with time. This result indicates that the TBA concentration simply decreased as time elapsed and thus can be interpreted to mean that once a TBA molecule is captured by DNase it is degraded rapidly. When TBAA20 was examined in the same manner, the band became smeared and shifted downward (to lower molecular weight) during the first 24 h. Even after 48 h, there was some amount of DNA remaining for TBAA20, corresponding to TBA (without the poly(dA) tail), while TBA completely disappeared after 24 h. Comparison of these two results indicates considerable difference in degradation pattern between TAB and TBAA20.

We examined the thrombin-binding ability for degraded aptamers with gel shift assay and the obtained image is presented as (c) and (d). Even after 48 h TBAA20/thrombin complex was observed although the band brightness attenuated considerably, indicating that TBAA20 maintained binding ability after 48 h. On the other hand, the binding ability of TBA was rapidly eliminated. Combining the results of (b) and (d), it can be concluded that attachment of a poly(dA) tail at the 3′ end lowered the degradation of molecules due to hydrolysis, and the degradation presumably occurred from the 3′ end at the poly(dA) tail. This conclusion is consistent with the fact

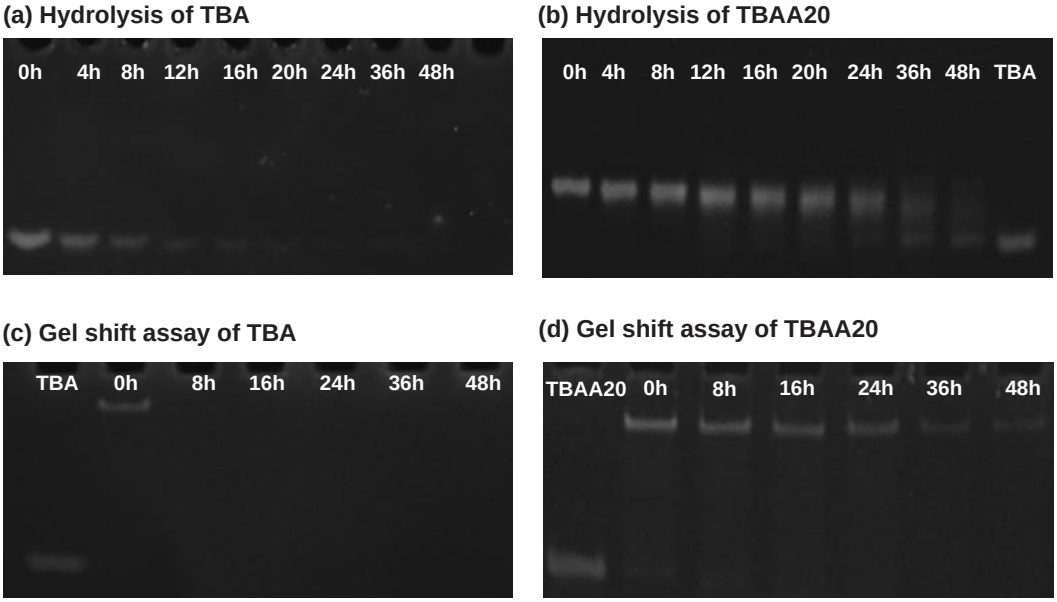

Figure 2. Comparison of the time course of hydrolysis in human plasma for TBA and TBAA20 and thrombin binding ability assay. (a) & (b): 8.5 μg of sample was immersed in 500 μL of human plasma at 37 °C and examined with 15% acrylamide gel at RT applying 100 V. (c) & (d): final concentration of 9.0×10^{-6} M of thrombin was added to each hydrolyzed aptamer and examined with 15% acrylamide gel.

Table 2. Half Life of Degradation of Several Aptamers in Human Serum

Sample	TBA	TBAA10	TBAA20	TBAT20	TBAC20	A20TBA
T_h/h	8.3 ± 0.3	24 ± 0.4	29 ± 0.9	10 ± 0.6	8.0 ± 0.4	9.4 ± 0.5

that the major DNases in plasma are 3′ exonucleases.[15] When we examined A20TBA [TBA with a poly(dA) tail attached at the 5′ end] for both degradation and the thrombin binding assay, it degraded as rapidly as TBA. This result also agrees with 3′ exonuclease mediated degradation occurring.

MW for each band was determined from the migration length and the poly(dA) tail average length (n_A) was evaluated by subtracting the MW of TBA from each obtained MW. The integrated band brightness is related to the population of the surviving aptamer, where we summed up all visible bands when they were smeared. Both brightness and n_A are plotted against incubation time in Figure 3, comparing between TBA and TBAA10. The value of n_A almost linearly decreased and became zero after 24 h, while the line connecting the brightness data points bends downward at 24 h. These results indicate that the degradation rate of the aptamer was relatively slow as long as it had the dA tail, once the dA tail was digested it was rapidly hydrolyzed. The half life (T_h), required time for the concentration of surviving aptamer to decay to half of its initial value, was evaluated from a plot similar to Figure 3 and listed in Table 2 as well as the results of other samples.

Table 2 compares T_h of TBA with various types of tailed TBAs. TBAC20 and TBAT20 showed no significant prolongation. The table shows that attachment of a poly(dA) tail at the 3′ end provides a significant increase in T_h: 3-fold at $n_A = 10$ and 3–4 fold at $n_A = 20$. More than 30 years ago, Yanagawa et al.[16,17] reported that poly(dA) is less degradable than other homo deoxynucleotides. Even at the present time, we do not have a clear explanation for this strange anti-nuclease resistance for poly(dA). It is interesting that the poly(dA) tail is much more stable than highly structurally organized portions of TBA.

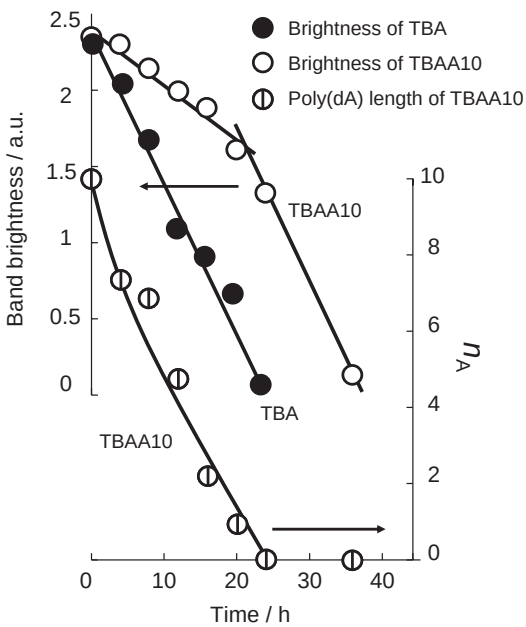

Figure 3. The hydrolysis time dependence of the band brightness and poly(dA) length for TBAA10 and TBA.

One of the drawbacks of DNA (or RNA) aptamers is their short circulation time in biological fluids. In the blood stream,

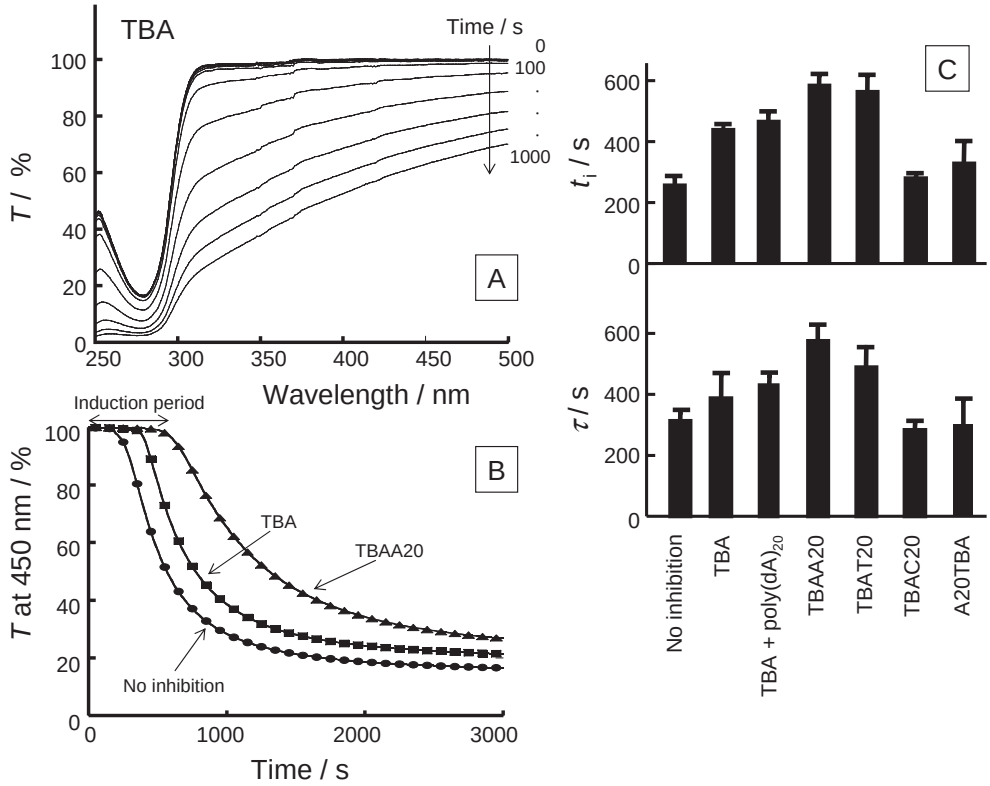

Figure 4. Thrombin clotting behavior observed with the transmittance change (A) and the time course of the transmittance at 450 nm comparing TBA and TBAA20 (B), and comparison of the induction period (t_i) and the specific time of clotting (τ): both are defined by eq 1. To a fibrinogen solution (2.0 mL, 3.0×10^{-6} M), 0.1 NIH unit of thrombin and an aptamer solution (3.7×10^{-8} M) were added and the transmittance was measured at interval at 37 °C.

Table 3. Comparison of the Thrombin Inhibitory Parameters for TBA Aptamers[a)]

	No inhibition	TBA	TBAA 20	A20 TBA	TBA+ Poly(dA)$_{20}$	TBA C20	TBA G20	TBA T20	T20 TBA	TBA31
t_i/s	2.5 ± 0.3	4.3 ± 0.1	5.7 ± 0.4	3.2 ± 0.7	4.5 ± 0.3	2.7 ± 0.1	3.2 ± 0.2	5.5 ± 0.5	4.8 ± 0.3	7.7 ± 0.2
τ/s	3.1 ± 0.3	3.8 ± 0.8	5.7 ± 0.5	3.0 ± 0.8	4.7 ± 0.3	2.8 ± 0.3	3.5 ± 0.4	4.8 ± 0.7	5.1 ± 0.1	7.4 ± 0.6

a) unit: $\times 10$ s.

they are enzymatically degraded as well as being rapidly eliminated by the reticuloendothelial system (RES). PEG modification (PEGylation) of aptamers helps to stabilize them in solution and also facilitates clinical delivery. PEGylation provides a shielding "stealth" effect, suppressing recognition of the aptamers by RES and nucleases, to prolong the circulation time. In most cases, the stealth effects also decrease the binding ability of the aptamers to its target proteins, which is a problem in PEGylation. According to previous studies,[18] the half-life of TBA in human blood is about 2 min, whereas attachment of 40 kDa PEG increased the half-life time to several hours. Compared with the present poly(dA) tail and PEG results, the poly(dA) tail attachment may be less effective in incrementing the half-life. However, it should be noted that the attachment of poly(dA) tail increased the anticoagulation activity of TBA as mentioned later.

Anticoagulation Activity of Poly(dA)-Tailed Thrombin Aptamer. Thrombin converts fibrinogen to a monomer that assembles into fibrin and the resultant fibrin eventually leads to coagulation. The coagulation can be detected by decrease in transmittance as presented in Figure 4A. When the transmittance at 450 nm (T_{450}) was plotted against time, T_{450} changed in the initial period (induction time) and decreased exponentially (Figure 4B).

The exponential decay could be well fitted by the following equation given a specific time (τ) and an induction time (t_i).

$$T_{450} = (100 - T_\infty) \exp\left(-\frac{t - t_i}{\tau}\right) + T_\infty \quad (1)$$

Here, T_∞ is an asymptotic value of the transmittance after a prolonged period of reaction.

When we added TBA in the same assay system, the coagulation was slowed with τ and t_i increasing by 173% and 123%, respectively, confirming the reported inhibitory activity of the thrombin aptamer. It is interesting that TBAA20 is more clotting-inhibitory than TBA, and the two parameters increased by 231% and 184% compared with the non-inhibition, respectively. We examined other tailed TBA aptamers and the obtained parameters are summarized in Table 3 and as a bar chart in Figure 4C. For comparison, we examined a simple mixture

of poly(dA) and TBA and found no significant change. When a poly(dT)$_{20}$ tail was attached to the 3′-end (TBAT20), it provided the same effect with TBAA20. And when the poly(dA) tail was attached to the 5′-end (A20TBA), no inhibitory activity was observed. These comparisons indicate that it is essential to attach poly(dA) or poly(dT) tail to 3′ end in order to enhance the inhibition ability. When we increased the TBA or TBAA20 concentrations at fixed thrombin and fibrinogen concentrations, both τ and t_i increased almost proportionally to the aptamer concentration. Figure 5 plots τ and t_i against n_A. With increasing n_A, both τ and t_i gradually decrease and come close to those of TBA at $n_A = 40$. Considering that the base number of the TBA body itself is 15, longer poly(dA) tails may spatially hinder interactions between thrombin and aptamer.

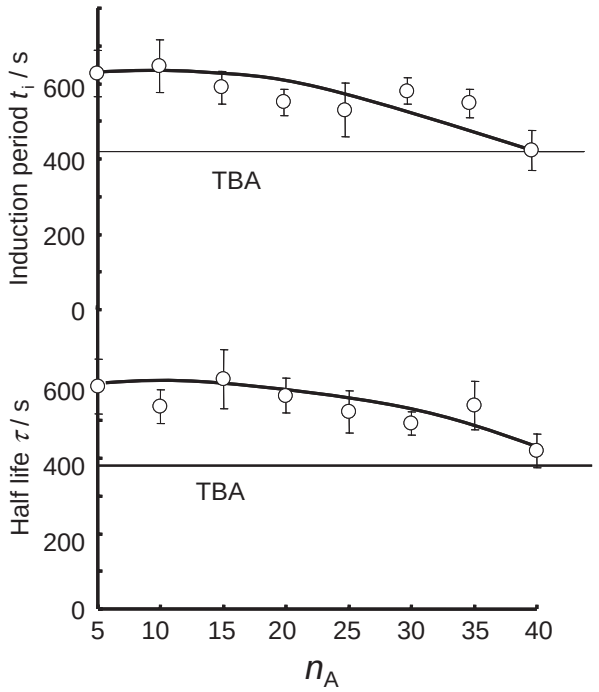

Figure 5. Poly(dA) tail length (n_A) dependence of t_i and τ for TBAAx.

We tried to estimate the dissociation constant (K_d) for TBA and TBAA20 with the gel electrophoresis mobility assay. However, as shown in the Supporting Information, the bound aptamer fraction increased in a sigmoidal manner in increase of thrombin concentration and thus the relation could not be fitted with a simple bimolecular reaction model.[13] In dilute thrombin concentrations ($<1 \times 10^{-6}$ M, while the aptamer concentration was fixed at 3×10^{-6} M), K_d of TBA seemed larger than that of TBAA20. On the other hand, in large concentrations ($>1 \times 10^{-6}$ M), K_d of TBA was slightly smaller than that of TBAA20. These obtained values are in a similar range with previously reported results.[12] We carried out the inhibitory activity assay within that dilute concentration range and this may be related to the higher activity of TBAA20 than TBA.

Ikebukuro et al.[12] improved the SELEX technique and obtained a new thrombin aptamer with a higher inhibitory activity than TBA. For their aptamer (denoted by TBA31, the sequence is shown in Table 1), the τ and t_i values were obtained in the same way as mentioned above and compared with the others in Table 3, confirming its high activity. According to them, TBA31 showed a slightly higher dissociation constant than that of TBA. They postulated that the aptamer showing the strongest binding activity is not always the best enzyme inhibitor and thus there might be a better way to optimize DNA sequences than SELEX. Referring to their results, the difference in K_d between TBA and TBAA20 may not be directly related to the activity difference and more data is needed to understand the origin of it.

Figure 6 presents the CD spectra of TBAA20, A20TBA, and a mixture of TBA and poly(dA)$_{20}$ as well as comparing those of TBA and poly(dA)$_{20}$ in the inset. The positive band at 290 nm and negative band at 260 nm are characteristic for anti-parallel G-quartets,[19,20] confirming the presence of an intramolecular tetraplex with an anti-parallel orientation compacted in the chair-like conformation presented in Figure 1. The spectra of TBAA20 and TBA + poly(dA)$_{20}$ mixture are almost identical to each other and those coincided with that of a numerical summation of TBA and poly(dA)$_{20}$ spectra. This fact means that neither 3′-tailed poly(dA) nor free

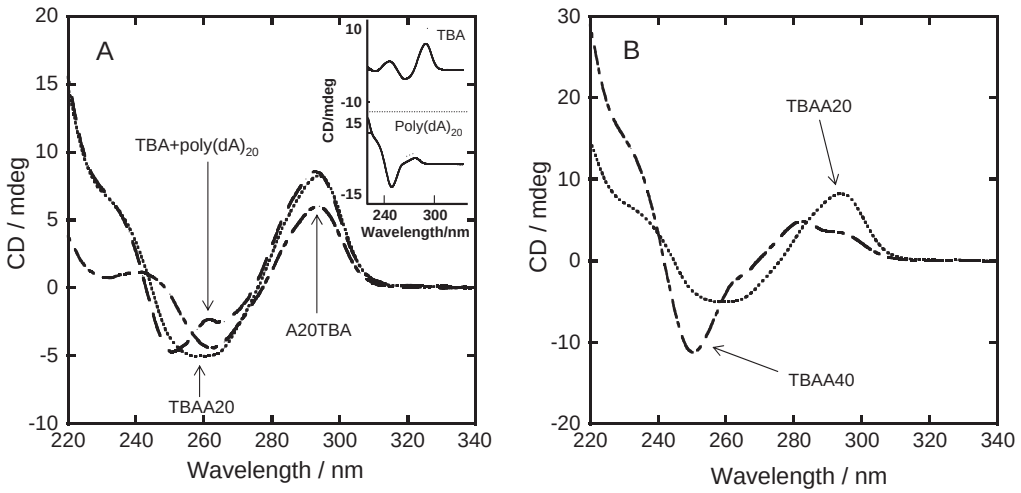

Figure 6. CD spectra of TBAA20, A20TBA, and a mixture of TBA and poly(dA)$_{20}$ (A), and of TBAA20 and TBAA40 (B). The inset of the panel (A) shows the CD spectra of TBA and poly(dA)$_{20}$.

poly(dA) perturb the original conformation of the aptamer. A20TBA spectrum shows a lower intensity around 290 nm than that of TBAA20 and is deviated in the range of 206–220 nm. Both facts can be interpreted by deformation of the original anti-parallel G-quartet conformation and thus rationalize lost activity of A20TBA. When poly(dC) was attached on the 3′ or 5′ end, CD showed a heavily deformed anti-parallel G-quartet due to duplex formation between G and C and the activity was completely diminished. The same deformation was observed for longer poly(dA) tails (Figure 6B), probably because of interaction between the tail dA and dT. As expected, with increasing tail length, the deformation became more obvious and thus the activity decreased. At this moment, we do not have a clear explanation why the poly(dA) tail attached on the 3′ end does not interfere with the entire conformation, although 3′ and 5′ are located at almost the identical position in the TBA structure as presented in Figure 1. Incidentally, when we measured the melting behavior of those aptamers by elevating temperature, all of them lost the quartette structure above 60 °C and there was no difference in the thermal stability among them.

We postulate that the lower activity in A20TBA can be related to deformation of the G-quartet due to interaction between the tail A and body T moieties. If the T moieties on the thrombin binding sites are interacting with the poly(dA) tail, such interaction might spatially obstruct the recognition between aptamer and thrombin. This may be another reason that A20TBA shows a lower activity than TBA.

TBAT20 did not improve anti-DNase resistance at all (Table 2), while it increased the anticoagulation activity in the same degree as TBAA20 (Figure 4). The K_d of TBAT20 was determined in the same manner as mentioned above and found to be in the same range as TBAA20 (see Supporting Information). The CD spectrum of TBAT20 showed that the anti-parallel G-quartet is maintained. These results imply that the attachment of poly(dT) to the 3′ end of TBA provides anticoagulation activity by the same mechanism as TBAA20, while it cannot increase the resistance against DNase because the poly(dT) is degraded more rapidly than poly(dA).

Figure 7 compares the clotting time of mouse blood inhibited by TBA and TBAA20. The clotting time of TBAA20 at 3 nM dose was prolonged by about 2-fold of TBA at 9 nM dose. When the dose was increased to 9 nM for TBAA20, the clotting time was prolonged by about 3-fold of TBA at the same dose. These results can be interpreted by both the increased stability and enhanced activity of the poly(dA) tailed TBA, confirming the conclusions from the in vitro studies.

Application of the Poly(dA) Attachment to Other Aptamers. We examined whether the advantage of poly(dA) attachment is extendable to other DNA and the results are summarized in Table 4. For INF-γ, L-selectin, and tenascin aptamers, T_h was increased by 2–3 fold.

Conclusion

We attached a poly(dA) tail to the 3′ end of thrombin DNA aptamer to increase its survival time in human plasma as well as enhancing the clotting inhibitory activity. Gel shift assay showed that degraded TBAA20 maintained the thrombin-binding ability even after left in plasma for 48 h, while TBA lost its ability after 1 h. Gel electrophoresis data indicated the degradation rate of the aptamer was relatively slow as long as it had the dA tail, once the dA tail was digested it was rapidly hydrolyzed. When poly(dA) tail was attached to the 5′ end (such as A20TBA), DNase resistance was not improved and the clotting inhibitory activity was decreased. The loss of the activity was consistent with the large change in CD spectrum of A20TBA while TBAA20 showed a similar spectrum to that of a simple mixture of TBA and poly(dA)$_{20}$. A preliminary in vivo assay using BALB/c mice confirmed a considerably elongated clotting time for the tailed aptamer. When the poly(dA) tail was attached to three other DNA aptamers, increased DNase resistance was observed, indicating that the present finding can be universally applied to increase stability of DNA aptamers.

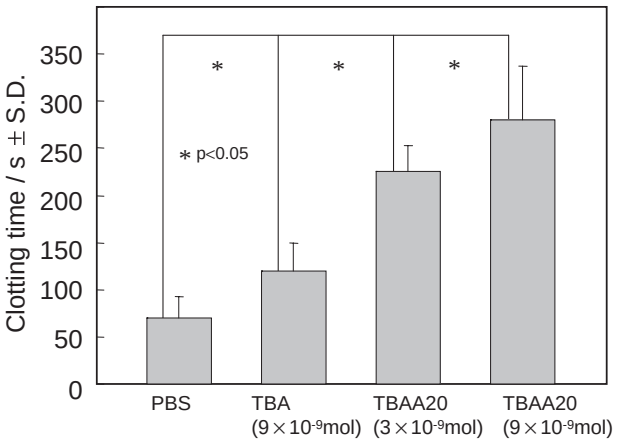

Figure 7. Comparison of the clotting time for mouse blood inhibited by TBA and TBAA20.

Table 4. Stability of Other Poly(dA)$_{20}$-Attached DNA Aptamers in Human Serum

Target protein	Number of bases	T_h•dA$_{20}$/T_h	References
IFNγ	26 (46) mer	3.0	21
L-selectin	39 (59) mer	2.4	22
Tenascin-c	69 (89) mer	3.3	23

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Supporting Information

Estimation of dissociation constant with electrophoresis mobility shift assay and circular dichroism spectra. This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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