Development of the Convenient Method for Artificial Nucleic Acid Aptamer Preparations Using CE-SELEX

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Preface

Specific binders comprised of nucleic acids, that is, RNA/DNA aptamers, are attractive functional biopolymers owing to their potential broad application in medicine, food hygiene, environmental analysis, and biological research. Despite the large number of reports on selection of natural DNA/RNA aptamers, there are not many examples of direct selection of chemically modified nucleic acid aptamers. This is because of (i) the inferior efficiency and accuracy of polymerase reactions involving transcription/reverse transcription of modified nucleotides compared with those of natural nucleotides, (ii) technical difficulties and additional time and effort required when using modified nucleic acid libraries, and (iii) ambiguous efficacies of chemical modifications in binding properties until recently; in contrast, the effects of chemical modifications on biostability are well studied using various nucleotide analogs. Although reports on the direct selection of a modified nucleic acid library remain in the minority, chemical modifications would be essential when further functional expansion of nucleic acid aptamers, in particular for medical and biological uses, is considered.

The present thesis consists of five chapters. Chapter 1 describes general introduction of chemically modified nucleic acid aptamers. In Chapter 2, improvement of nuclease resistance of thrombin–binding aptamer by enzymatically addition of sugar–modified nucleotides is described. In Chapter 3, capillary electrophoresis–systematic evolution of ligands by exponential enrichment (CE-SELEX) selections using a

DNA-based library that contains 2'-*O*,4'-*C*-methylene-bridged/linked bicyclic ribonucleotides (B/L nucleotides) over the full length are presented. In Chapter 4, CE–SELEX selections of DNA-based chimeric aptamers with base and sugar modifications are described. Finally, these works are summarized in Chapter 5.

In the present study, CE–SELEX selections of chemically modified DNA aptamers were first demonstrated. The author wishes that the developed methodology for aptamer selections will find vast applications in the field of medicine and bioanalysis.

Contents

Abbreviations

15TBA	15-mer thrombin-binding aptamer
2',4'-BNA/LNA	2'-*O*,4'-*C*-methylene bridged/locked nucleic acid
29TBA	29-mer thrombin-binding aptamer
6-FAM	6-carboxyfluorescein
76TBA	76-mer thrombin-binding aptamer
ADCC	antibody-dependent cell-mediated cytotoxicity
AMD	age-related macular degeneration
ANA	arabinonucleic acid
ATP	adenosine triphosphate
B/L nucleotide	2'-*O*,4'-*C*-methylene-bridged/linked bicyclic ribonucleotide
BNA/LNA	2'-*O*,4'-*C*-methylene
CDC	complement-dependent cytotoxicity
CE	capillary electrophoresis
CeNA	cyclohexenyl nucleic acid
CST	compartmentalized self-tagging
C^{me}	5-methylcytosine
CD	circular dichroism
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EOF	electro-osmotic flow
ESI	electrospray ionization
f29TBA	29-mer thrombin-binding aptamer
FAB	fast atom bombardment
FANA	2'-fluoro- arabinonucleic acid
HPLC	high-performance liquid chromatography
HNA	1,5- anhydrohexitol nucleic acid
K_d	dissociation constant
*KOD*2	*KOD* mutant DNA polymerase
LIF	laser-induced fluorescence
M-SELEX	microfluidic-SELEX
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NMR	nuclear magnetic resonance

MPLC	medium-pressure liquid chromatography
NTPs/dNTPs	substrate triphosphates
NECEEM	non-equilibrium capillary electrophoresis of equilibrium mixtures
ODN	oligodeoxyribonucleotide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RSV	Rous sarcoma virus
SELEX	systematic evolution of ligands by exponential enrichment
ssBNA/LNA	single stranded 2',4'-BNA/LNA
TBA	thrombin binding aptamer
TBAF	tetra-*n*-butylammonium fluoride
T^bTP	B/L thymidine-5'- triphosphate
TdT	terminal deoxynucleotidyl transferase
THF	tetrahydrofuran
TNA	α-L-threofuranosyl nucleic acid
UV	ultraviolet
VEGF	vascular endothelial growth factor
XNA	xeno-nucleic acid

Chapter 1

General introduction

1. Background

For living organisms, nucleic acids like DNA and RNA are fundamental biomacromolecules that function to preserve, transfer, and express genetic information. The potential of nucleic acids to behave as functional molecules had not attracted much interest due to the classic notions that proteins are functional molecules playing an important role in the body, whereas DNA is a blueprint of proteins and RNA is a mediator. However, in the 1980s, Cech *et al*. and Altman *et al*. discovered RNA enzymes (ribozymes) that catalyze RNA self-cleavage or RNA transesterification in splicing.[1,2] Since this discovery, much interest has been focused on the creation and application of functional nucleic acids with new activities that are different from the original activities of nucleic acids in the classical sense.

Around 1990, Szostak *et al*., Joyce *et al*., and Gold *et al*. independently developed a selection methodology to select RNA molecules that can catalyze a specific reaction (ribozyme) or bind to a specific molecule (aptamer) from the RNA library (RNA pool of miscellaneous random sequences).[3–5] This method is called *in vitro* selection; often called SELEX (systematic evolution of ligands by exponential enrichment) when used especially for selecting aptamers. First, these selections were performed for RNA, following which the

creation of the DNA enzyme as catalyst and the DNA aptamer as specific binder was attempted using the *in vitro* selection method.[6]

Single-stranded RNA/DNA enzymes and aptamers with a particular sequence, which were selected using the random libraries, can exert their activities by forming a specific steric structure with intramolecular hydrogen bonding, stacking interactions, electrostatic interactions, and metal coordination. These RNA/DNA enzymes and aptamers can exhibit activity and functionality that are similar to those of protein enzymes and antibodies, respectively, though functional nucleic acids with activity superior to the corresponding protein have not been reported yet. Thus far, various RNA/DNA enzymes were reported, including the enzyme that catalyzes the isomerization of a bridged biphenyl and the one that catalyzes alkylation.[7,8] In addition, using *in vitro* selection, various RNA/DNA aptamers have been created, which are specific for a broad spectrum of targets involving small molecules like ATP or amino acids (*e.g.*, arginine), macromolecules like thrombin, and particles like Rous sarcoma virus (RSV).[9]

However, some limitations on the ability of nucleic acid molecules to function as catalysts and specific binders were revealed. Although many examples of artificial molecular evolution of functional nucleic acids have been reported,[10–13] the reason for these limitations is the fact that nucleic acids consist of a combination of only four nucleotides, while proteins consist of a combination of twenty amino acids with diverse functional groups in their side chains. Therefore, researchers have attempted recovering by *in vitro* selection of modified nucleic acid enzymes and aptamers from chemically modified libraries, in which

different functionalities that cannot be found in a nucleotide are incorporated.

In particular, nucleic acid aptamers have attracted keen interest because of their potential medical uses. For applications in the medical field, improvement of nuclease resistance, *i.e.*, biostability in serum or cells as well as affinity to target molecules, became an important issue. An effective solution is selection of a specific binder from the modified nucleic acid library and the subsequent post-SELEX chemical modification of the selected aptamers. Regarding binding affinity, some modified nucleic acid aptamers can bind to proteins like enzymes or growth factors relative to a specific disease, with a dissociation constant of subnanomolar to subpicomolar, comparable to those reported for antibodies. Thus, the development of chemically modified aptamers and the related technologies have recently received much attention.

2. The First Aptamer Drug

Although RNA/DNA aptamers do not cause antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), their specific binding abilities are expected to neutralize actions on the target and relieve symptoms. Indeed, the first example of an aptamer drug, “Macugen (pegaptanib sodium injection)” is being used for age-related macular degeneration (AMD) therapy.[14] Pegaptanib is a RNA-based aptamer that involves 2'-fluoropyrimidine nucleotides (U, C) and 2'-methoxy purine nucleotides (A, G) to remain intact under physiological conditions. In addition, a branched

polyethylene glycol strand (40 kDa) and 3'-thymidylic acid are introduced at its 5' and 3' ends, respectively. The 5'-end modification is known to prolong circulation time *in vivo* as well as to enhance nuclease resistance. Pegaptanib tightly binds to the vascular endothelial growth factor (VEGF) in a Ca^{2+}-dependent fashion with a dissociation constant (K_d) of 200 pM, while the corresponding aptamer, which lacks the 5'- and 3'-end capping, has much higher affinity (K_d = 49 ± 6 pM at 37°C in phosphate buffered saline containing 2 mM Ca^{2+}). Incidentally, the K_d value of the anti-VEGF antibody, "Avastin (bevacizumab)," which is used for cancer therapies, is 1.1 nM at 25°C. The natural type of anti-VEGF RNA aptamers also shows high binding affinity at a picomolar range (K_d = 140 ± 4 pM at 37°C in phosphate-buffered saline containing no Ca^{2+}),[15] indicating that the effects of chemical modifications on binding affinity are not significant, considering the different Ca^{2+} concentrations used. In contrast, the effects on biostability are remarkable; pegaptanib was found to be stable after incubation at ambient temperature for 18 h in human plasma containing ethylenediaminetetraacetic acid, whereas unmodified oligoribonucleic acids are known to degrade within a few minutes *in vivo*[16] of the RNA polymerase (T7 RNA polymerase) used for SELEX, the 2'-methoxy (–OMe) groups need to be replaced with 2'-hydroxy (–OH) groups of natural purine nucleotides after obtaining the precursor from a modified RNA library involving 2'-fluoro (–F) analogs of uridine and cytidine and natural adenosine and guanosine (Figure 1-1). The post-SELEX modifications have been successful in rendering nuclease resistance but required considerable time and

effort because binding affinities could be markedly decreased or eliminated, depending on the position of the replacement. To overcome this problem, T7 RNA polymerase double mutant Y639F/H784A was used for enzymatic preparation of the modified RNA library in the SELEX processes, and 2'-OMe RNA aptamers specific to VEGF have been successfully screened directly.[17] One of the 2'-OMe RNA aptamers that could be minimized to 23-mer (which is an unusual short length) was found to be quite stable, and no degradation was observed after incubation at 37°C for 96 h in plasma. Despite being successful for direct selection, structural minimizing, and biostability enhancing, these aptamers were found to have binding affinities in a low nanomolar range that were inferior to those of pegaptanib and its precursors.

This may be because the potential binding ability of the chemical library used was inherently low, and/or the unusual polymerase reaction would cause unfavorable critical biases in the sequences of the chemical library constructed. Conversely, it may also be possible that differences in the selection outcomes would not be sufficient to clarify their causes because only a part of all possible sequences were screened. This is a characteristic difficulty in SELEX when chemical modification is involved. Regardless of this difficulty, a polymerase reaction involving modified nucleotides is a key step that should be improved and optimized to construct desirable direct selection systems for modified RNA/DNA aptamers when the SELEX methods are applied.

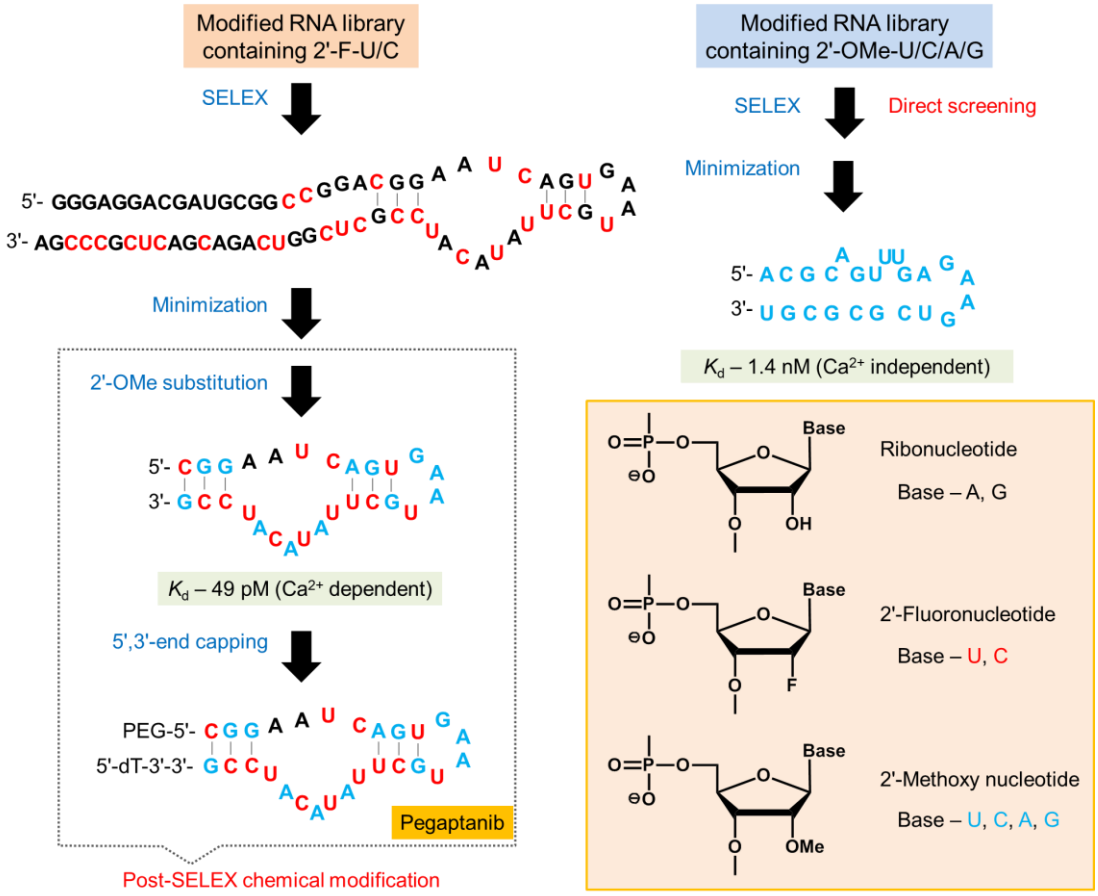


Figure 1-1. Preparation scheme for chemically modified nucleic acid aptamers that bind to VEGF. High nuclease-resistant 2'-methoxy nucleotides were introduced through Post-SELEX modification process (left), and fully modified 2'-OMe RNA aptamers were directly selected from a library of 2'-OMe transcripts (right).

3. Enzymatic Synthesis of Modified Nucleic Acids

RNA/DNA polymerases incorporate substrate triphosphates (NTPs/dNTPs) corresponding to the type of bases on the template strand and successively add them to the 3' end of the extending strand to form 3',5'-phosphodiester linkage. Some polymerases are known to accept chemically modified NTPs/dNTPs as substrates and can produce nucleic acid polymers containing foreign functionalities. Such

polymerase reactions are applied to DNA sequencing,[18–21] fluorophore, and redox labeling,[22,23] expanding the genetic alphabet,[24–26] and preparing library for SELEX.[27–57] Unlike enzymatic functional labeling of DNA, for which modified dNTP is often used in the presence of the corresponding natural dNTP to increase product yields, modified NTP/dNTP is generally used in the absence of the corresponding natural NTP/dNTP when modified natural nucleotide needs to be completely replaced with the corresponding modified nucleotide at all sites incorporated into the extending strand. In general, total replacement could decrease the product yield because the catalytic efficiencies of the polymerase may be affected by the modifications not only for substrate triphosphate but also on the extending strand and template. Previously reported kinetic studies using a base modified nucleotide showed that the reaction efficiencies of single modified nucleotide incorporation are drastically decreased when the modifications exist on the 3' terminus of the extending strand, although the single incorporation of the modified substrate proceeds smoothly at almost the same rate as the corresponding natural substrate.[48] The results indicate that the successive incorporation of modified nucleotides is the most difficult aspect of strand extension. Therefore, the inefficiency of modified RNA/DNA polymerization could naturally bias the outcomes of the selection; it could unintentionally lead to the exclusion of the sequences with the highest binding affinity. To reduce this influence, reactions are often conducted under very high enzyme and/or substrate concentrations to achieve large reaction velocities. However, it should be noted that such conditions are prone to result in

a high frequency of misincorporations. As the solution strategy, polymerase variants, triphosphate analogs, and their combinations that improve the reaction efficiency have been developed and are still being studied. Furthermore, when modified DNA is used in SELEX, the modified DNA is normally amplified indirectly by a polymerase chain reaction (PCR) to prepare the next library. After affinity selection, the selected modified DNA is reverse transcribed and PCR amplified to natural DNA, and then transcribed to modified DNA, even when PCR amplification was available for the modification.

For SELEX using modified DNA, certain thermophilic DNA polymerases, for example, *Pwo*, *Pfu*, *Vent*(*exo*$^-$), *Deep Vent*(*exo*$^-$), and *KOD Dash*, that belong to the evolutional family B, were found to be preferable to other types of DNA polymerases.[42] Especially in successive incorporations of modified nucleotides, those polymerases were found to exhibit much superior performance than family A DNA polymerases such as *Taq*, *Tth*, and thermo sequenase. In addition, a family D DNA polymerase derived from Pyrococcus horikoshii did not show any tolerance for chemical modification in the experiments using C5-substituted pyrimidine nucleoside triphosphates.[50] The efficiency of enzymatic production using modified dNTP varies depending on the site where the substituent is introduced. As for base modification, dNTP analogs with pyrimidine substituted at the 5th position and purine substituted at the 7th position of the base moiety tend to be acceptable for DNA polymerases and act as good substrates.[38,43] Modified purine nucleotide analogs at the 8th position can also be incorporated but with lower efficiency.[21,51] In addition, sugar

modifications such as 2'-fluoro, 2'-fluoro-D-arabino, and 2'-*O*,4'-*C*-methylene (BNA/LNA) were also found to be acceptable.[53–55] Furthermore, phosphate modified dNTPs with 5'-(α-thio)triphosphates and 5'-(α-borano)triphosphates were found to work as alternative substrates. Certain types of DNA polymerases were also found to accept some modifications of the leaving group of the phosphate moiety,[20] although those analogs have been applied for advanced DNA sequencing and not for SELEX.

Among the modifications, DNA polymerases could endure substitution at the base moieties, and various functional groups could be introduced into those positions with relatively high efficiency. When artificially created proteinlike functional nucleic acids were considered, researchers would first envisage introducing proteinous amino acids into nucleic acids. Indeed, base-modified dNTP analogs bearing various proteinous amino acids or their side chains have been reported to date, and their substrate properties in polymerase reactions such as PCR and primer extension have been investigated (Figure 1-2).[49] For example, PCR assays using *KOD Dash* DNA polymerase showed that triphosphates containing amino acyl group with basic (Arg, His, Lys), aromatic (Phe, Trp), aliphatic (Leu, Pro), and neutral hydrophilic (Gln, Ser, Thr) side chains act as good substrates, while those with acidic (Asp, Glu) and thiol (Cys) side chains act as poor substrates. Production of DNA-containing cysteinyl residue necessitated the addition of dithiothreitol as a reduction reagent. To introduce plural functionalities with high density, it was found that four natural nucleotides (A, G, C, T) are totally replaced with four base-modified nucleotides by the

addition of manganese chloride and betaine.[43,46] Those additives could improve efficiency and yield in the enzymatic production of modified DNA, although they could raise the frequency of misincorporation at the same time.[58,59]

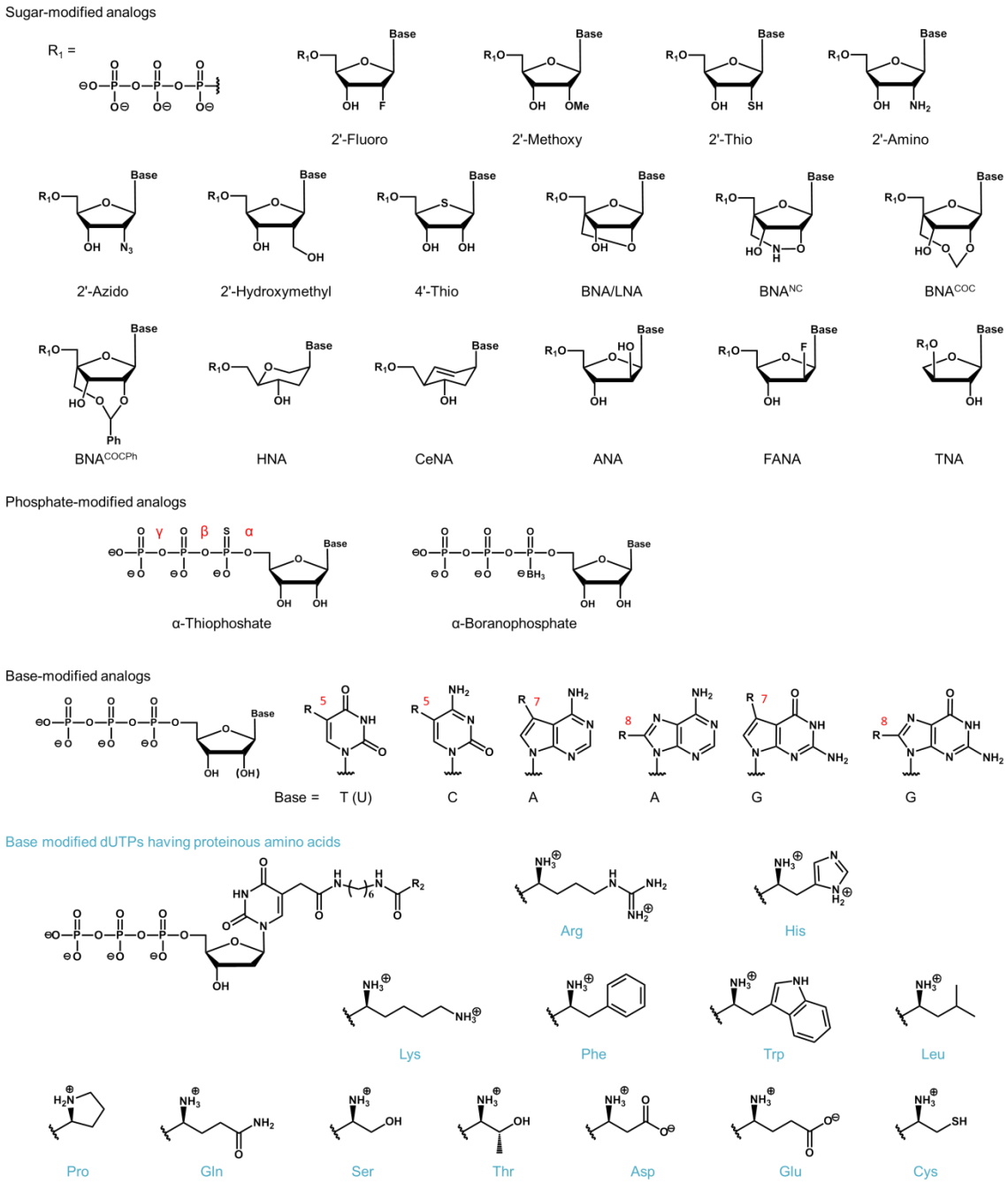


Figure 1-2. Examples of modified nucleoside triphosphates that act as substrates for polymerase reactions.

4. CE–SELEX Selection

Unlike many other alternative SELEX techniques based on the principal of affinity chromatography involving molecular interactions at the solid–liquid interface (Figure 1-3A), CE-SELEX can separate active species form non-active species in the liquid phase by non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[60] (Figure 1-3B). Furthermore, in NECEEM, electro-osmotic flow (EOF), which occurs from the anode to the cathode, can carry the target–aptamer complexes prior to unbound free oligonucleotides. Hence, CE-SELEX may be one of the best methods to enable efficient aptamer enrichment because it maximally excludes contamination of non-active species; in the typical SELEX methods, species non-specifically bound to the solid support cannot be completely washed out while maintaining the desirable specific binding, resulting in inevitable contamination of non-active species in the elution of active species. Thus, in this study, CE-SELEX was used for selecting modified DNA aptamers (Chapters 3 and 4).

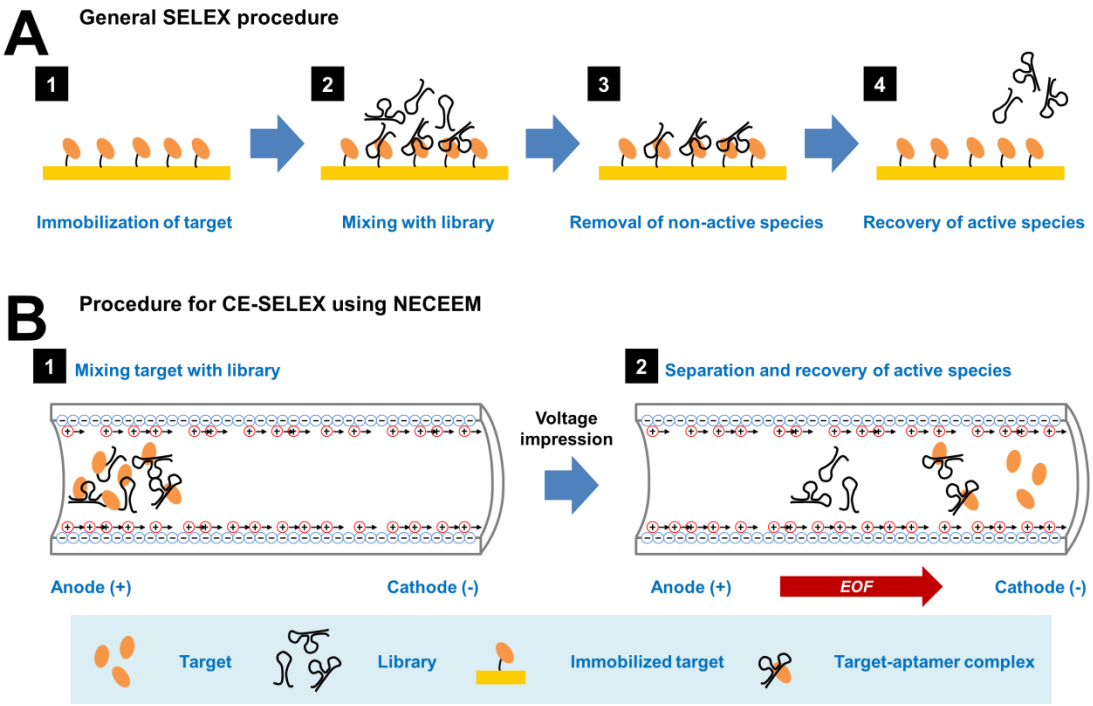


Figure 1-3. Procedure for standard SELEX based on the principal of affinity chromatography (A), and that for CE-SELEX using NECEEM employed for 2',4'-BNA/LNA aptamer selection (B).

Chapter 2

Improvement of Nuclease Resistance of Thrombin-binding Aptamer by Enzymatically Addition of Sugar-modified Nucleotides

1. Introduction

Researchers have created a variety of nucleic acid aptamers with the systematic evolution of ligands by exponential enrichment (SELEX) method[4,5,61] and studied them in order to apply them as research tools, biosensors, therapeutic agents and so on. With this technique, nucleic acid aptamers can be selected from a library of single-stranded oligonucleotides, differing in nucleotide sequence. In general, after being selected from an initial library, individual aptamers are isolated from an enriched pool by cloning and researchers determine their sequences. For practical use, nuclease resistance is a necessary property of aptamers. Therefore, in order to gain stability in cell cultures and *in vivo*, isolated monoclonal aptamers should be modified with proper substituents, based on their sequence information and the correlations between modifications and activities, which may involve costly and monotonous processes. If an enriched pool, functioning as the polyclonal family, could gain nuclease resistance without cloning, sequencing and the aforementioned modifications, the enriched pool, that is, the polyclonal aptamer,[62] could be used as a polyclonal antibody.

Until now, 2'-*O*,4'-*C*-methylene bridged/locked nucleic acid (2',4'-BNA[63,64]/LNA[65]) and its analogues,[66–70] have been developed, and

some of them showed excellent nuclease resistances. Recently, we reported the convenient conferring of nuclease resistance on nucleic acid by using 2',4'-bridged nucleoside-5'-triphosphates[54,71] and terminal deoxynucleotidyl transferase (TdT).[72] I was easily able to add the 2',4'-bridged nucleotides to the 3'-ends of oligodeoxy-ribonucleotides (ODNs) with an enzymatic reaction. After the addition, the nuclease resistances of the ODNs were enhanced, depending on the chemical structures of the bridged moieties. In this work, I have designed and synthesized a new 2',4'-bridged nucleoside-5'-triphosphate with a 2'-CH(Ph)OCH_2-4' bridged linkage, QTP, expecting that the bulkiness of the phenyl group would interfere with the nuclease degradation of the ODN. Furthermore, the substrate properties of QTP for TdT and the characterizations of thrombin binding aptamers (TBAs) capped with various 2',4'-bridged nucleotides for nuclease resistances, biostabilities in human serum and binding affinities to the target were investigated.

2. Results and Discussion

First, I studied the incorporation of the triphosphate QTP during the enzyme reaction with TdT, using a 26-mer single-stranded ODN with 5'-FAM-GGC GTT GAG TGA GTG AAT GAG TGA GT-3' (ODN1); the 5'-end of ODN1 was labelled with 6-carboxyfluorescein (6-FAM) for detection. Syntheses and characterizations of QTP and its intermediates are described in detail in Experimental procedures. As I have reported,[66] ODNs capped with K, L and M-type bridged nucleotides were quantitatively produced using KTP, LTP and MTP (see

Figure 2-1), respectively, after incubating the reaction mixture containing 0.4 μM of ODN1, 200 μM of KTP, LTP or MTP, 0.2 U/μL of TdT and the reaction buffer (an enzyme at 1 × concentration) for 1 h at 37°C. However, the use of QTP did not provide the corresponding ODN capped with a single Q-type bridged nucleotide under the same conditions. If I increased the enzyme concentration to 10 U/μL and extended the incubation time for a few hours, the addition reaction was almost completed (see Figure 2-2). I then assessed the nuclease resistance of the capped ODN for snake venom phosphodiesterase I, which has a strong 3'–5' exonuclease activity. It was about 736-fold more stable than uncapped ODN1 and 51-, 19- and 2.3-fold more stable than the other capped ODNs with K, L and M-type bridged nucleotides, respectively, under the above reaction conditions (see Table 2-1). These results indicated that the bulky phenyl group on the bridged linkage affected the addition reaction with TdT but improved resistance for phosphodiesterase I.

The addition of the Q-type bridged nucleotide at the 3'-end greatly enhanced the nuclease resistance; however, ODN1 is not an oligodeoxyribonucleotide with a special function, such as antisense ODNs,[73] decoy ODNs[74] or DNA aptamers.[75] Therefore, I then performed experiments with the same conditions using a thrombin binding aptamer,[76] 5'-FAM-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3' (TBA1) and its capped aptamers, TBA-K, TBA-L, TBA-M and TBA-Q, instead of ODN1 (see Figure 2-1). As shown in Figure 2-3, the band of the intact TBA1 disappeared after 120 minutes of incubation (lane 6, image A) while the bands of the intact TBA-M and TBA-Q were thick

(lanes 6, images D and E). Compared to the decay curves of intact aptamers in Figure 2-4, the capped aptamers were most stable in the order TBA-Q > TBA-M > TBA-L > TBA-K in the solution containing the nuclease. The initial reaction rates estimated from the decay curves of TBA1, TBA-K, TBA-L, TBA-M and TBA-Q were 8.5, 2.4, 1.6, 0.46 and 0.32, respectively (see Table 2-2). The capping effect of TBA1 was smaller than that of ODN1 presumably because the aptamer formed a specific conformation, that is, G-quadruplex,[77,78] which would have decreased the accessibility of the nuclease to the aptamer. However, the capping effect with bridged nucleotides clearly reflected in the nuclease resistances and the correlations between the chemical structures of the bridged linkages and the nuclease resistances were extremely consistent with the case of ODN1.

Next, I examined the biostabilities of these capped TBAs in human serum containing various types of nucleases. As shown in Figure 2-5, TBA-M and TBA-Q were apparently more stable than the others; more than 20% of the intact TBA remained in the cases of these two aptamers while the intact TBA was completely gone in the case of the uncapped TBA1 (see Figure 2-6) after incubating in 80% v/v human serum for 8 hours at 37°C. The relative rates of the degradation showed that TBA-M and TBA-Q were about 4.0 and 3.3-fold more stable than TBA1 (see Table 2-3), but on the other hand, TBA-Q was more stable than TBA-M in solution containing phosphodiesterase I. The difference in the stabilities was small, but I observed this tendency had good reproducibility, indicating that it may be due to the difference of the substrate specificities between phosphodiesterase I and 3'–5'

exonuclease contained in human serum and not to any measurement errors. The capping effect for the stability in human serum compared to that in phosphodiesterase I solution was significantly smaller because of the action of endonuclease contained in the serum. However, the effect on the nuclease resistance was still clearly observed, indicating that the action of 3' to 5' exonuclease would be significantly dominant compared to that of any other types of nuclease in human serum. Recently, Peng *et al*. reported an effort of enhancing the nuclease resistance of a thrombin binding aptamer in the sequence d($G_2T_2G_2TGTG_2T_2G_2$) by modification with 2'-deoxy-2'-fluoro-$_D$-arabinonucleotide.[79] Some modified aptamers exhibited increased nuclease resistances (4–7-fold) in 10% v/v fetal bovine serum at 37°C without losing their binding abilities. However, gaining stability in the serum required at least four replacements with the modified nucleotide. Furthermore, some modified aptamers almost lost their binding abilities completely depending on the numbers and positions of the replacement points. Thus, conferring nuclease resistance on aptamers by postmodification[80] is not very easy in general.

Finally, I measured the binding affinities of capped TBAs to human thrombin using non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[81] in order to assess the capping effect on the binding ability of the aptamer[82] (Figure 2-7). The K_d values for the target-TBA interactions were determined from the biding saturation curves of TBA with different concentrations of thrombin as seen in Figure 2-8. The K_d values of TBA1, TBA-K, TBA-L, TBA-M and TBA-Q for the target were 0.25 ± 0.06, 0.18 ± 0.04, 0.27 ± 0.05, 0.21 ±

0.06 and 0.28 ± 0.09 nM, respectively; those affinities were found to be almost the same level.

In conclusion, the capping of the 3'-ends of thrombin binding aptamers with bridged nucleotides increased the nuclease resistances 3.6–27-fold and the stabilities in human serum 1.5–4.0-fold. Also, the binding abilities of the aptamers were not affected by the capping. The capping could be simply executed *via* a one-step enzymatic process using 2',4'-bridged nucleoside 5'-triphosphate and TdT. The merit of this method is in developing a method that will enable us to modify and use aptamers without isolating them from the polyclonal family. The chemical structure of the bridged linker should be improved further. Particularly, it is interesting that phosphodiesterase I nucleases in human serum may have different substrate specificities, as shown in the experiment using TBA-M and TBA-Q. The bulkiness of the bridged ring structure likely enhanced the biostabilities; however, enlarging the ring structure would make its triphosphate analogue poorer as a substrate for TdT. Molecular design based on the steric structure of the substrate-binding site in TdT and the nuclease should lead to optimized chemical structures of the analogue in future.

3. Experimental Procedures

3.1. General

^{1}H- and ^{31}P-nuclear magnetic resonance (NMR) spectra were recorded on a JNM-EX270 or JNM-LA500 Fourier-transform-NMR spectrometer. Tetramethylsilane and 85% phosphoric acid were used

as the internal standards for ^{1}H- and ^{31}P-NMR, respectively. The sodium salt of QTP, generated from the corresponding triethylammonium salt using Dowex 50WX8 (Na^+ form), was used for NMR measurements. Mass spectral analyses for nucleoside analogues and oligonucleotides were performed on an ABI MDS-Sciex API-100 spectrometer (ESI), JEOL JMS-600 (FAB), JEOL JMS-700, or Bluker Daltonics Autoflex II TOF/TOF (MALDI-TOF) mass spectrometer. IR spectra were recorded on a JASCO FT/IR-200 or JASCO FT/IR-4200 spectrometer. Optical rotations were recorded on a JASCO DIP-370 instrument. Ultraviolet (UV) analyses were performed on a Shimadzu UV-1200 spectrometer. For column chromatography, Fuji Silysia silica gel PSQ-100B (0.100 mm) and FL-100D (0.100 mm) was used. For flash column chromatography, silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) was used. Reversed-phase high-performance liquid chromatography (HPLC) was performed using a JASCO Gulliver system with UV detection at 260 nm and a packed Wakosil 5C18 (φ4.6 × 250 mm; Wako) or TSKgel ODS-80Ts (φ20 × 250 mm; Tosoh) column. Reversed-phase medium-pressure liquid chromatography (MPLC) was performed using an YFLC-Wprep system (Yamazen) with a glass column (φ33× 250 mm) filled with Wakosil 40C18 (Wako). Ion exchange column chromatography was performed using an ECONO system (Bio-Rad) with a glass column (φ25× 500 mm) filled with diethylaminoethyl (DEAE) A-25-Sephadex (Amershambiosciences).

3.2. Synthesis of QTP[54]

The synthetic route of QTP was shown in Scheme 2-1. Compound 4

was kindly provided by Prof. Obika and coworkers. Compound 4 (106 mg, 0.282 mmol) and *N,N,N′,N′*-tetramethyl-1,8-naphthalenediamine (Proton Sponge®; 119 mg; 0.555 mmol; 1.5 eq) was dried in a flask under vacuum overnight. Trimethylphosphate (2.6 mL) was added to the flask under argon, and the solution was cooled to 0°C. Distilled phosphorus oxychloride (42 µL; 0.444 mmol; 1.2 eq) was then added dropwise using a micro syringe, and the reaction mixture was stirred at 0°C. After 45 min, n-tributylamine (328 µL; 1.37 mmol; 3.7 eq) and n-tributylamine pyrophosphate (3.7 mL of a 0.5 M solution in DMF; 1.85 mmol; 5 eq) were added at 0°C, and the reaction mixture was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched with triethylammonium bicarbonate (1.0 M aqueous solution). The solvents were removed in *vacuo*, and the remaining crude mixture was dissolved in water. The product was purified using a Sephadex DEAE A-25 column with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate buffer (pH 8). The corresponding fractions were combined and evaporated under reduced pressure. To remove the excess of pyrophosphate, the residue was purified by reversed-phase MPLC with a linear gradient of 0% to 20% acetonitrile in 10 mM triethylammonium acetate buffer (pH 7). Further purification was performed on reversed-phase HPLC (φ20 × 250 mm) with a linear gradient of 0% to 4.9% acetonitrile in 50 mM triethylammonium acetate buffer to give the triphosphate QTP (6.58 µmol) in 2.3% yield starting from 4: ^{31}P NMR (500 MHz, D_2O) δ -10.56 (d), -11.37 (d), -22.94 (t); ESI-MS (negative ion mode) *m/z* , found = 615.1, calculated for $[(M-H)^-]$ = 615.03.

3.3. Enzymatic 3'-End Capping of Oligodeoxyribonucleotides with 2',4'-Bridged Nucleotides

Enzymatic capping of 2',4'-bridged nucleotide to 3'-end of oligodeoxyribonucleotide (ODN) was performed by use of TdT (Roche Diagnostics, Basel, Switzerland), the triphosphate QTP and 26-mer single-stranded oligodeoxyribonucleotide ODN1 with 5’-GGC GTT GAG TGA GTG AAT GAG TGA GT-3' (JBioS, Saitama, Japan). To detect extension products, the 5'-ends of ODN1 were labeled with 6-carboxyfluorescein (6-FAM). The volume of the total reaction mixture was 20 μL, containing 0.4 μM of ODN1, 200 μM of QTP, 10 U/μL of TdT, and the reaction buffer supplied with an enzyme (at 1× concentration). Reaction mixture was incubated for ~180 min at 37°C. The reaction product was resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and the gel image was recorded with excitation of the 5’-labeled fluorophore at 488 nm using Molecular Imager® FX (Bio-Rad, Hercules, CA, USA). The capped ODN, *i.e.*, ODN-Q was quantitatively produced as shown in Figure 2-2. I have previously reported enzymatic 3'-end capping of oligodeoxyribonucleotides with 2',4'-bridged nucleotides by using triphosphate analogs, *i.e.*, KTP, LTP, and MTP (Figure 2-1)[72]. The corresponding ODNs, *i.e.*, ODN-K, ODN-L, and ODN-M were obtained from ODN-1 according to the aforementioned method.

3.4. Resistances of Capped ODNs for Snake Venom Phosphor-diesterase I

Reactions were performed in a 5 μL reaction volume, containing 0.4

μM of ODN1, ODN-K, ODN-L, ODN-M, or ODN-Q, 50 μU of snake venom phosphodiesterase I (Worthington Biochemical Corporation, NJ, USA) with 3' to 5' exonuclease activity, and reaction buffer supplied with an enzyme (at 1× concentration). All reactions were incubated for ~120 min at 37°C. The reaction products were resolved by denaturing PAGE and gel images were recorded using Molecular Imager® FX. The band intensity was quantified using Quantity One® software. The decay curves of intact ODNs were fitted from band intensities at appropriate intervals of reaction time. Initial rates of degradation v_0 and relative rates that obtained from the decay curves were listed in Table 2-1.

3.5. Resistances of Capped TBAs for Snake Venom Phosphor-diesterase I

Reactions were performed in a 5 μL reaction volume, containing 2 μL of 1 M TBA-K, TBA-L, TBA-M, TBA-Q or TBA1 purchased from GeneDesign Inc. (Osaka, Japan), 2 μL of 20 U/μL snake venom phosphodiesterase I (Worthington Biochemical Corporation, Lakewood, NJ, USA) with 3' to 5' exonuclease activity and 1 μL of the reaction buffer at 5× concentration (250 mM Tris-HCl, 50 mM $MgCl_2$, 2.5% Tween 20 pH 8.0, purchased from TCI, Tokyo, Japan). All reactions were incubated for approximately 120 min at 37°C. The reaction products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and gel images were recorded using a Molecular Imager® FX (Bio-Rad, Hercules, CA, USA) as seen in Figure 2-3. The band intensity was quantified using Quantity One® software (Bio-Rad, Hercules, CA, USA). The decay curves of intact TBAs were fitted from band intensities

at appropriate intervals of reaction time by the least squares method using OriginPro ver.8 (Northampton, MA, USA) (see Figure 2-4). Table 2-2 lists initial rates of degradation v_0 and relative rates obtained from the decay curves.

3.6. Stabilities of Capped TBAs in 80% v/v Human Serum

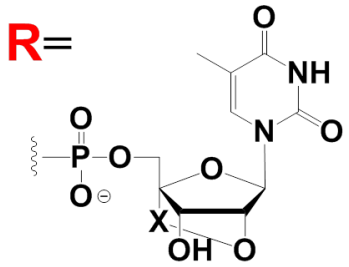
Reactions were performed in a 5 μL reaction volume containing 0.5 μL of 4 μM TBA-K, TBA-L, TBA-M, TBA-Q or TBA1, 4 μL of human serum from human male AB plasma (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 μL of the reaction buffer at 10× concentration (500 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0). All reactions were incubated for approximately 480 min at 37°C. The reaction products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and gel images were recorded using a Molecular Imager® FX as seen in Figure 2-6. The band intensity was quantified using Quantity One® software. The decay curves of intact TBAs were fitted from band intensities at appropriate intervals of reaction time by the least squares method using OriginPro ver.8 (see Figure 2-5). Table 2-3 lists initial rates of degradation v_0 and relative rates obtained from the decay curves.

3.7. Affinity Analyses of Capped TBAs to Thrombin by CE

The TBAs were dissolved in the incubation buffer (20 mM Tris-HCl buffer containing 1 mM $MgCl_2$, pH 7.4), reconstituted by denaturing at 94°C for 0.5 min and then cooled down to 25°C at a rate of 0.5°C/min using a TC-312 thermal cycler (Techne, Stone, Staffordshire, UK). The TBA solutions, each containing a TBA at a concentration of 1 nM, respectively, and thrombin from human plasma (Sigma-Aldrich) in the

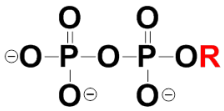
range of 0–10 nM, were dissolved in the incubation buffer (40 μL). Those sample solutions were incubated at 37°C for 30 min.

All separations were performed on a P/ACE MDQ capillary electrophoresis (CE) instrument (Beckman Coulter, Fullerton, CA, USA) equipped with a laser-induced fluorescence (LIF) detector ($\lambda_{ex}/\lambda_{em}$ = 488/520 nm). Fused-silica capillaries (eCAP™ capillary tubing, Beckman Coulter) of length 30.2 cm (20 cm to the detector) with an internal diameter of 75 μm and an external diameter of 375 μm were used for this experiment. The capillaries were pretreated with a separation buffer (100 mM sodium borate buffer, pH 8.35) prior to use everyday and also rinsed between runs. The premixed TBA/thrombin samples were introduced by hydrodynamic injection at 0.5 psi for 7.8 s. Separations were performed at 25°C for 4 min by applying a voltage of +12 kV. The data were recorded at 4 Hz and analyzed with 32 Karat software (version 8.0, Beckman Coutler) as seen in Figures 2-7 and 2-8.



5′-FAM-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-O**R**-3′

TBA-N : **R** = H
TBA-K : **X** = 2′-CH_2-4′
TBA-L : **X** = 2′-CH_2OCH_2-4′
TBA-M : **X** = 2′-$NHCH_2$-4′
TBA-Q : **X** = 2′-CH(Ph)OCH_2-4′



KTP : **X** = 2′-CH_2-4′
LTP : **X** = 2′-CH_2OCH_2-4′
MTP : **X** = 2′-$NHCH_2$-4′
QTP : **X** = 2′-CH(Ph)OCH_2-4′

Figure 2-1. Chemical structures of 2',4'-bridged nucleotides used in this experiment

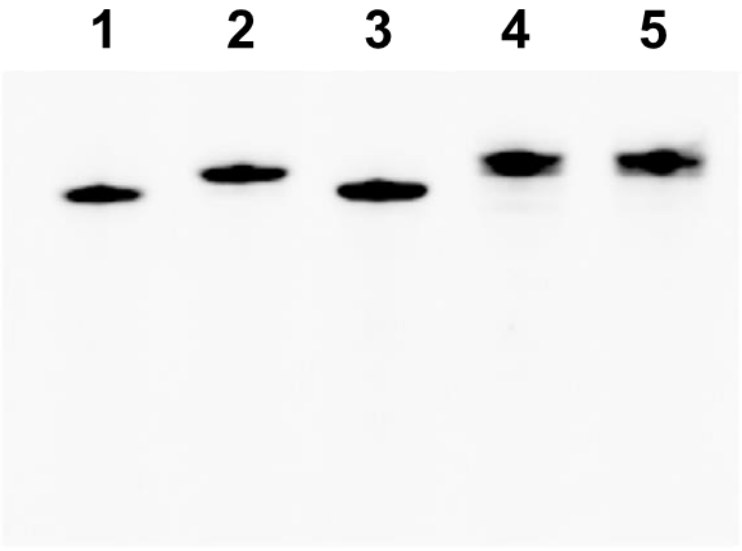


Figure 2-2. Representative gel images of reactions using the 2',4'-bridged nucleoside triphosphates with TdT. The reaction mixtures for the positive control with LTP and TdT (0.2 U/μL) incubated for 1 h (lane 2), those containing QTP and TdT (0.2 U/μL) incubated for 1 h (lane 3), QTP and TdT (10 U/μL) incubated for 2 h (lane 4), and QTP and TdT (10 U/μL) incubated for 3 h (lane 5). ODN1 migrated only in lane 1.

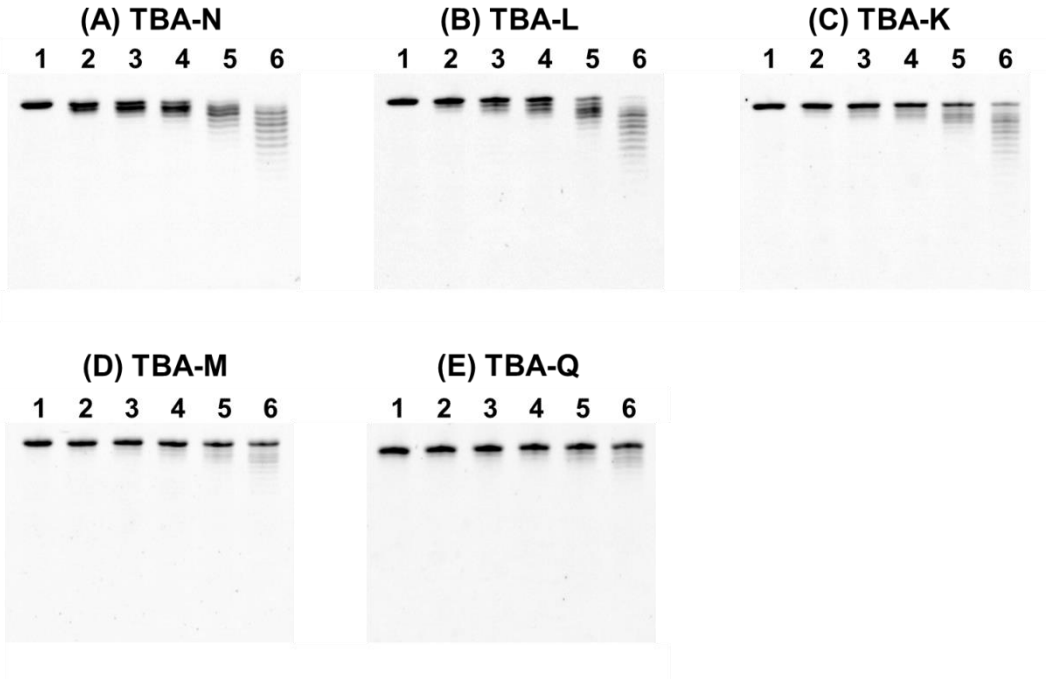


Figure 2-3. Representative gel images of degradation of TBAs with phosphodiesterase I. Reactions using TBA1 (image A), TBA-K (image B), TBA-L (image C), TBA-M (image D), and TBA-Q (image E). The reaction mixtures at 0, 10, 20, 30, 60, and 120 min are in lanes 1–6, respectively.

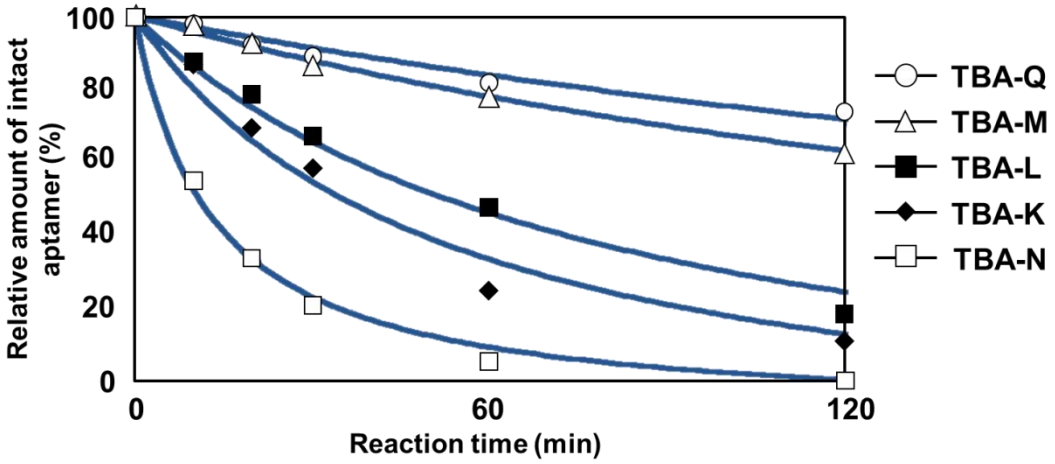


Figure 2-4. The time course of the degradation of thrombin binding aptamers (TBAs) by phosphodiesterase I; reaction using TBA1 (open squares), TBA-K (closed diamonds), TBA-L (closed squares), TBA-M (open triangles), and TBA-Q (open circles). The x-axis indicates the reaction time (min), and the y-axis represents the relative amount of intact TBAs (%). Total quantities of the products were set at 100% in each reaction mixture.

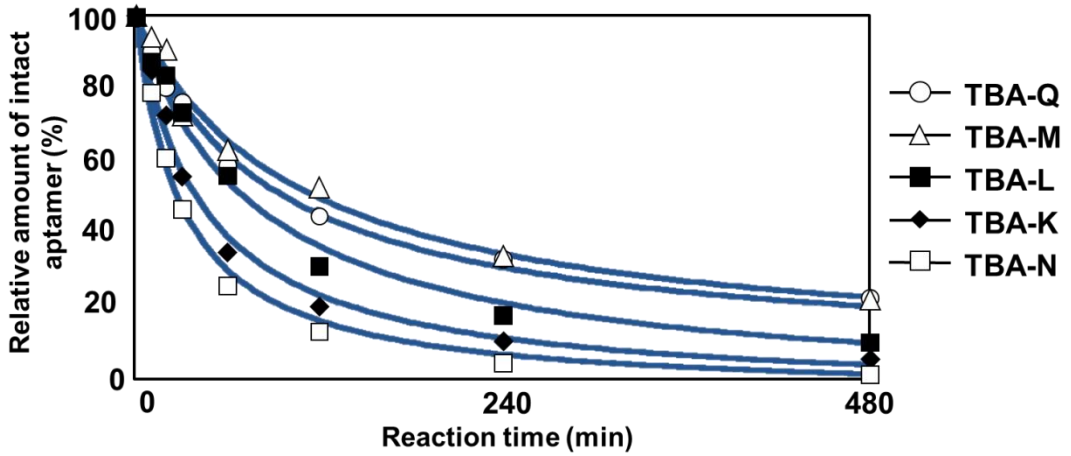


Figure 2-5. The time course of degradation of thrombin binding aptamers (TBAs) in 80% v/v human serum; reaction using TBA1 (open squares), TBA-K (closed diamonds), TBA-L (closed squares), TBA-M (open triangles), and TBA-Q (open circles). The x-axis indicates the reaction time (min), and the y-axis represents the relative amount of intact TBAs (%). Total quantities of the products were set at 100% in each reaction mixture.

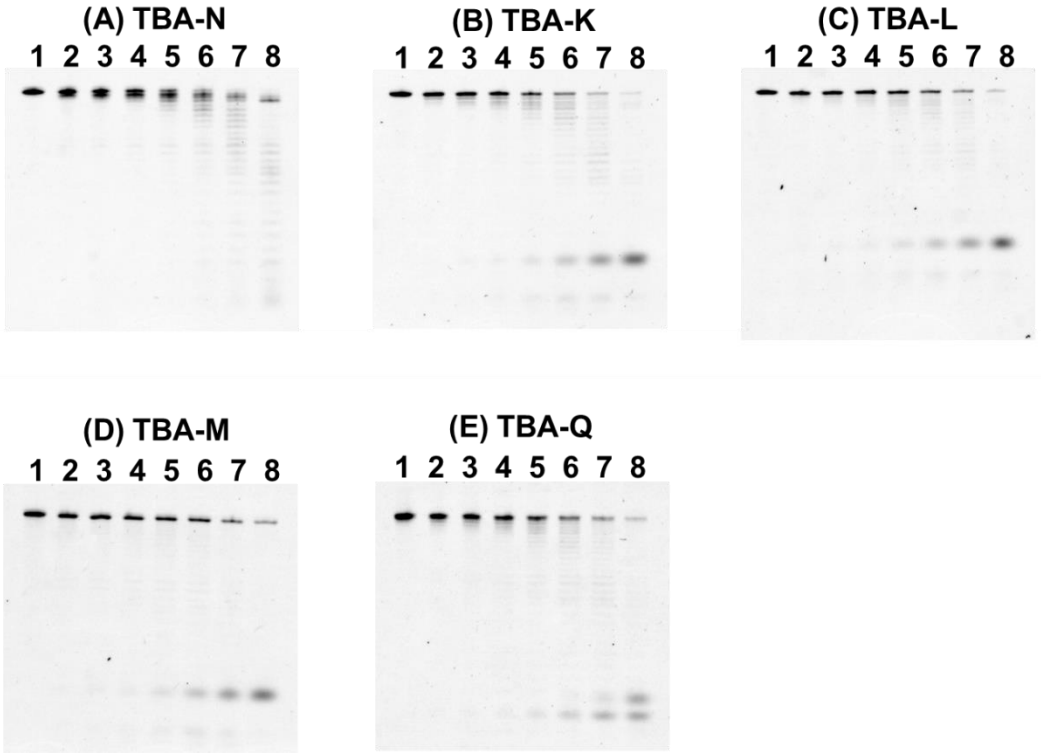


Figure 2-6. Representative gel images of degradation of TBAs in 80 % v/v human serum; reactions using TBA1 (image A), TBA-K (image B), TBA-L (image C), TBA-M (image D), and TBA-Q (image E). The reaction mixtures at 0, 10, 20, 30, 60, 120, 240 and 480 min in lanes 1, 2, 3, 4, 5, 6, 7 and 8, respectively.

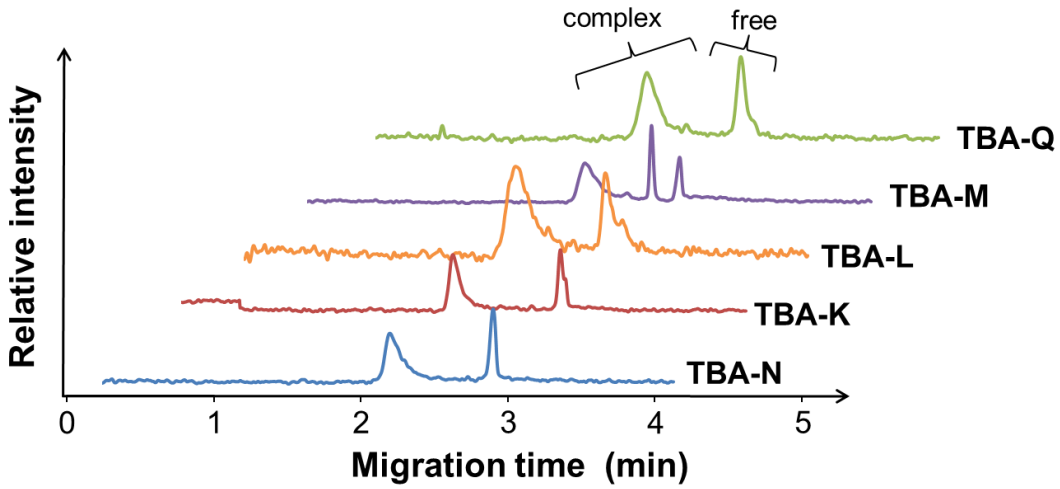


Figure 2-7. Representative capillary electropherograms for individual thrombin-TBA complexes. The x-axis indicates the migration time (min), and the y-axis represents the relative intensity of signals from emission of 5'-FAM detected at 520 nm (excited at 488 nm). The peak with the asterisk (*) corresponds to TBA-M dissociated from thrombin during CE, which might conform a particular structure. The concentrations of TBAs and thrombin were 1 nM and 4 nM. The sample buffer and running buffer were 20 mM Tris–HCl (1 mM $MgCl_2$) at pH 7.4 and 100 mM sodium borate at pH 8.35, respectively. Experimental conditions: separation voltage, +12 kV; injection 0.5 psi × 7.8 s.

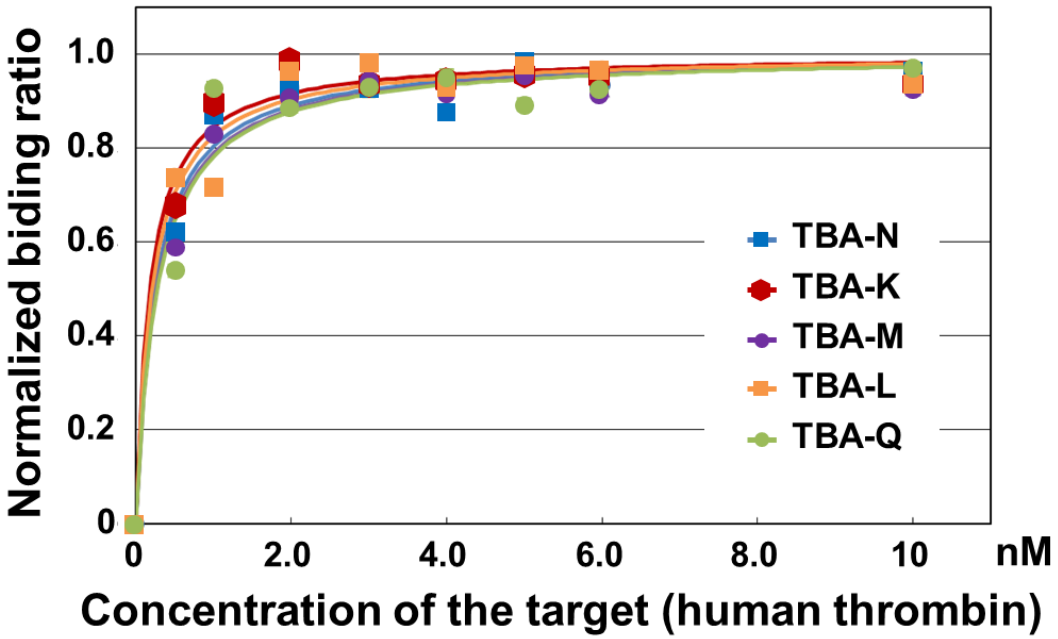


Figure 2-8. Binding saturation curves of TBA-1, TBA-K, TBA-L, TBA-M and TBA-Q with the target (human thrombin), which were obtained from electropherograms by non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).

Table 2-1. Degradation of oligonucleotides (ODNs) with snake venom phosphodiesterase I.

ODNs [a]	v_0 (%/min)[b]	Relative rate
ODN1	130	736
ODN-K	7.1	51
ODN-L	2.7	19
ODN-M	0.32	2.3
ODN-Q	0.14	1

[a] Sequences of ODNs used were 5'-GGC GTT GAG TGA GTG AAT GAG TGA GTX-3'. Here, X = none, 2',4'-bridged nucleotide K, L, M, and Q in ODN1, ODN-K, ODN-L, ODN-M, and ODN-Q, respectively. The 5'-ends of ODNs were labelled with 6-carboxyfluorescein (6-FAM) for detection of intact ODNs. [b]Initial rates of degradation v_0 were calculated by curve fittings, respectively.

Table 2-2. Degradation of thrombin binding aptamers with snake venom phosphodiesterase I

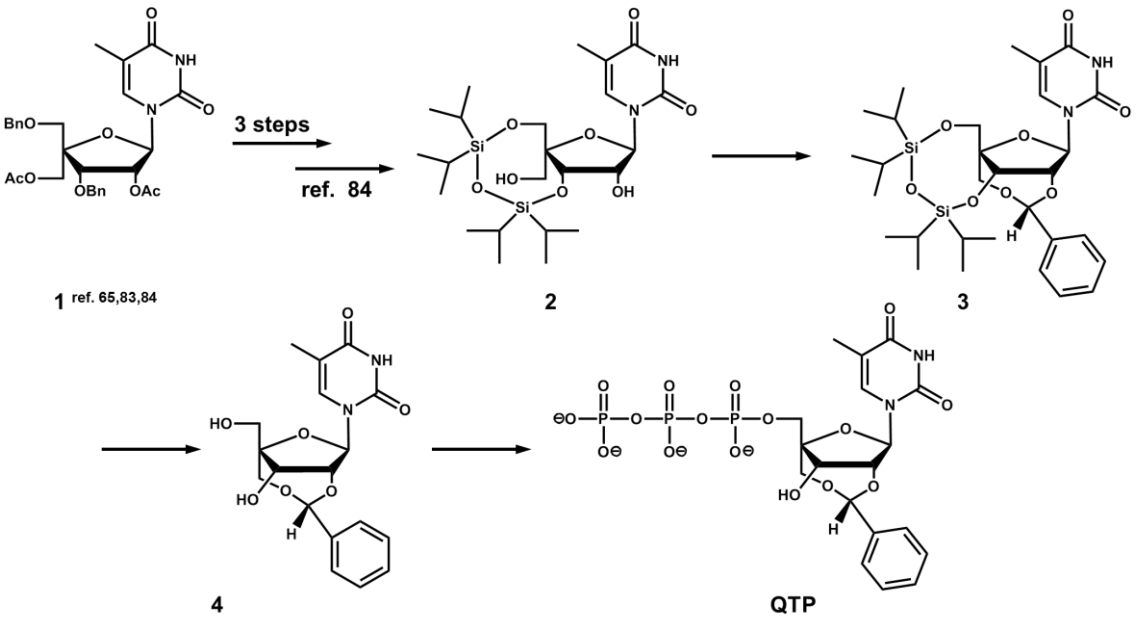
Thrombin binding aptamers	v_0 (%/min)[a]	Relative rate
TBA1	8.5	27
TBA-K	2.4	7.5
TBA-L	1.6	5.0
TBA-M	0.46	1.5
TBA-Q	0.32	1

[a] Initial rates of degradation v_0 were calculated by the fitting of their decay curves, respectively.

Table 2-3. Degradation of thrombin binding aptamers in 80% v/v human serum

Thrombin binding aptamers	v_0 (%/min)[a]	Relative rate
TBA1	3.5	4.0
TBA-K	2.4	2.7
TBA-L	1.3	1.5
TBA-M	0.88	1
TBA-Q	1.1	1.2

[a] Initial rates of degradation v_0 were calculated by the fitting of their decay curves, respectively.



Scheme 2-1. Synthetic routes of the 2',4'-bridged thymidine 5'-triphosphate analog, *i.e.* QTP. a) benzaldehyde, $ZnCl_2$, rt, 14 h (78%); b) TBAF, THF, 0 ˚C, 1 h (96%); c) $POCl_3$, *N*,*N*,*N'*,*N'*-tetramethyl-1, 8-naphthalendiamine, trimethyl phosphate, 0 ˚C, 45 min, followed by *n*-tributylamine pyrophosphate, DMF, room temperature (2.3%). TBAF = tetra-*n*-butylammonium fluoride.

Chapter 3

CE-SELEX Selections Using a DNA-based Library That Contains B/L Nucleotides

1. Introduction

Since the invention of 2',4'-BNA/LNA in the late 90s,[63–65] 2',4'-BNA/LNA analogues have received continued research attention due to their potential therapeutic and diagnostic applications. Biostability is a critical property of medicinal and biological agents, and 2',4'-BNA/LNA analogs[69,70,85,86] are well known to have superior nuclease resistance than other types of sugar-modified nucleotides. Therefore, to create nucleic acid aptamers that are extremely stable under physiological conditions, attempts to use 2',4'-BNA/ LNA in SELEX experiments[4–6,8] have been made. However, both inefficiency of polymerase reactions involving 5'-thiphosphates of B/L nucleotides and inadequate accuracy of incorporation into polynucleotide strands have reduced the progress of this approach, and may introduce selection biases that interfere with the enrichment of active species.

In our previous study, we demonstrated that *KOD Dash* DNA polymerase and *KOD* mutants could elongate modified DNA strands with base- and sugar-modified nucleotides using natural DNA as a template and vice versa.[36,48,54] These results, which were also confirmed by other researchers,[55,88,89] indicated that *KOD*-related polymerases are suitable for SELEX experiments that produce xeno-nucleic acid (XNA) aptamers with unconventional sugars. Very

recently, mutants of *Tgo* DNA polymerase, which have more than 90% amino acid sequence similarity to the non-exonuclease domains of *KOD* DNA polymerase,[90,91] were created using a random screening method known as compartmentalized self-tagging (CST),[92] and were shown to synthesize the six types of XNA 1,5-anhydrohexitol nucleic acid (HNA),[33] cyclohexenyl nucleic acid (CeNA),[47] α-L-threofuranosyl nucleic acid (TNA),[35] arabinonucleic acid (ANA),[34] 2'-fluoro-arabinonucleic acid (FANA),[93] and 2',4'-BNA/LNA. Subsequently, aptamers comprising only non-natural nucleotides were directly recovered from a HNA library. Thus, engineering of DNA polymerases, particularly those belonging to evolutionary family B, such as *KOD* and *Tgo*, is expected to expand the applications of SELEX methods to include artificial nucleic acids with foreign functionalities.

2. Results and Discussion

Protocols of 2',4'-BNA/LNA aptamer selection by CE-SELEX[80,94–96] are illustrated in Figure 3-1, and selection procedures and experimental conditions are described in Experimental Procedures. The modified DNA library contained B/L thymidines (**t**'s) instead of the natural thymidines in the non-primer region and 8 B/L nucleotides placed at intervals of two residues in the primer region. The initial 2',4'-BNA/LNA library was enzymatically prepared from 70-mer oligodeoxyribonucleotides (ODNs) comprising the variable region (30 bases) flanked by the forward and reverse primer regions (20 bases each) (steps 1–3). The library was mixed with human thrombin,

injected into a separation capillary, and fractionated into thrombin bound active and free inactive 2',4'- BNA/LNAs using non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[81,97] (step 4). Active species were then amplified by PCR using four natural dNTPs to yield a double stranded DNA (dsDNA) pool (step 5). Using the dsDNA pool as template, dATP, dCTP, dGTP, B/L thymidine-5'-triphosphate (T^bTP) instead of TTP, and the 5'-(6-carboxyfluorescein)-labeled primer (5'-/6-FAM/TCG CCT TGC^{me} CGG ATC^{me} GCA GA-3') that contains B/L nucleotides bearing adenine, guanine, thymine, and 5-methylcytosine (bold capitals), primer extension was catalyzed by a 19:1 enzyme mix of *KOD Dash* and *KOD* mutant DNA polymerases (*KOD*2)[98] (step 6). To obtain a library of single stranded 2',4'-BNA/LNA (ssBNA/LNA) from the resulting precursory 2',4'-BNA/LNA library, an AT_8 tail was attached to the 3'-end of the forward primer-elongated strand (step 7), and the extended 2',4'-BNA/LNA strands were separated from natural dsDNA strands using denaturing polyacrylamide gel electrophoresis (PAGE) (step 8). These were then used as the library for the next round. As shown in Figure 3-2 and Figure 3-3, 11 rounds of selection saturated the enrichment of active species. Usually, an obvious peak of the aptamer–target complex is observed in electrograms of CE-SELEX. However, a substantially broadened peak was observed prior to free 2',4'-BNA/LNA migration. Forty 2',4'-BNA/LNA aptamers were isolated from the enriched pool using a cloning method, and their sequences were identified and classified into six groups as shown in Table 3-1. Consensus sequences containing **t**'s were seen among aptamers of groups I, IV, V, and VI. In

contrast, a sequence motif comprising the three natural nucleotides A, G, and C was observed in group II. Whereas four aptamers of group III had identical sequences, no similar sequences were found among the other 36 aptamers.

Binding affinities of these 2',4'-BNA/LNA aptamers were assessed using NECEEM, and apparent dissociation constants (K_d) of thrombin–aptamer complexes were determined according to previous studies[99,100] (Figure 3-4). Incidentally, the peak strength of the complex was confirmed to increase depending on the concentration of the target (Figure 3-5). Aptamers from groups V and VI, namely A#17, A#18, A#23, and A#40, had K_d values of 23, 27, 20, and 18 nM, respectively, while those from the other groups, namely A#1, A#14, A#20, and A#26, were inactive. The K_d value of 5'-(6-FAM)-labeled 29-mer ODNs of the known thrombin-binding aptamer[76] (29TBA) 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3' was 0.29 nM, as determined by CE affinity analyses. As mentioned above, inactive species were enriched, presumably due to weak binding that cannot be detected under the conditions employed, or due to accidental PCR-amplification in a selection round. Normally, such undesirable sequences are eliminated through repeated selection rounds. However, in this case, these may have remained in the site due to the low separation resolution of active and inactive species. Among active species, the t-containing motif C**t**C **tt**(A/G) ACA GGA C was commonly found in groups V and VI. In addition, aptamers in group V had guanine (G)-rich sequences at the 3'-side of the variable region, unlike those in group VI. These observations unexpectedly indicate that G-rich

sequences, which are often identified as the active sites of various DNA aptamers,[101–103] were not necessary for binding activity. Binding specificity and the effects of B/L nucleotides on binding activity were assessed using the aptamer A#40, which had the strongest binding affinity. As shown in Figure 3-6, A#40 distinguished human thrombin from other proteins such as lysozyme, soybean lectin, and streptavidin. However, it bound rat thrombin with higher affinity (K_d = 4.2 nM) than human thrombin, indicating that its binding specificity was not sufficient to discriminate between proteins from different animal species. To investigate the effects of B/L nucleotides, two variants of A#40, A#40a, and A#40b, with identical sequences were enzymatically prepared. The former comprises a 2',4'-BNA/LNA primer region and a natural ODN non-primer region, and the latter is a fully natural ODN. Neither of these variants exhibited binding activities (Figure 3-4), indicating that B/L nucleotides were indispensable for aptamer activity. Hence, this library of full-length B/L nucleotides may provide an inherent peak for strong binders in the fitness landscape that is different from that of natural ODNs.

I demonstrated that 2',4'-BNA/LNA aptamers can be obtained by the CE-SELEX method using an enzyme mix of *KOD*-related polymerases. In the present study, the AT_8 tail was attached for separation of ssBNA/LNA library, but may confer additional functions such as biostability,[104] cell permeability,[105] and photo-reactivity[106] via the 3'-tail, using modified nucleoside triphosphates with corresponding foreign functionalities. In the present experiments, NECEEM conditions and DNA polymerase combinations were not well optimized. Hence,

further improvements of these selection systems will produce various BNA (LNA) aptamers with higher affinity and specificity.

3. Experimental Procedures

3.1. Materials

Sample solutions and buffers were prepared using ultrapure water obtained using the Ultrapure Water system CPW-100 (Advantec Co. Ltd., Tokyo, Japan). The initial DNA library, DNA primers and templates, known 29-mer oligodeoxyribonucleotides (ODNs) of thrombin-binding aptamer (TBA)[76] labeled with 6-carboxyfluorescein (6-FAM) at the 5'-end (*i.e.*, 29TBA) were purchased from Japan Bio Services Co. Ltd. (Saitama, Japan) or Hokkaido System Science Co. Ltd. (Hokkaido, JAPAN). The bridged/locked nucleic acid (BNA/LNA) primer (P1fb; 5'-/6-FAM/TC**G** CC**T** TG**C**^{me} CG**G** AT**C**^{me} GC**A** GA-3'), which contained 2'-*O*,4'-*C*-methylene-bridged/linked bicyclic ribonucleotides (B/L nucleotides) bearing adenine (A), guanine (G), 5-methylcytosine (C^{me}), and thymine (T) as shown in bold capitals, was purchased from GeneDesign Inc. (Osaka, Japan). The B/L nucleotide bearing C^{me} not cytosine (C) was used because the C analog was not in the supplier's regular assortment. Primers P1fb and P1f (5'-/6-FAM/TCG CCT TGC CGG ATC GCA GA-3') were labeled with 6-FAM at the 5'-end to facilitate fluorescence detection. The initial DNA library T1 comprised random regions (30 bases) flanked by the primer sequence (20 bases each) 5'-/6-FAM/TCG CCT TGC CGG ATC GCA GA-(30 random bases)-TGG TCC GTG AGC CTG ACA CC-3'. Primer P1p

(5'-/phosphate/TCG CCT TGC CGG ATC GCA GA -3') was labeled with monophosphate at the 5'-end to prepare single stranded DNA using lambda exonuclease after PCR amplification of selected 2',4'-BNA/LNAs. Thrombin from human plasma, thrombin from rat plasma, tris(hydroxymethyl)aminomethane (Trizma®base), chicken egg lysozyme were purchased from Sigma-Aldrich, Inc. (MO, USA). Lambda exonuclease, soybean lectin, streptavidin type II, ethanol, aqueous HCl, boric acid, sodium tetraborate, NaCl and $MgCl_2 \cdot 6H_2O$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fused-silica capillary (eCAP™ capillary tubing, 75 μm I.D., 375 μm O.D.) was purchased from Beckman Coulter, Inc. (CA, USA). The 4 dNTPs (dATP, dGTP, dCTP, and TTP) were purchased from Roche Diagnostics K.K. (Tokyo, Japan). *KOD Dash* DNA polymerase was purchased from Toyobo Co. Ltd. (Osaka, Japan) and *Taq* DNA polymerase from Invitrogen Co. Ltd. (CA, USA). The *KOD* mutant (*KOD* 2)[98] was generous gifts from Toyobo Co. Ltd. (Osaka, Japan). The plasmid DNA purification kit was purchased from Cosmo Genetech Co. Ltd. (Seoul, Korea). The binding buffer contained 20 mM Tris-HCl buffer (pH 7.40), $MgCl_2$ (1 mM) and NaCl (10 mM). The running buffer for separation contained 100 mM sodium borate (pH 8.35). Binding and running buffers were filtered through a 0.45-μm membrane filter (EMD Milipore Co., MA, USA).

3.2. CE-SELEX Using BNA/LNA Library

3.2.1. *Preparation of BNA/LNA Library*

Complementary DNA library was enzymatically prepared from T1

prior to construction of BNA/LNA library as follows. The reaction mixture (800 μL) containing T1 (80 nM), the reverse primer P2 (5'-GGT GTC AGG CTC ACG GAC CA-3'; 0.4 μM), four natural dNTPs (0.2 mM each), *KOD Dash* DNA polymerase (0.005 U/μL), and 1× polymerase reaction buffer was prepared, and then subjected to one-primer PCR (eight thermal cycles at 94°C for 30 s, 54°C for 30 s, and 74°C for 60 s) in a TC-312 thermal cycler (Techne, Staffordshire, UK). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs to yield roughly purified 70-mer ODN products. To isolate the P2-elongated products (*i.e.*, complementary DNA library), the AT_8 tail was added to the 3'-end of T1; 800 μL of reaction mixture containing the ODN products (0.4 μM), T2 (0.4 μM 5'-AAA AAA ATG GTG TCA GGC TCA CGG ACC A/phosphate/-3'), dATP, TTP (0.2 mM each), *KOD Dash* DNA polymerase (0.01 U/μL), and the reaction buffer was prepared, and then subjected to eight thermal cycles at the abovementioned conditions. Complementary DNA library was separated from the T1-elongated product having the AT_8 tail by denaturing polyacrylamide gel electrophoresis (PAGE).

As shown in Figure 3-1, one-primer PCR was subsequently performed using the complementary DNA library as a template, the forward primer P1fb, dATP, dGTP, dCTP, T^bTP in place of TTP (0.2 mM each), enzymes (0.25 U/μL 19:1 mix of *KOD Dash* and *KOD* mutant DNA polymerases), and the reaction buffer. The reaction mixture (3,200 μL) was subjected to 22 thermal cycles at the abovementioned conditions (Step 1). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs to yield roughly purified

P1fb-elongated products (*i.e.*, precursory BNA/LNA library). To remove the complementary DNA library, the AT_8 tail was added to the 3'-end of the precursory BNA/LNA library; 800 μL of reaction mixture containing the precursory BNA/LNA library (0.4 μM), T2 (0.4 μM), dATP, TTP (0.2 mM each), *KOD Dash* DNA polymerase (0.01 U/μL), and the reaction buffer was prepared, and then subjected to eight thermal cycles at the abovementioned conditions (Step 2). Single-stranded BNA/LNAs having the AT_8 tail were purified by denaturing PAGE, and used as the initial BNA/LNA library (Step 3).

After affinity separation using CE (Step 4), a fraction containing the BNA/LNA–thrombin complex was subjected to symmetric PCR amplification (16–22 PCR cycles at the abovementioned conditions) using the forward primer P1p (0.4 μM) and the reverse primer P2 (0.4 μM) (Step 5). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs to yield roughly purified natural double stranded DNA (dsDNA). To degrade the P1p-elongating product, dsDNA (ca. 320 pmol) was incubated at 37°C for 30 min with 1× lambda exonuclease reaction buffer (760 μL) containing lambda exonuclease (0.025 U/μL). The reaction mixture was subjected to ethanol precipitation to remove degradation products to yield roughly purified P2-elongated products. Then, 800 μL of reaction mixture containing the P2-elongated products as a template (80 nM), the primer P1fb (0.4 μM), dATP, dGTP, dCTP, T^bTP (0.2 mM each), the enzyme mix (0.25 U/μL), and the reaction buffer was prepared, and then subjected to 22 thermal cycles at the abovementioned conditions (Step 6). The reaction mixture was then subjected to ethanol

precipitation to remove dNTPs to yield roughly purified P1fb-elongated products (*i.e.*, precursory BNA/LNA library). Similarly, the single-stranded BNA/LNAs having the AT_8 tail was prepared from the precursory BNA/LNA library (Step 7), purified by denaturing PAGE (Step 8), and used as a library for the next round.

3.2.2. *Affinity Separation Using Capillary Electrophoresis (CE)*

All CE separations were performed using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with 32 Karat software. A laser-induced fluorescence (LIF) detector was used to monitor the separation. Excitation was generated using the 488 nm line of an Ar^+ laser (Beckman Coulter) and emission was collected at 520 nm. At the start of daily use, the separation capillary (bare fused silica, 75 μm I.D., 375 μm O.D., total length = 80 cm, length to detector = 70 cm) was rinsed with 0.1 M aqueous NaOH for 10 min and with the running buffer for another 10 min. Before each separation, the capillary was flushed with 0.1 M aqueous NaOH for 10 min followed by a 10 min rinse with the running buffer. Human thrombin (200 nM) was incubated with the BNA/LNA library (500 nM) in the binding buffer (20 μL total volume) for 30 min at 37°C before loading onto the CE instrument for separation. All samples were housed in the P/ACE MDQ sample chamber which was held at 15°C. The incubation mixture (ca. 60 nL) was injected into the separation capillary using pressure injection (16 s, 0.5 psi). A voltage of +18 kV was applied across the capillary for 11.6–12.1 min to migrate ODNs that bind to human thrombin into a collection vial containing 20 μL of the running buffer. Unbound ODNs remaining in the capillary were

later washed into a waste vial using pressure. The temperature of the capillary was automatically maintained at 25°C for all separations. CE was performed 3 times for every round of separation to obtain sufficient active species. 11 rounds of selection saturated the enrichment of active species (Figures 3-2 and 3-3).

After 11 cycles of separation and amplification, enriched BNA/LNAs were amplified by PCR using *KOD Dash* DNA polymerase with non-labeled forward/reverse primers P1/P2, and the four natural dNTPs. The resulting dsDNA was further amplified by PCR using *Taq* DNA polymerase. Subsequently, amplified dsDNA was cloned into a TA vector by the TA cloning method[107] according to the manufacturer's protocol. Plasmid DNAs were selected from different clones and isolated using the plasmid DNA purification kit. Sequences inserted into the prepared plasmids were analyzed using DNA sequencing services at Operon Biotechnologies Co. Ltd. (Tokyo, Japan) and Takara Bio Inc. (Shiga, Japan). Forty aptamers were isolated from BNA/LNA library.

3.3. Preparation of Isolated TBAs, Their Chemical Variants

TBAs used for affinity analysis (*i.e.*, A#1, A#14, A#17, A#18, A#20, A#23, A#26 and A#40) were enzymatically prepared using the respective isolated plasmid DNAs as templates in compliance with the protocol for preparation of the BNA/LNA library. Chemical variants of A#40 (*i.e.*, A#40a, and A#40b) were prepared in compliance with the protocol for preparation of A#40 using TTP instead of $T^{b}TP$, and using TTP and P1f instead of $T^{b}TP$ and P1fb, respectively.

3.4. Affinity and Specificity Analyses by CE

TBAs were dissolved in binding buffer, and refolded by denaturing at 94°C for 0.5 min and cooling to 25°C at a rate of 0.5°C/min using the thermal cycler. Sample solutions (40 μL) containing TBA and human thrombin (1 nM each in the binding buffer) were prepared for K_d value determination. Similarly, those containing TBA (10 nM) and human thrombin (40 nM) were prepared for activity assays (Capillary electrograms in Figure 3-4). These solutions were incubated at 37°C for 30 min prior to CE analysis. For specificity analyses, sample solutions (40 μL) containing TBA and a protein, *e.g.* lysozyme, soybean lectin, streptavidin or rat thrombin (1 nM each in the binding buffer) were prepared (Capillary electrograms in Figure 3-6).

Analysis was performed using the P/ACE MDQ CE instrument equipped with a LIF detector ($\lambda_{ex}/\lambda_{em}$ = 488/520 nm). Fused-silica capillaries having a length of 30.2 cm (20 cm to the detector), an internal diameter of 75 μm and an external diameter of 375 μm were used for the experiment. These were pretreated with running buffer before daily use and rinsed between runs. The premixed TBA–protein samples were introduced by hydrodynamic injection at 0.5 psi for 7.8 s. Separations were performed at 25°C for approximately 5 min by applying a voltage of +12 kV. Electrograms were recorded at 4 Hz and analyzed with 32 Karat software ver. 8.0.

The apparent dissociation constants (K_d) of the thrombin–TBA complex were determined according to the literature.[81] These K_d values were calculated using equation 1, where A_1, A_2 and A_3 are peak areas of DNA in the protein–DNA complex, DNA dissociated from the complex

during electrophoretic separation, and free DNA in a non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) electropherogram, respectively. Initial concentrations of protein and DNA are represented by $[P]_0$ and $[D]_0$, respectively.

$$K_d = \frac{[P]_0\left\{1 + \frac{A_3}{A_1 + A_2}\right\} - [D]_0}{1 + (A_1 + A_2)/A_3} \qquad eq.1$$

Precision of determination of K_d values (% relative standard deviation) was <5% for all analytes measured.

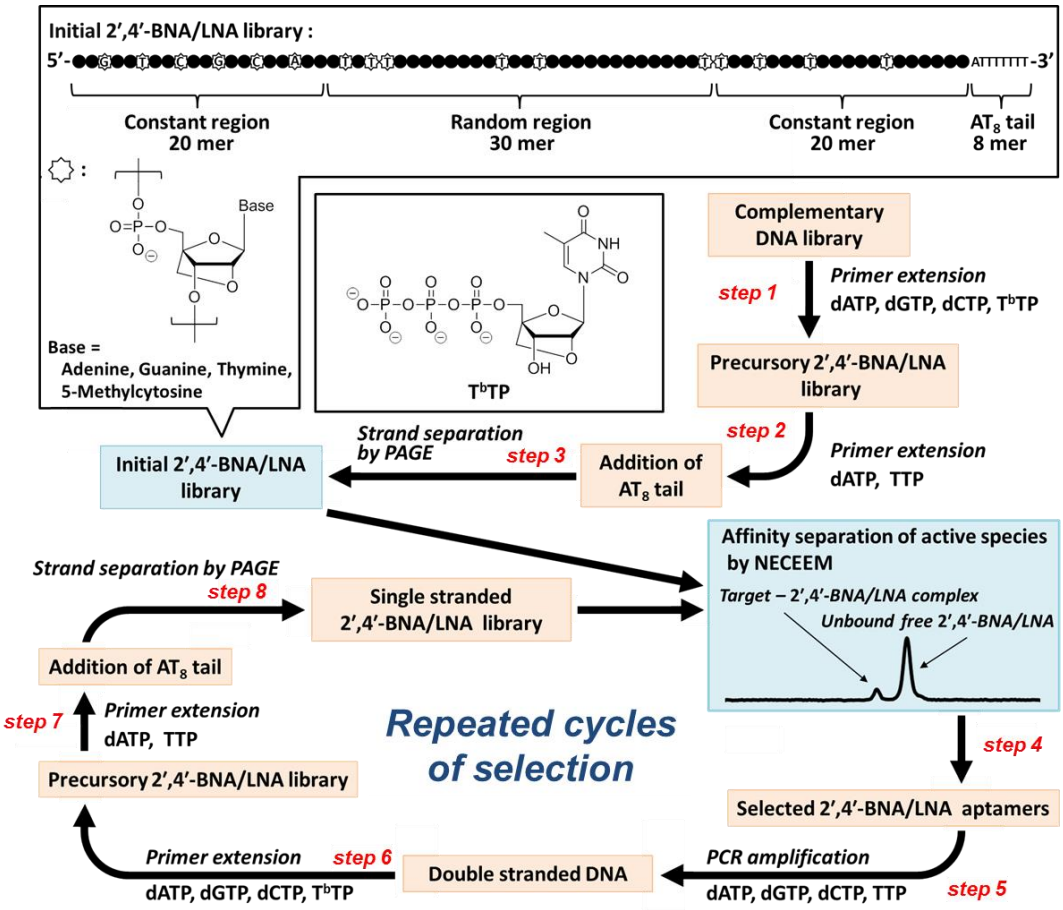


Figure 3-1. The schematic illustration of thrombin-binding aptamer (TBA) selection from the 2',4'-BNA/LNA library by CE-SELEX.

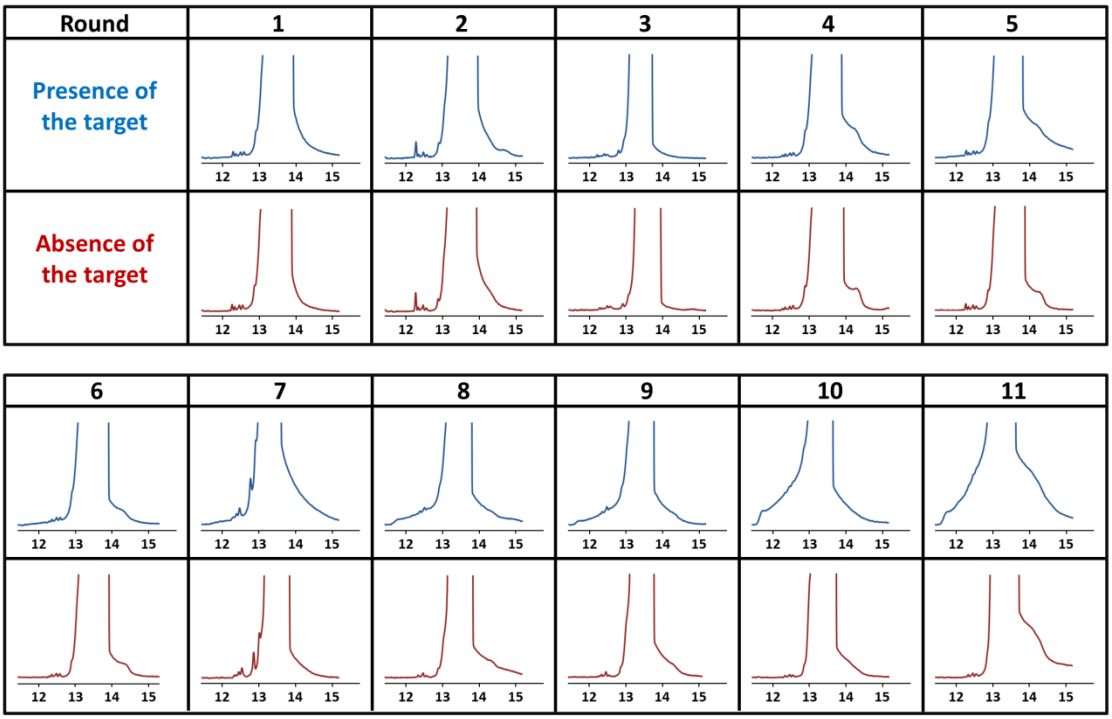


Figure 3-2. Capillary electrograms for the 2',4'-BNA/LNA library with or without human thrombin in each round of selection. All electrograms recorded fluorescent intensity of 5'-labeled 6-FAM versus migration time (min).

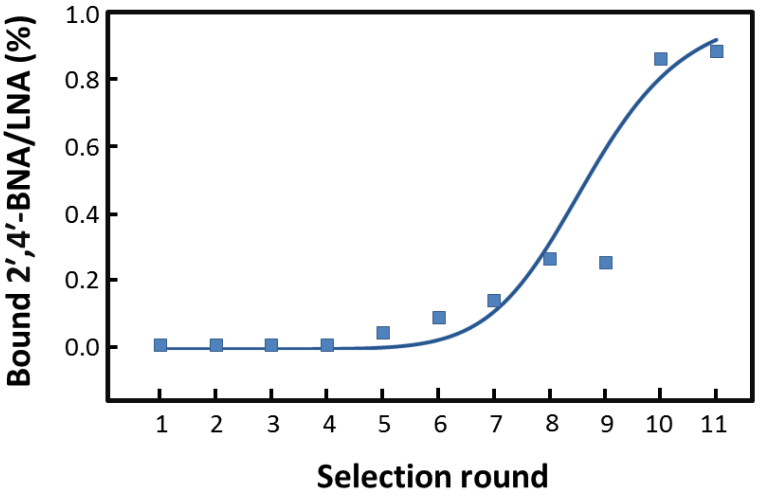


Figure 3-3. Process of active species enrichment in selection rounds.

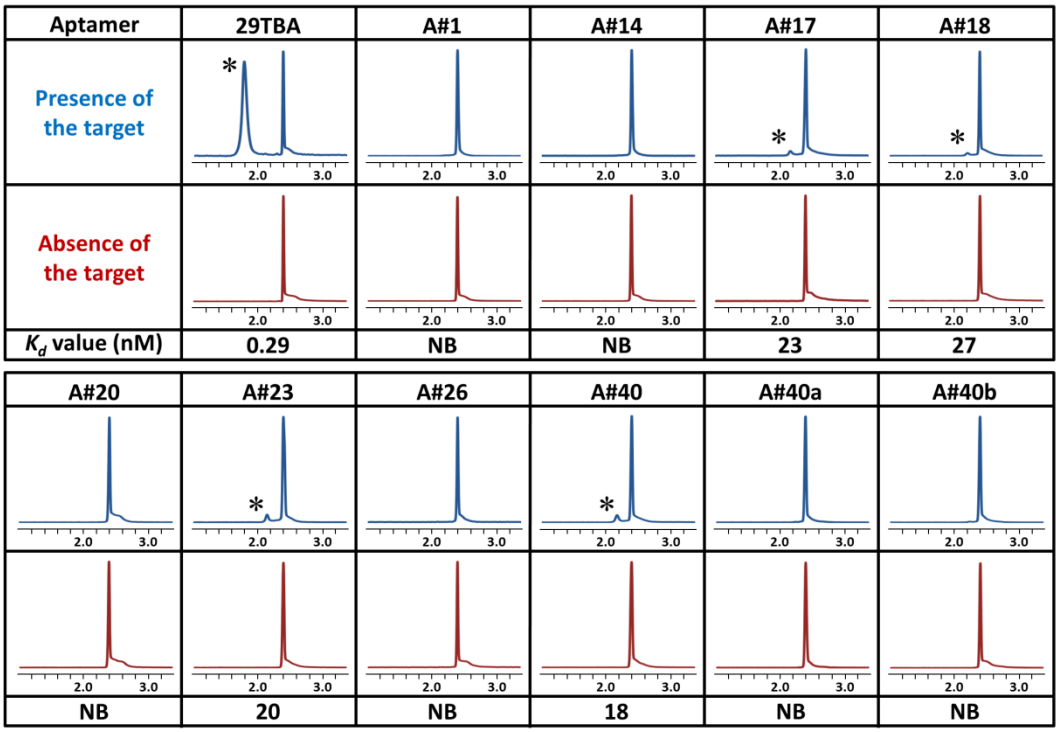


Figure 3-4. Capillary electrograms for 29TBA and 2',4'-BNA/LNA aptamers with or without human thrombin. All electrograms recorded fluorescent intensity of 5'-labeled 6-FAM versus migration time (min). The asterisk indicates the peak of the thrombin–aptamer complex. K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <5% for all analytes measured. NB, no binding.

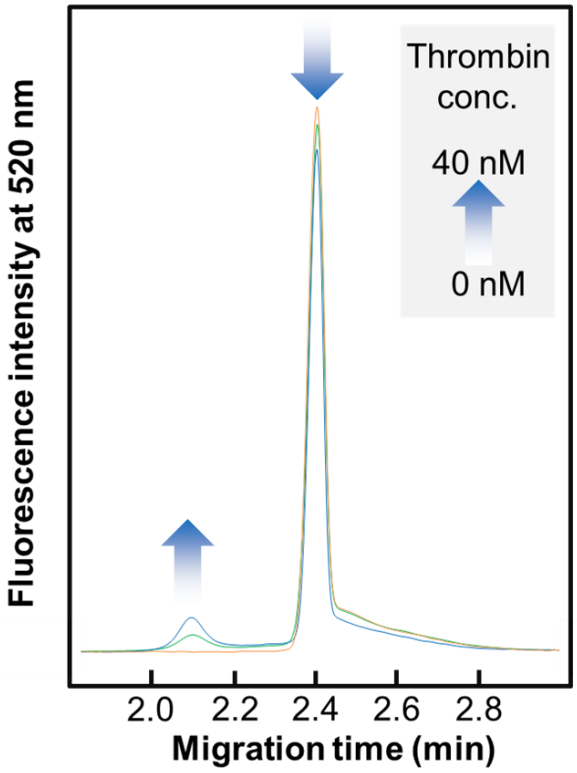


Figure 3-5. Capillary electrograms of the assays using A#40 (10 nM) in addition to 0- to 4-fold equivalents of human thrombin (0, 10, and 40 nM). The asterisk indicates the peak of the thrombin–aptamer complex. As the target concentration was raised, the peak strengths of the complex and free aptamers increased and decreased, respectively.

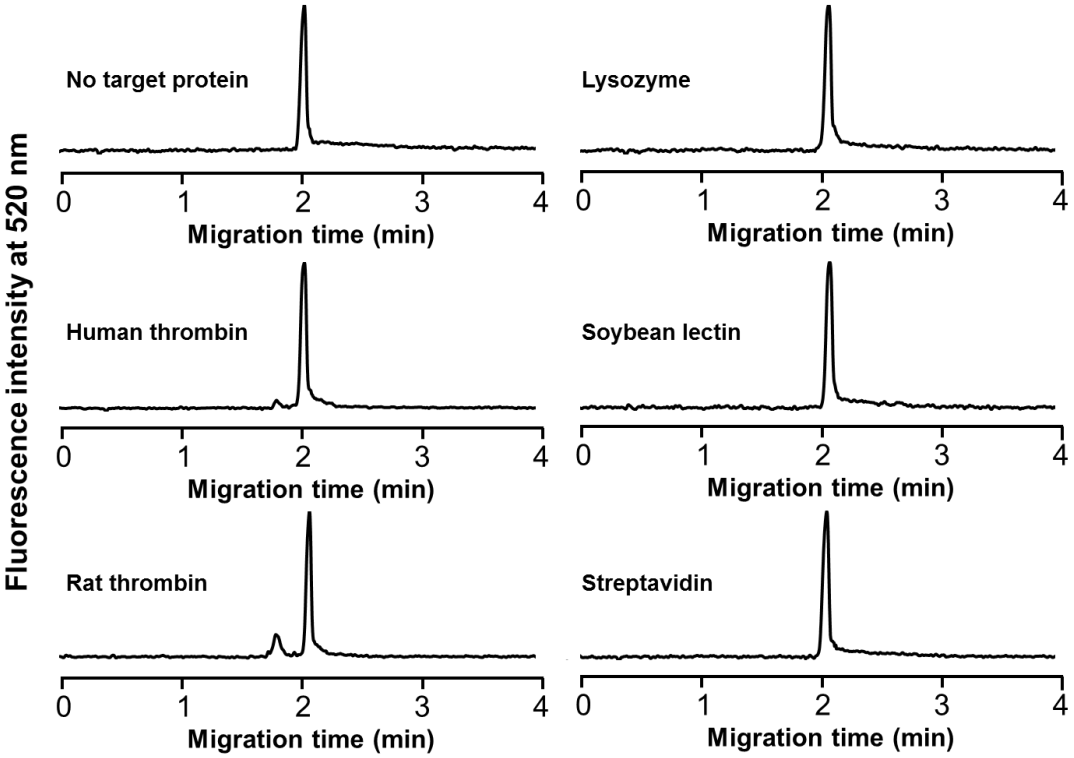


Figure 3-6. Binding specificity of A#40 to protein targets. The aptamer–target complex was observed when human thrombin and rat thrombin were used as targets. The peak of the aptamer–target complex was observed prior to that of the free aptamer in NECEEM.

Table 3-1. Sequences in the variable region of 2',4'-BNA/LNA aptamers obtained by CE-SELEX

Group	Aptamer	Sequence[a]
I	A#1,8,11,12,13,30,35,37	GGAGCAAGA**t**G**t**CACGG**t**CCGCA**t**GCAGAG
	A#21	GGAGCAAG**tt**G**t**CACGG**t**CCGCA**t**GCAGAG
	A#5	GGAGCAAGG**t**G**t**CACGG**t**CCGCA**t**GCAAC
	A#4	GGAGCAAG**tt**G**t**CAC**t**GACCGCACGCAGAG
II	A#14,38	GAGGCAA**t**G**t**G**t**GGG**t**ACCGCA**tt**ACCCAG**t**
	A#27	GAGGCAAGGACGC**t**CCAAGCCGCAACCGG**t**
	A#31	GAGGGA**t**GCGGGAGGCC**t**GA**t**ACG**t**GCAC
III	A#6,20,25,33	AG**t**GGC**t**GGC**t**CCGCG**t**GGGGGG**tt**GGG**tt**
IV	A#26	CACACAGG**ttt**GCGGCCGC**tt**C**t**GGGCAGC
	A#39	AACACAGG**ttt**GCGGCCGC**tt**C**t**GGGCAGC
	A#22	ACACAGG**ttt**GCGGCCGC**tt**C**t**GGGCAGC
V	A#3,18	C**t**C**tt**GACAGGAC**t**CA**t**GCACGGGGGGC**t**
	A#15	C**t**C**tt**GACAGGACACA**t**GCACGGGGGGC**t**
	A#23,28,32	C**t**C**tt**GACAGGACACA**t**GCACGCGGGGC**t**
	A#36	C**t**C**tt**GACAGGAC**t**CA**t**GCACGGAGGGC**t**
	A#40	C**t**C**tt**AACAGGAC**t**CA**t**GCACGGGGGGC**t**
	A#9	C**t**C**tt**GACAGGAC**t**CA**t**GCACGGGGGGA
	A#2	C**t**C**tt**GACAGGACACA**t**GCACGGGGGGC**t**
	A#10	C**t**C**tt**GACAGGACACA**t**GCACGGGGGGCG
	A#16	C**t**C**tt**AACAGGAC**t**CA**t**GCACGGGGGGCC
	A#7	C**t**C**tt**AACAGGAC**t**CA**t**GCGCGGGGGGCC
	A#29	C**t**C**tt**AACAGGAC**t**CA**t**GCACGGGGGC
	A#24	C**t**C**tt**AACAGGAC**t**CAAGCACGGGGGG**t**
	A#19	C**t**C**tt**AACAGGAC**t**CA**t**GCCACGCGGGGCC
VI	A#17	C**t**C**tt**AACAGGACAAG**t**GAAACAAACCCGC
	A#34	C**t**C**tt**GACAGGACCAG**t**GACAAA**t**C**t**GACA

[a] Sequences are aligned in the 5' to 3' direction. The B/L thymidine is shown in bold letters (**t**). Consensus sequences in each group are marked with gray boxes.

Chapter 4

CE–SELEX Selections of DNA-based Chimeric Aptamers with Base and Sugar Modifications

1. Introduction

Enzymatic syntheses of xeno-nucleic acids (XNAs)[33,47,53,54,107–111] and *in vitro* selection of XNA aptamers are hot topics in a broad area of fundamental and applied science.[92] Owing to potential therapeutic and diagnostic applications of XNA aptamers, attempts have been made to use XNAs in systematic evolution of ligands by exponential enrichment (SELEX) experiments.[4,5,87] Among XNAs having unconventional sugars, 2'-*O*,4'-*C*-methylene-bridged/locked nucleic acid (2',4'-BNA/LNA)[63–65] and its analogues[69,70,85,86] have superior nuclease resistance and are expected to provide nucleic acid aptamers that are extremely stable under physiological conditions encountered during practical applications. Therefore, it is worthwhile to develop direct selection systems for BNA/LNA aptamers, despite some technical difficulties in polymerase reactions involving B/L nucleotides, which are inevitable processes in SELEX experiments. Wengel and co-workers are currently working on *in vitro* and *in vivo* selection of aptamers that contain B/L nucleotides in either the 5'-primer region or the random region.[113–115]

Meanwhile, I have very recently succeeded in creating 2',4'-BNA/LNA aptamers using a DNAbased library that contains B/L nucleotides in both the 5'-primer region and the random region.[116]

Prior to this, I had already established an *in vitro* selection system employing capillary electrophoresis for DNA aptamers containing B/L nucleotides in the 5'-primer region. However, our experimental results indicated that B/L nucleotides in the primer region had marginal effects on either the binding activity or the biostability of the selected aptamers. Instead, sequence analyses demonstrated that G-quadruplex motifs were predominantly selected, which implies that G-quadruplexes would nullify the effects of the contained B/L nucleotides.

G-quadruplex motifs are frequently found in DNA aptamers reported so far.[102] This phenomenon suggests that DNA aptamers with G-quadruplexes can target a broad range of molecular structures and retain specificity as well. At present, it is accepted that the structural diversity of G-quadruplexes, *i.e.*, parallel, antiparallel, mixed parallel/antiparallel-stranded configurations with loops and tails of various sequence combinations, can achieve specific recognition of a variety of molecules, even with a similar scaffold.[101,103,117] However, there have been many examples of G-quadruplex DNA aptamers binding totally unrelated targets, and not many have achieved subnanomolar affinity for protein targets.[118] Despite a number of outcomes in SELEX experiments implying that among miscellaneous patterns of DNA sequence G-quadruplex arrangements are most effective nucleic acid receptors, these implications are likely to bring about inherent limitations to the binding ability of DNA aptamers.

In this chapter, I investigated an approach to introduce chemical modifications that would suppress the frequency of G-quadruplex

motifs and selected alternative arrangements for strong binding to human thrombin. Human thrombin is well-known to favor G-quadruplexes[6] and even binds to a short-chain 15-mer oligodeoxyribonucleotide (ODN) (15TBA), which forms a two-tiered G-quadruplex with a dissociation constants (K_d) value of 113 nM.[119] I conducted random independent selections from four different libraries A, B, C, and D using a capillary electrophoresis (CE)−SELEX protocol[81,82,94−96,120] (Figure 4-1). Here, A is a common DNA library, whereas B and D are DNA-based libraries containing B/L nucleotides at intervals of two residues in the forward primer region. Libraries C and D contain the C5-modified thymidine (t) in the random region and the reverse primer-binding region at the 3'-end.

The CE−SELEX method was first reported by Mendonsa and Bowser in 2004. It can be used to separate high-affinity aptamers from free low-affinity aptamers and nonbinding species with high resolution by using nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).[94] This method has distinct advantages over many other SELEX methods developed on the basis of affinity chromatography strategies. Targets do not need immobilization on a solid support, and highly pure fractions of active species, which include minimal contamination by nonbinding species, can be obtained during each selection round because active species are eluted before nonbinding species. Furthermore, the selection processes can be automated using a CE instrument, which could easily provide aptamers with minimal influence of manipulator skills.

I speculated that introduction of a bulky substituent at the C5

position of thymine may destabilize G-quadruplex, because replacement of thymine with uracil in the linker parts is known to stabilize the structure.[121] Thus, it is anticipated that generation of G-quadruplex containing aptamers would be decreased in these experiments. If so, B/L nucleotides in primers may retain structural stability instead of G-quadruplex; B/L nucleotides are considered to stabilize active forms and give rise to unusual conformational folding of DNA aptamers, because the B/L nucleotides-containing DNA duplex is known to be more stable than the corresponding DNA duplex and is more likely to form A-type helix due to its C3'-endo sugar puckered ribose.[122,123] Although B/L nucleotides are contained only in the primer regions, it is possible that DNA aptamers that definitively require B/L nucleotides in primers for activity may be obtained because the sequences of constant regions often affect outcomes of aptamer selection.[124,125] The BNA/LNA primer-dominated binding would provide precursory models of DNA-based molecular switches with binding activities that can be controlled by locking/unlocking of sugar puckering through chemical conversions of the 2',4'-bridged ring.

I chose *N*^{6}-ethyladenine as a base-modified group with the expectation that it increases the probability of inter- and intramolecular hydrogen-bond formation and stacking interactions. In addition, introduction of these base and sugar modifications was expected to improve not only the binding affinity but also the biostability of the DNA aptamer. In our previous work,[54] it was confirmed that reverse transcription of DNA templates containing B/L nucleotides at intervals of a few residues proceeds smoothly and

accurately under the standard conditions of enzyme/substrate concentration, reaction time, and temperature using *KOD Dash* DNA polymerase. This polymerase can efficiently transcribe and reverse-transcribe DNA involving C5-modified thymidine.[48,126,127] The SELEX experiments have been successfully implemented based on this technical knowledge and methodology.

2. Results and Discussion

2.1. Sequence Analyses of the Obtained Aptamers.

Every sequence selected from library A has the potential to form two tiered G-quadruplex motifs; $T(G_2N_n)_3G_2T$, here n = 1–5 and thymine content in N residues was not less than 40% (Table 4-1 and Table 4-2). An identical motif was observed in every sequence from library B, indicating that B/L nucleotides in primers do not suppress the frequency of G-quadruplex motifs (Table 4-3). In contrast, no G-quadruplex motifs were found in sequences from libraries C and D, except for C#7 and C#8 (clone no. 7 and clone no. 8) (Tables 4-4 and 4-5). Instead, the consensus sequence G$\mathbf{t}_3AC_3\mathbf{t}$ was found in 12.5% of aptamers from library C (including C#4, C#5, C#12, C#13, C#14, C#16, C#19, and C#20). In addition, the consensus sequence $\mathbf{t}_5G\mathbf{t}_2G_2$ was found in aptamers recovered from both libraries C and D (including C#1, C#10, D#7, D#11, and D#13). Base modification was found to mainly contribute to suppressing the frequency of G-quadruplex motifs and generating alternative motifs containing plural **t**'s. Two identical sequences appeared separately from libraries A and B: first is the

sequence of A#1, A#2, A#3, A#4, A#6, B#3, B#5, B#6, B#10, B#12, and B#14, while the other is that of A#8, A#9, A#11, A#12, A#13, A#16, B#1, and B#8. Contrary to our expectations, this result indicates that the inserted B/L nucleotides barely affected the selection outcome as long as the four natural nucleotides were used in the nonprimer region of the library. In contrast, obtained sequences seem to be influenced by B/L nucleotides in the primer when the C5-modified thymidine-containing libraries were used. Moreover, no aptamers with an identical sequence were observed in both populations recovered from libraries C and D. In the random region of aptamers from libraries A and B, guanine (G) and thymine (T) contents were found to be higher than adenine (A) and cytosine (C) contents (Figure 4-4). The A content tended to be suppressed in selection outcomes of all four libraries used. Interestingly, when compared with library A and B selections, library C and D selections generally brought about higher C content instead of lower G and T contents. This implies that the probability of G–C pair formation rather than that of G-quadruplex formation would be increased in conformation of aptamers from libraries C and D.

2.2. Binding Affinity and Specificity Analyses of the Obtained Aptamers.

The apparent dissociation constants (K_d) of aptamers selected from these libraries were found to be in the range of 10^{-11}–10^{-8} M, and in many cases, several nanomolar (10^{-9} M) (Table 4-1–5). Seven aptamers from libraries C and D showed strong binding affinity to the target within a subnanomolar range. Among these, the four aptamers C#1,

D#7, D#11, and D#13 contained the motif $\mathbf{t}_5\mathrm{G}\mathbf{t}_2\mathrm{G}_2$. D#12 shared a 67% sequence similarity with D#6 in the random region, and it had the highest binding affinity (K_d = 0.093 nM), which was much stronger than that of TBAs with a G-quadruplex arrangement furnished by conventional SELEX[76] and microfluidic-SELEX (M-SELEX);[128] their K_d values were 0.5 and 0.33 nM, respectively. In a previous study by Latham *et al.*,[129] modifications that caused destabilization of G-quadruplex were considered to be unfavorable for TBA selection; TBAs containing 5-pentynyl 2′-deoxyuridine instead of thymidine had the potential to form hairpin loops but not G-quadruplex motifs, and their K_d values were estimated to be approximately 400–1000 nM. However, our results demonstrated that even though G-quadruplex motifs disappeared, alternatives could emerge depending on the chemical structure of the introduced functionality. The high-affinity modified DNA aptamers D#6 and D#12 showed binding specificity and distinguished human thrombin from other proteins such as lysozyme, soybean lectin, and streptavidin (Figure 4-5). However, both of these aptamers strongly bound rat thrombin with almost the same affinity as human thrombin, indicating that their binding specificity was not sufficient to discriminate between proteins from different animal species.

2.3. Effects of the Chemical Modifications on Binding Affinities.

Base modification was found to be essential for the binding ability of aptamers from libraries C and D (Tables 4-4 and 4-5, Figure 4-6). Chemical variants of these aptamers, in which all **t**'s were completely

replaced with natural T's, had no binding affinity to the target in all assessed cases (C#1a, C#2a, C#4a, C#5a, C#7a, C#8a, C#9a, D#1a, D#2a, D#6a, D#7a, D#11a, D#12a, and D#13a). Among these, even though C#7 and C#8 had G-quadruplex motifs and they could form G-quadruplex structures, they were unable to retain binding activity without base modification. Conversely, chemical variants A#1c and A#5c (derived from A#1 and A#5, respectively), in which natural T's were replaced with t's, also had no binding activity, indicating that base modification interferes with target binding of these natural DNA aptamers (Table 4-2). From the comparisons of K_d values, sugar modification seemed to contribute little to the target binding affinity in many cases. Except for A#5b/A#5, D#1/D#1c, D#6/D#6c, and D#12/D#12c, the binding affinities were almost unchanged or decreased up to 12-fold when the 2',4'-BNA/LNA primer was replaced with the natural DNA primer (Figure 4-7). In contrast, D#1 and D#6, which contain the motif $\mathbf{t}_2G_2\mathbf{t}C(A/G)A_2G_2t$ that is highly complementary to a part of the primer sequence, completely lost their binding activities in the absence of B/L nucleotides. Similarly, D#12 which contains a similar sequence ($\mathbf{t}_2AG\mathbf{t}CGA_2G_2\mathbf{t}$), was significantly affected by the replacement of B/L nucleotides and showed a 940-fold decrease in its binding activity. Considering the identified sequence motif, these losses in binding activity may be caused by the destabilization of putative hairpin structures due to the absence of sugar modification (Figure 4-8).[130] Conversely, the binding activity of A#5 was increased by the replacement of the natural DNA primer with the 2',4'-BNA/LNA primer, indicating that conversions of natural

nucleotides to B/L nucleotides could interfere with formation of the active form, though this change in binding activity was not dramatic as that of aptamers D#1, D#6, and D#12. Thus, the introduction of B/L nucleotides in the primer provided the very sharp 2',4'-BNA/LNA-dependent binding activity demonstrated by D#1 and D#6, which contained the motif **t**$_2$G$_2$**t**C(A/G)A$_2$G$_2$**t**. It should be noted that such aptamers could be recovered only from library D, indicating that the combined use of B/L nucleotides and **t**'s leads to the expression of this unique function. The primer parts of D#1 and D#6 were predicted to form the hairpin structure (Figure 4-8). It is, therefore, conceivable that thermostability of the hairpin structure could retain the binding activity. This property could be controlled using B/L nucleotide derivatives that are sensitive to photoirradiation, acids, and reductants.[131,132] Namely, library D could provide precursory models of DNA-based molecular switches having unconventional sugars, *i.e.*, XNA switches such as riboswitches and aptazymes,[133–138] and such chimeric ODN libraries will enable us to establish direct selection methodologies for them.

2.4. CD Measurements.

To analyze G-quadruplex formation, I examined the structural changes in aptamers using CD spectroscopy. These analyses included A#1, C#1, and D#6 in addition to 15TBA (5'-GGT TGG TGT GGT TGG-3'), 29TBA (5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'), and 76TBA (5'-AGA TGC CTG TCG AGC ATG CTC TTT GGA GAC AGT CCG TGG TAG GGC AGG TTG GGG TGA CTT CGT GGA AGA AGC GAG A-3') in which

G-quadruplex core sequences (underlined) are considered to form antiparallel G-quadruplexes[76] because of K^+ addition (Figure 4-9). In the presence of K^+ (50 mM), 15TBA and 29TBA exhibited a typical CD spectra of an antiparallel form with positive bands at approximately 245 and 295 nm and a negative band at approximately 265 nm[139–141] (Figure 4-9, parts A and B). For long-chain TBAs, the contribution of a G-quadruplex to CD signals would be counterbalanced with those of ODN structures in the other parts. However, significant increases in signal intensity were observed at 295 and 245 nm in the CD spectra of 76TBA as the concentration of K^+ increased (Figure 4-9C). Similarly, addition of K^+ to A#1 caused signal increases at 245 and 295 nm and a decrease at 265 nm, indicating G-quadruplex formation for A#1 (Figure 4-9D). In contrast, significant spectral changes were not observed after K^+ addition to either C#1 or D#6, although D#6 had a sequence that could form a G-quadruplex while C#1 did not (Figure 4-9, parts E and F). This indicated that both modified DNA aptamers did not form G-quadruplexes; rather, they generated alternatives that tightly bound to the target.

2.5. Effects of the Base and Sugar Modifications on Biostability.

As shown in Figure 4-10A, they were found to be stable in the order of D#6, D#2, D#2c, D#6c, B#1, and A#8; half-lives of the intact forms were 10.5, 6.0, 5.0, 4.2, 2.0, and 1.9 h, respectively. As indicated by the differences between D#6 and D#6c, the effect of the sugar modification seemed to improve stability by more than 2.5-fold. A large proportion of short 6-FAM-labeled fragments was observed in the reaction mixture

of D#6c, indicating that the primer region was degraded (Figure 4-10B). In contrast, the half-life of D#2 did not differ much from that of D#2c, presumably because of the presence of 10 and 6 successive natural nucleotide sequences in the random region, whereas two and six successive natural nucleotide sequences were present in D#6. On comparing the electrograms of D#2 and D#6, degraded fragments, which migrated between 4.3 and 6.1 min, were more prevalent in D#2 than in D#6. Conformational factors could be another reason; D#2c retained binding activity, whereas D#6c did not. This means that the steric structure of D#2c is close to that of D#2, while D#6c forms a conformation that is different from D#6; this make nucleases much more accessible to the natural nucleotide 20-mer primer region of D#6c. Similarly, there was only a slight difference in the time taken for full-length aptamer disappearance between A#8 and B#1, both of which have the same sequence and retain binding activity. This is because the nonprimer region of B#1 was as readily attacked by nucleases as that of A#8, whereas the primer region of B#1 was not degraded similar to that of A#8. Thus, the sugar modification affords only minimal effects on the biostability of the entire structure as long as it retains its active form, although the nuclease resistance of the sugar-modified part was certainly confirmed by the amounts of short fragments generated (<20-mer), as observed in the electrograms of degradation products. On the other hand, the base modification can provide significant effects depending on density of the functionality inserted and/or conformation of the aptamers.

3. Conclusion

The base modification could suppress the frequency of G-quadruplex motifs, but alternately, they could also generate alternatives to yield very high affinity receptors; moreover, by cooperating with the base modification, the sugar modification in the primer could form a core domain, which indispensably retains the entire structure in an active form, to yield precursory models of XNA switches. Furthermore, significant effects by base and sugar modifications on resistance to biological degradation were confirmed. Thus, our results demonstrated modifications that evade the adverse effects of limited structural DNA topologies on functional activity and suggest the potential of chimeric ODN libraries for developing new classes of DNA aptamers for omics research and for diagnostic and therapeutic applications.

4. Experimental Procedures

4.1. Materials

Sample solutions and buffers were prepared using ultrapure water obtained using the Ultrapure Water system CPW-100 (Advantec Co. Ltd., Tokyo, Japan). The initial DNA library, DNA primers and templates, a fragment of aptamer A#1 (*i.e.* A#1_36–53), known 29-mer ODNs of thrombin-binding aptamer (TBA)[76] 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3' labeled with/without 6-carboxyfluorescein (6-FAM) at the 5'-end (*i.e.* f29TBA and 29TBA), and non-labeled 15-mer and 76-mer TBAs (15TBA and 76TBA) were purchased from Japan Bio Services Co. Ltd. (Saitama, Japan) or Hokkaido System Science Co. Ltd.

(Hokkaido, JAPAN). 2',4'-BNA/LNA primers were purchased from GeneDesign Inc. (Osaka, Japan). Cytosine-type 2',4'-bridged/locked nucleosides and their amidite derivatives were synthesized according to previously published procedures.[63,142] The initial DNA library and forward primers were labeled with 6-FAM at the 5'-end to facilitate fluorescence detection. The initial DNA library T1 comprised random ssDNA (30 bases) regions flanked by the primer sequence (20 bases each) 5'-/6-FAM/TCG CCT TGC CGG ATC GCA GA-(30 random bases)-TGG TCC GTG AGC CTG ACA CC-3'. Some primers were labeled with monophosphate at the 5'-end to prepare ssDNA using λ exonuclease after PCR amplification of selected ODNs. Thrombin from human plasma, thrombin from rat plasma, tris(hydroxymethyl) aminomethane (Trizma®base), chicken egg lysozyme, and human serum (from human male AB plasma) were purchased from Sigma-Aldrich, Inc. (MO, USA). Soybean lectin, streptavidin type II, λ exonuclease, ethanol, aqueous HCl, boric acid, sodium tetraborate, NaCl, and $MgCl_2 \cdot 6H_2O$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fused-silica capillary (eCAPTM capillary tubing, 75 μm I.D., 375 μm O.D.) and ssDNA 100-R kits were purchased from Beckman Coulter, Inc. (CA, USA). The 4 dNTPs (dATP, dGTP, dCTP, and TTP) were purchased from Roche Diagnostics K.K. (Tokyo, Japan). *KOD Dash* DNA polymerase was purchased from Toyobo Co. Ltd. (Osaka, Japan) and *Taq* DNA polymerase from Invitrogen Co. Ltd. (CA, USA). The plasmid DNA purification kit was purchased from Cosmo Genetech Co. Ltd. (Seoul, Korea). The binding buffer contained 20 mM Tris-HCl buffer (pH 7.40), $MgCl_2$ (1 mM), and NaCl (10 mM). The running buffer

for separation contained 100 mM sodium borate (pH 8.35). Binding and running buffers were filtered through a 0.45-μm membrane filter (EMD Milipore Co., MA, USA).

4.2. Preparation of natural/modified DNA libraries

Detail procedures are illustrated in Figure 4-3. For the initial library A, T1 was used without further purification. As shown in Figure 4-3A, after affinity separation using CE, the fraction containing the DNA–thrombin complex was subjected to symmetric PCR amplification (16-24 PCR cycles at 94°C for 30 s, 54°C for 30 s, and 74°C for 60 s) in a TC-312 thermal cycler (Techne, Staffordshire, UK) using the forward primer P1f (5'-/6-FAM/TCG CCT TGC CGG ATC GCA GA-3'; 0.4 μM), the reverse primer P2p (5'-/phosphate/GGT GTC AGG CTC ACG GAC CA-3'; 0.4 μM), four natural dNTPs (0.2 mM each), *KOD Dash* DNA polymerase (0.005 U/μL), and 1× polymerase reaction buffer (step 1). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified natural dsDNA. To degrade the P2p-elongating product, dsDNA (ca. 300 pmol) was incubated at 37°C for 30 min with 1× λ exonuclease reaction buffer (760 μL) containing λ exonuclease (0.025 U/μL) (step 2-1). Single-stranded natural DNAs were purified by denaturing polyacrylamide gel electrophoresis (PAGE) (step 2-2) and used as the natural DNA library for the following round.

For preparation of initial library B, symmetric PCR was performed using T1, the forward primer P1fb (5'-/6-FAM/TC[G] CC[T] TG[C] CG[G] AT[C] GC[A] GA-3'), which contained B/L nucleotides as shown in capitals enclosed in squares, and the reverse primer P2p. The reaction mixture

(800 μL) contained the template T1 (8 nM), primers P1fb and P2p (0.4 μM each), the four natural dNTPs (0.2 mM each), *KOD Dash* DNA polymerase (0.005 U/μL), and the reaction buffer. The reaction mixture was subjected to 20 PCR cycles at the abovementioned conditions. The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified dsDNA. To degrade the complementary strand (*i.e.* P2p-elongating product), dsDNA was treated with λ exonuclease under the same conditions as used above. Single-stranded modified DNAs were purified by denaturing PAGE and used for the initial library B of modified DNA. The modified DNA library for the next round was prepared according to the abovementioned protocol for preparation of library A using the forward primer P1fb, instead of P1f (Figure 4-3B).

The library C was constructed as follows. One-primer PCR was performed using T1 and the non-labeled reverse primer P2. The reaction mixture (800 μL) contained the template T1 (80 nM), reverse primer P2 (0.4 μM), four natural dNTPs (0.2 mM each), *KOD Dash* DNA polymerase (0.005 U/μL), and the reaction buffer. Six PCR cycles were performed at the abovementioned conditions. The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified natural dsDNA. Subsequently, one-primer PCR was performed using natural dsDNA as a template, dATP, dGTP, dCTP, (*E*)-5-(2-(*N*-(2-(*N*^{6}-adeninyl)ethyl))carbamylvinyl)-2'-deoxyuridine-5'-triphosphate (dUmdTP) in place of TTP (0.2 mM each), the forward primer P1f (0.4 μM), *KOD Dash* DNA polymerase (0.005 U/μL) and the reaction buffer. The reaction mixture (800 μL) was subjected to 8 PCR

cycles at the abovementioned conditions, to produce modified DNA strands. Single-stranded modified DNAs were separated from byproducts by denaturing PAGE and used for the initial library C of modified DNA. As shown in Figure 4-3, after affinity separation using CE, a fraction containing the modified DNA–thrombin complex was subjected to symmetric PCR amplification (16–24 PCR cycles at the abovementioned conditions) using the 5'-phosphate-labeled forward primer P1p having sequence identical to P1f (0.4 μM) and the reverse primer P2 (0.4 μM) (step 1*). The reaction mixture was then subjected to ethanol precipitation to remove dNTPs and yield roughly purified natural dsDNA. To degrade the P1p-elongating product, dsDNA was treated with λ exonuclease under the same conditions as used above (step 2*). Using the intact complementary strand as a template, one-primer PCR (8 PCR cycles under the abovementioned conditions) was performed using a reaction mixture (800 μL) containing the primer P1f (0.4 μM), dATP, dGTP, dCTP, dUmdTP (0.2 mM each), *KOD Dash* DNA polymerase (0.005 U/μL), and the reaction buffer (step 3*-1). Single-stranded modified DNAs were purified by denaturing PAGE (step 3*-2) and used as the modified DNA library for the next round.

The library D was constructed according to the abovementioned protocol for preparation of library C using the forward primer P1fb, instead of P1f (Figure 4-3D). One-primer PCR was performed for 24 cycles (step 3*-1).

4.3. Affinity Separation Conditions of CE–SELEX.

Experiments were conducted in the same manner as in Chapter 3

(see 3.2.2.). The initial libraries provided relative peak areas of the target–aptamer complex with approximately 0.03% for libraries A and B and 0.1% for libraries C and D, respectively. Thus, the affinities of those initial libraries to the target are very low, with K_d values of more than 0.1 mM (Figure 4-2).

4.4. Isolation and Sequencing of Selected Natural/Modified DNA Aptamers.

Four cycles of separation and amplification were required for sufficient enrichment of active species in libraries A–C, while six cycles were required for enrichment in library D (Figures 4-2 and 4-3). Subsequently, enriched natural/modified DNAs were amplified by polymerase chain reaction (PCR) using *KOD Dash* DNA polymerase with nonlabeled forward and reverse primers (P1, 5'-TCG CCT TGC CGG ATC GCA GA-3', and P2, 5'-GGT GTC AGG CTC ACG GAC CA-3') and the four natural dNTPs. The resulting dsDNA was further amplified by PCR using Taq DNA polymerase. Subsequently, amplified dsDNA was cloned into a TA vector by the TA cloning method46 according to the manufacturer's protocol. Plasmid DNAs were selected from different clones and isolated using the plasmid DNA purification kit. Sequences inserted into the prepared plasmids were analyzed using DNA sequencing services at Operon Biotechnologies Co. Ltd. (Tokyo, Japan) and Takara Bio Inc. (Shiga, Japan). Twenty aptamers were isolated from each library (A–D); a total of 80 sequences for aptamers were identified (Table 4-1, 4-3–5).

4.5. Preparations of Isolated TBAs, Their Chemical Variants and Fragments

Natural/modified TBAs used for affinity analysis were enzymatically prepared using the respective isolated plasmid DNAs as templates in the protocol used for preparation of the corresponding libraries (Tables 4-2–4-5). Chemical variants of natural/modified TBAs were also enzymatically synthesized as follows. Preparation of B#2a, B#4a, B#11a, C#1a, C#2a, C#4a, C#5a, C#7a, C#8a, C#9a, D#1a, D#2a, D#6a, D#7a, D#11a, D#12a, and D#13a was performed in compliance with the protocol for preparation of library A, which employed the natural primer P1f and the four natural dNTPs. Preparation of A#5b, D#1b, D#2b, D#6b, D#7b, D#11b, D#12b, and D#13b was performed in compliance with the protocol for preparation of library B, which employed the 2',4'-BNA/LNA primer P1fb and the four natural dNTPs. Preparation of A#5c, D#1c, D#2c, D#6c, D#7c, D#11c, D#12c, and D#13c was performed in compliance with the protocol for the preparation of library C, which employed the natural primer P1f and triphosphates (dATP, dGTP, dCTP, and dUmdTP). Preparation of C#1d, C#5d, and C#8d was performed in compliance with the protocol for the preparation of library D, which employed the 2',4'-BNA/LNA primer P1fb and triphosphates (dATP, dGTP, dCTP, and dUmdTP). Similarly, fragments of TBAs obtained (including A#1_1-66, A#1_10-70, A#1_14-70, A#1_14-66, A#_24-70, C#1_26'-67, D#6_1-65, D#6_1-58, D#6_1-49, D#6_1-37, D#6_1-32, and D#6_35'-70) were prepared using corresponding 5'-(6-FAM)-labeled primers and templates (Table 4-6 and Figure 4-8). For CD measurements, TBAs (A#1, C#1, and D#6)

lacking 6-FAM label at the 5'-end were prepared using corresponding non-labeled primers and templates (Figure 4-9). All samples were purified by denaturing PAGE and used for CE analysis.

4.6. Affinity and Specificity Analyses by CE

Experiments were conducted in the same manner as in Chapter 3 (see 3.4.). Electrograms were recorded at 4 Hz and analyzed with 32 Karat software ver. 8.0 (Figures 4-5 and 4-6). The apparent dissociation constants (K_d) of the thrombin–TBA complex were determined according to the literature[28,99] (Tables 4-1–4-6). Five independent measurements (n = 5) were accordingly performed at a single concentration for all K_d determinations. The K_d values listed in Table 4-1 are the averages (μ) of those measurements (x_1, x_2, x_3, x_4, and x_5), and % relative standard deviations ($\%RSD$) were calculated using equation 2, where μ is the mean of the distribution, and σ is its standard deviation.

$$\%RSD = \frac{100 \cdot \sigma}{\mu} = \frac{100}{\mu} \cdot \sqrt{\frac{(x_1 - \mu)^2 + (x_2 - \mu)^2 + \cdots + (x_n - \mu)^2}{n}} \quad eq.2$$

4.7. Competitive Affinity Binding Assays

To analyse where the high-affinity modified DNA aptamers (D#6 and D#12) were recognized in human thrombin, competitive affinity binding assays were performed using non-labeled 29-mer TBA (29TBA), which is known to specifically bind to exosite 2[76] (Figure 4-11). The apparent K_d value of the 5'-(6-FAM)-labeled 29-mer TBA (f29TBA) as determined by the aforementioned CE affinity analyses was 0.29 nM (Figure 4-11A). Initially, D#6, D#12, f29TBA, 29TBA, and

human thrombin were separately dissolved in a binding buffer. Then, D#6, D#12, f29TBA, and

29TBA were refolded by denaturing at 94°C for 0.5 min and cooling to 25°C at a rate of 0.5°C/min. Sample solutions (40 μL) containing D#6, D#12 or f29TBA (1 nM) in addition to human thrombin (1 nM) and 29TBA (0.5–10 nM) were prepared for affinity analysis and incubated at 37°C for 30 min prior to CE analysis, which was performed as per the abovementioned protocol. Similarly, sample solutions containing D#6 or D#12 in addition to human thrombin and 29TBA were prepared by adding D#6 or D#12 after incubating the mixture of human thrombin and 29TBA at 37°C for 30 min. After the addition of D#6 or D#12, both mixtures were further incubated at 37°C for 30 min prior to CE analysis.

The competitive binding affinity assays showed that only 40%–50% replacements of D#6 and D#12 were observed when a tenfold excess of competitor (29TBA) was added, while nearly 90% replacement of f29TBA was observed under the same conditions (Figure 4-11B). The titration curves of D#6 and D#12 replacements reach horizontal asymptotes of 51% and 59%, respectively, and the equivalence points are roughly consistent with those at half-saturation. The same results were obtained when thrombin and 29TBA were mixed prior to the addition of D#6 or D#12 (data not shown). This indicates that binding modes rather than target binding affinities and kinetics are reflected in the titration profiles of D#6 and D#12, considering that the differences of binding affinities between the three aptamers (namely D#6, D#12, and f29TBA) were only a few fold. Thus, even though exosite 2 was

occupied with 29TBA, D#6, and D#12 could still firmly retain their target binding activities, indicating that they recognize not only exosite 2 but also other parts of thrombin. The steric structure of human thrombin closely resembles to that of rat thrombin, and they share a 89% amino acid sequence similarity. As shown in Figure 4-12, the common residues include many cationic and aromatic amino acids are displayed on the surfaces of both proteins. Structural differences around exosite 2 can be barely observed, whereas uncommon residues are more densely expressed on the opposite site. These structural differences are clearly visible in the center of each image at a rotation angle of (0, −120, 0), and in the right-hand side of each image at a rotation angle of (0, 120, 0). Notably, compared with 29TBA, D#6, and D#12 cover a broader portion of the structure containing exosite 2, and base modification may increase the access probability to the protein surface through hydrogen bonding, hydrophobic and stacking interactions, as expected.

4.8. CD Measurements

CD spectra were acquired with a JASCO J-820 spectrometer. CD spectra were measured over a wavelength range of 220–340 nm using a quartz cuvette with a 1.0-mm optical path length. The scanning speed was set at 100 nm/min, and the response time was 1 s. Each spectrum was an average of five measurements made at 25°C. Before CD measurements, each TBA was dissolved in 20 mM Tris-HCl buffer (pH 7.2) for a 20 μM solution followed by refolding by denaturing at 94°C for 0.5 min and cooling to 25°C at a rate of 0.5°C/min using the thermal

cycler. Sample solutions containing 10 μM (final conc.) of TBA in 20 mM Tris-HCl buffer (pH 7.2) with different KCl concentrations (0, 5, 10, 20, and 50 mM) were prepared and analysed.

4.9. Stability Test in 80% v/v Human Serum

I examined the effects of these modifications on the stability in human serum using B#1, A#8, D#6, D#6c, D#2, and D#2c (Figure 4-10); A#8, D#6c, and D#2c have sequences identical to B#1, D#6, and D#2, respectively, but they contain natural nucleotides instead of B/L nucleotides in the primer regions (Tables 4-2, 4-3, and 4-5). Degradation of TBAs was performed in a 4 μL reaction volume containing 0.4 μL of B#1, A#8, D#6, D#6c, D#2 or D#2c (400 nM), 3.2 μL of human serum (from human male AB plasma) and 0.4 μL of 10× reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$; pH 8.0). The reaction mixtures were incubated at 37°C for 0.5, 1, 2, 4, 8, 16, and 24 h and separated by capillary gel electrophoresis (CGE) using the ssDNA 100-R kit, whereas monitoring fluorescence (520 nm) of 6-FAM labeled 5'-ends of the aptamers. The data were recorded at 4 Hz and analyzed with 32 Karat software ver. 8.0 (Figure 4-10B). The relative amounts of intact aptamer were calculated from peak areas, and the decay curves of intact TBAs were fitted by the least squares method using OriginPro ver.8 to calculate the half-life of intact forms (Figure 4-10A).

4.10. Binding Activities of Aptamer Fragments

The binding activity of aptamers can be increased by minimising the length. For example, the K_d value of 29-mer TBA (5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3') was 0.5 nM, while that of the original

full-length 105-mer was 0.92 nM, as reported in the literature[76]. Therefore, the three aptamers namely A#1, C#1, and D#6 were attempted to minimize their length (Table 4-6).

The most stable secondary structure of A#1 was predicted by mfold[130] at 25°C in 10 mM Na^+ and 1 mM Mg^{2+} (Figure 4-8) and used to design fragments. The absence of four residues at the 3′-end (A#1_1-66) or nine residues at the 5'-end (A#1_10-70) had minimal effect on the binding activity. However, unexpectedly, the binding activity was substantially decreased in the absence of the 13 residues at the 5'-end (A#1_14-70) and was lost in the absence of the four residues at the 3'-end in addition to the 13 residues at the 5'-end (A#1_14-66). Accordingly, the primer sequence A#1_1-23, core sequence of G-quadruplex (A#1_36-53) and G-quadruplex motif flanked by sequences that form a stem structure with a dangling tail of four nucleotides at the 3'-end (A#1_24-70) were all inactive. These observations indicate the requirement of almost the entire aptamer sequence for target binding.

Similarly, the binding activity of D#6 fragments, in which the sequence at the 3'-end was shortened, was decreased as more residues were removed and finally lost when 33 residues were omitted (D#6_1-37). Unlike A#1, the secondary structures of modified DNA aptamers have not been correctly predicted as yet by any computer simulations. In the present study, although mfold regards input sequence as fully natural nucleotides, I compulsorily applied mfold to secondary structure predictions of modified DNA (Figure 4-8). As aforementioned, D#6 has a sequence motif that is highly

complementary to the primer, and it loses its binding activity without sugar modification. Hence, I expected that this part of the aptamer may form a hairpin structure similar to that of D#6_1–32, which was predicted as the most stable structure by mfold at 25°C, 10 mM Na^+ and 1 mM Mg^{2+}. Under these conditions except for Na^+ concentration (1 M), mfold predicted the secondary structure of D#6 with the hairpin structure as the third most stable structure. In this structure, the 31-mer sequence from the 35th to 65th residues formed a stem-bulge-loop structure, which may be a functional domain of the aptamer. Subsequently, D#6_35'-70 was designed and enzymatically prepared; however, it was found to be inactive. Incidentally, D#1 as well as D#6 was predicted to form the aforementioned hairpin structure, which was shown as the fourth most stable structure at 25°C, 10 mM Na^+ and 1 mM Mg^{2+}.

Similarly, the most stable secondary structure of C#1 predicted by mfold at 25°C, 10 mM Na^+ and 1 mM Mg^{2+} contained the consensus motif $t_5Gt_2G_2$ in the loop. Therefore, a stem-loop structure (C#1_26'-67) was designed and prepared as a potential functional domain; however, it was also inactive. Thus, approximately full-length nucleotides would also be required for these modified DNA aptamers to retain their maximum binding activities, as seen in the natural DNA aptamer A#1.

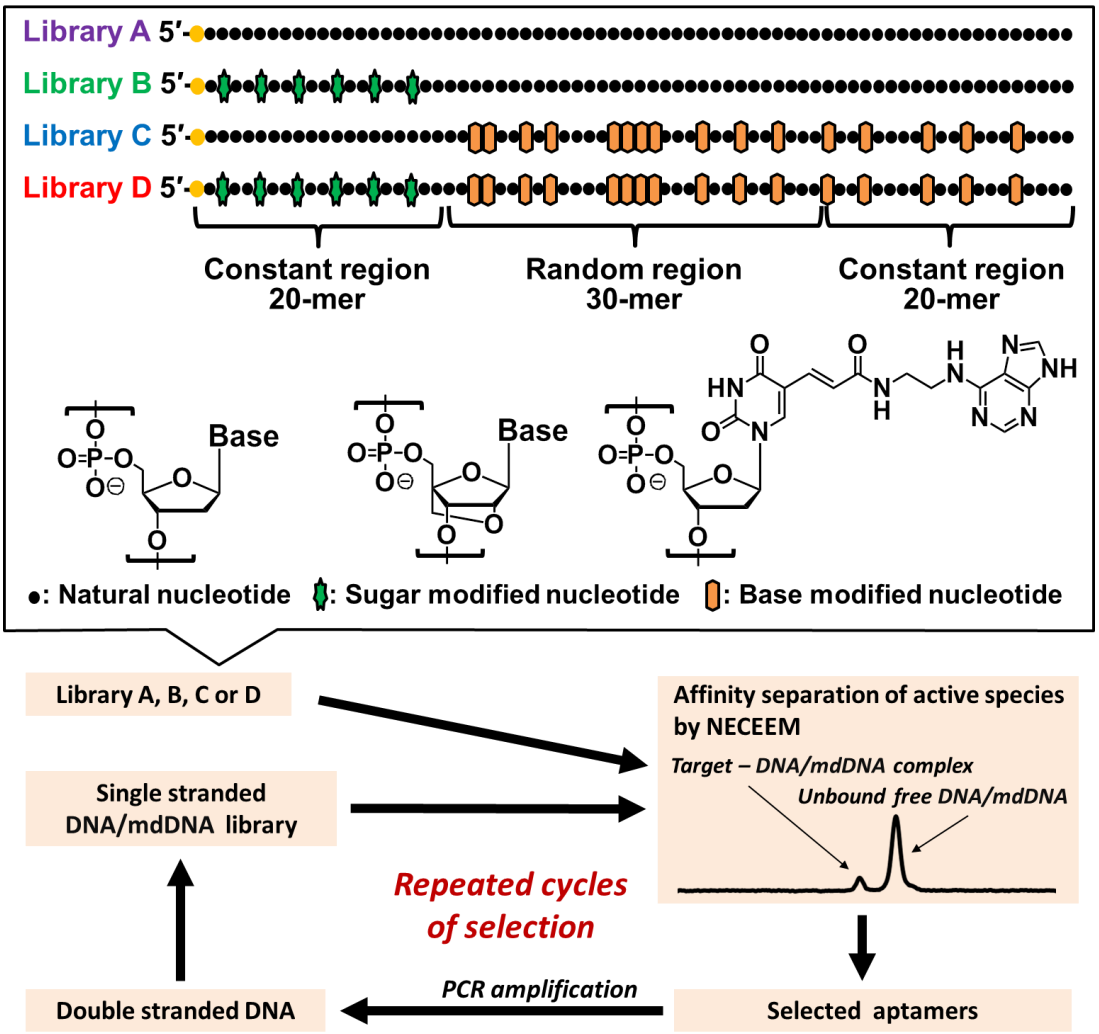


Figure 4-1. The four different ODN libraries prepared, and the schematic illustration of thrombin binding aptamer (TBA) selection from those libraries by CE–SELEX. Here, A, G, C, and T indicate 2'-*O*,4'-*C*-methylene-bridged/linked bicyclic ribonucleotides with adenine, guanine, cytosine, and thymine bases, respectively, and **t** indicates *N*-(2-(*N*^{6}-adeninyl)ethyl))-2'-deoxyuridine.

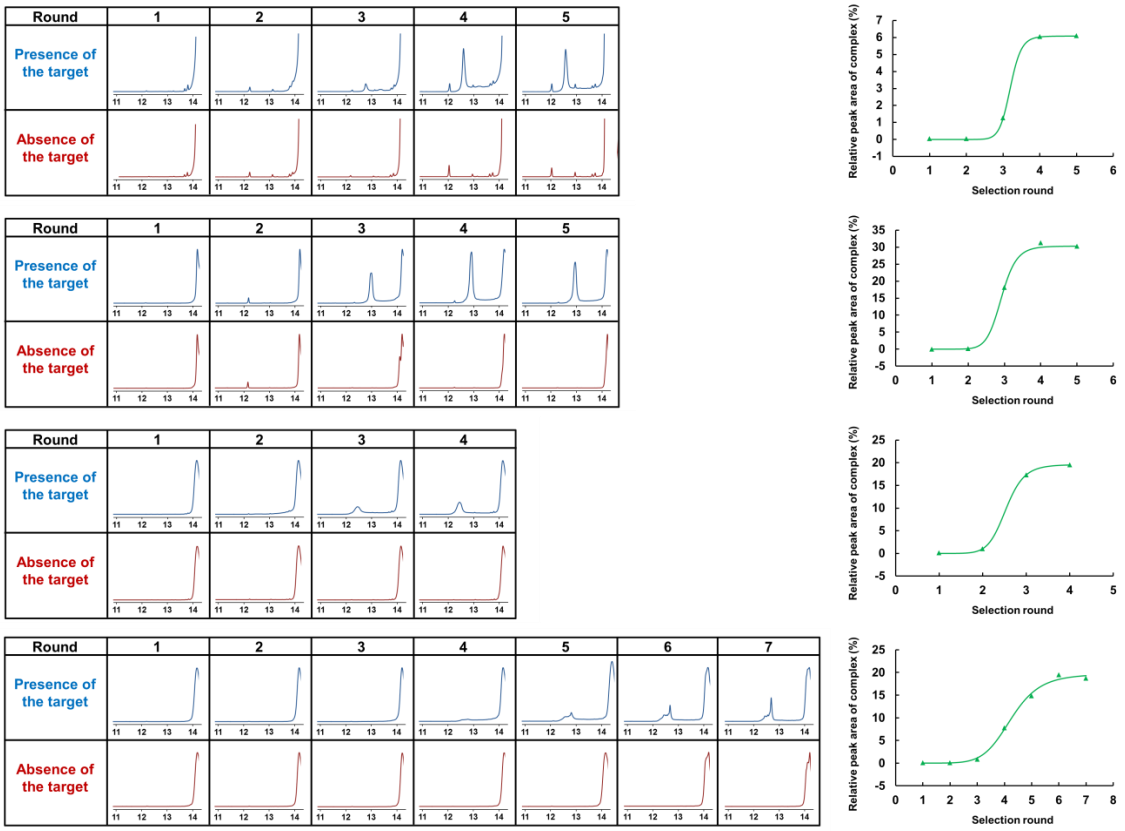


Figure 4-2. Process of active species enrichment in selection rounds. Capillary electrograms for (A) library A, (B) library B, (C) library C, and (D) library D of each round with or without human thrombin (left panels). All electrograms recorded fluorescent intensity of 5'-labeled 6-FAM versus migration time. The asterisk indicates the peak of the thrombin–aptamer complex. Saturation curves of the library enrichment for TBA acquisition (right panels). Concentrations of the target and ODN were 200 nM and 500 nM, respectively. The aptamers were isolated form enriched libraries of libraries A–C after the fourth round and from that of library D after the sixth round.

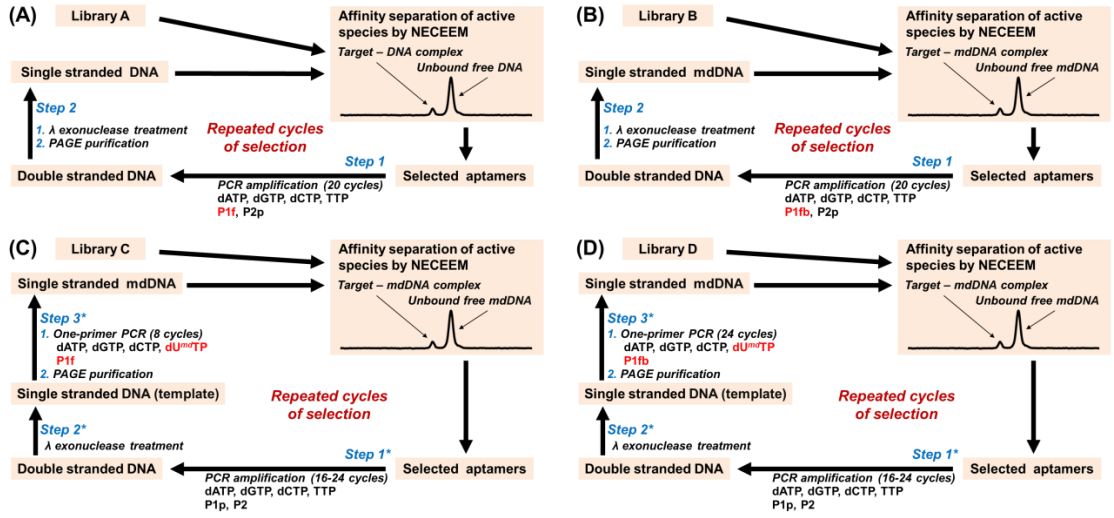


Figure 4-3. Schematic illustrations of SELEX experiments using (A) library A, (B) library B, (C) library C, and (D) library D, respectively. The primer P1fb contains six B/L nucleotides (A, G, C and T), and dU^{md}TP is a 5'-triphosphate analog of the C5-modified thymidine bearing N^6-ethyladenine (**t**).

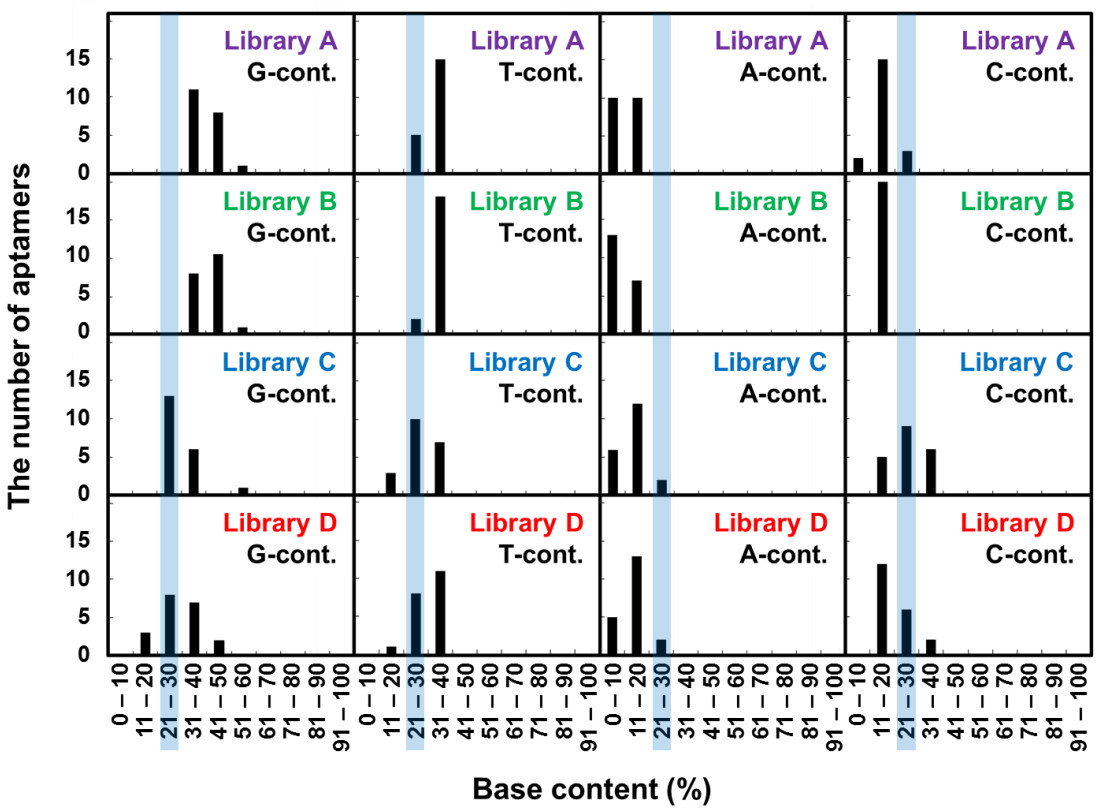


Figure 4-4. Base contents (%) in the random region of 20 aptamers recovered from each library (A–D). Percentage content of each base (G, T, A, C) would be approximately 25% highlighted with blue, if selection biases had a scarce effect on the base content in sequences selected.

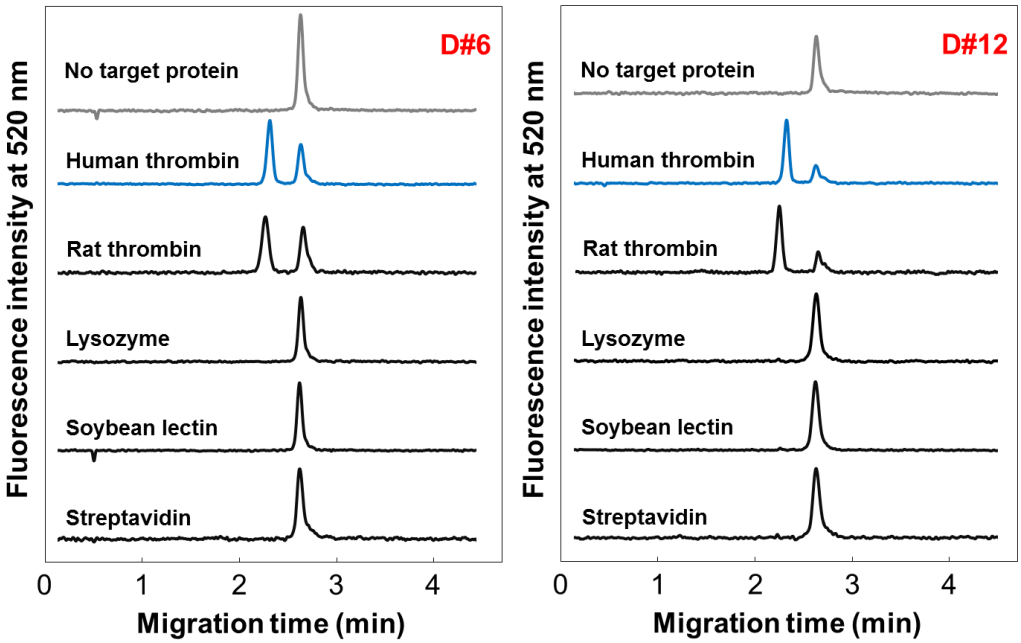


Figure 4-5. Binding specificity of D#6 (left) and D#12 (right) to protein targets. The aptamer–target complex was observed when human thrombin and rat thrombin were used as targets. The TBA concentration is equivalent to the target (1 nM). The peak of the aptamer-target complex was observed prior to that of the free aptamer in NECEEM.

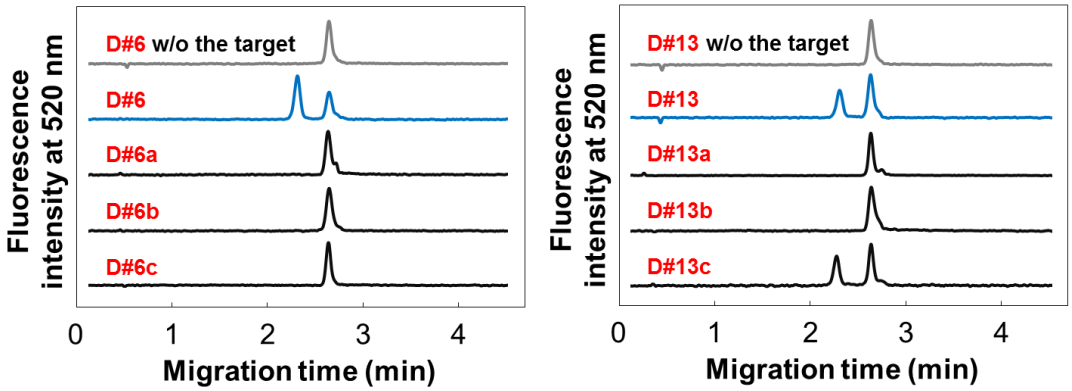


Figure 4-6. Capillary electrograms for D#6 and D#13 without human thrombin, D#6 and D#13, D#6a and D#13a, D#6b and D#13b, and D#6c and D#13c with 1 nM human thrombin (from top to bottom); the TBA concentration is equivalent to the target. Aptamers D#6a and D#13a, D#6b and D#13b and D#6c and D#13c were prepared by complete conversion of the modified nucleotides of D#6 and D#13 in the entire molecule, in all but the primer region, and in only the primer region, respectively, to the corresponding natural nucleotides. The peak of the aptamer–target complex was observed prior to that of the free aptamer in NECEEM.

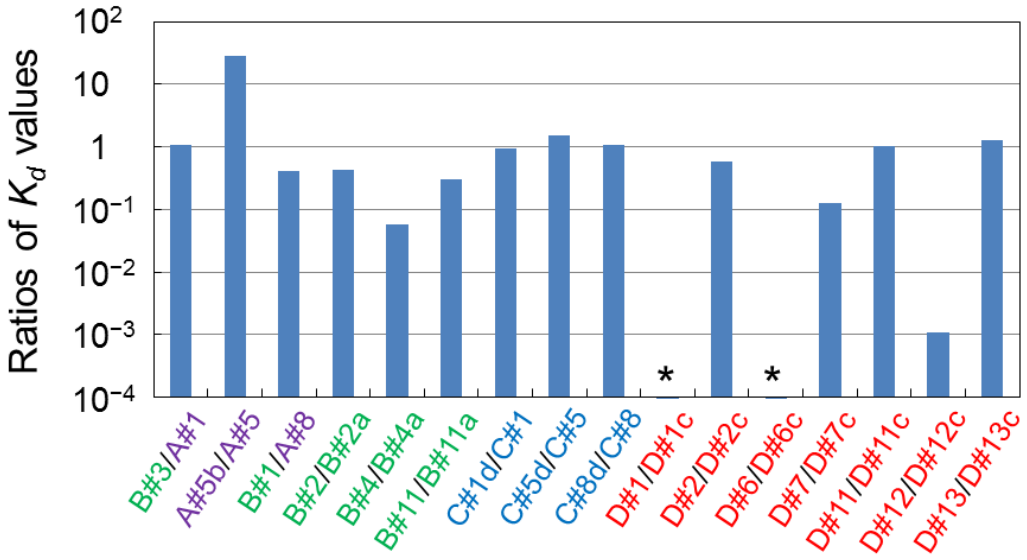


Figure 4-7. Effects of B/L nucleotides present in the primer on the binding activity; ratio of K_d values of 2',4'-BNA/LNA-primer aptamers to those of natural DNA-primer aptamers indicates increased or decreased binding activity when 2',4'-BNA/LNA-primer was replaced with natural DNA primer. Asterisks indicate that the binding activity was abolished by the replacement of primers.

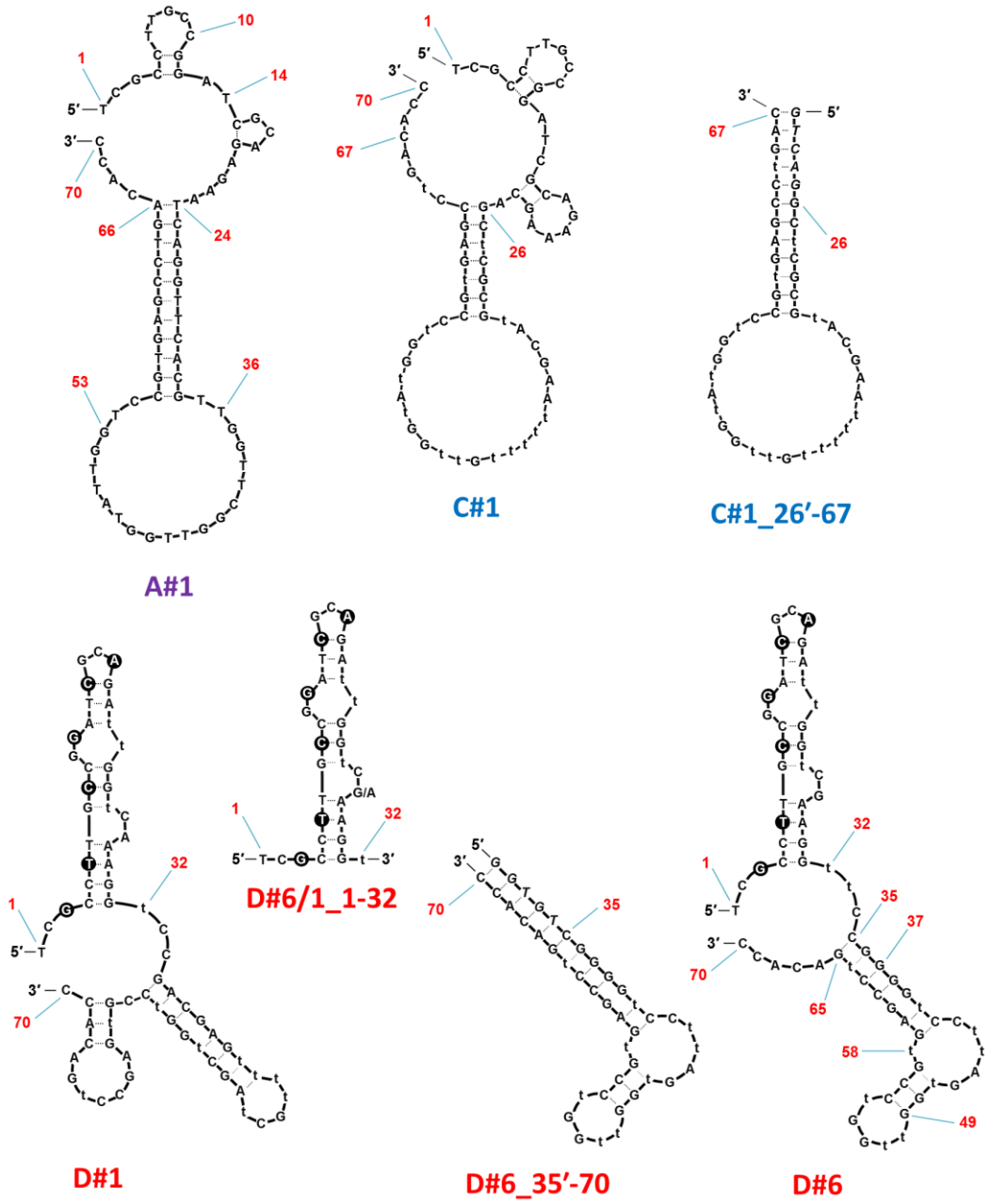


Figure 4-8. Predicted secondary structures of aptamers and fragments using mfold–DNA folding form. The B/L nucleotides, C5-modified thymidine and bases introduced so as to form stem structures are shown in outline characters, letters (**t**) and italic capitals, respectively. The G-quadruplex motif of A#1 TGG TTC GGT TGG TAT TGG T, and the alternative motif of C#1 $\mathbf{t}_5G\mathbf{t}_2G_2$ are involved in the loop domains of each predicted structure.

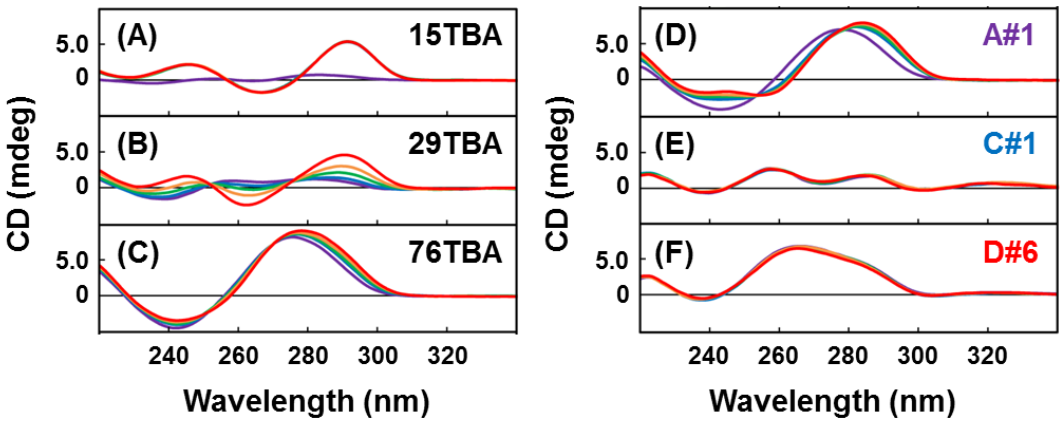


Figure 4-9. CD spectra for (A) 15 TBA, (B) 29 TBA, (C) 76 TBA, (D) A#1, (E) C#1, and (F) D#6 in 20 mM Tris-HCl buffer, pH 7.2, and with different K^+ concentrations: 0 mM (purple), 5 mM (blue), 10 mM (green), 20 mM (orange), and 50 mM (red).

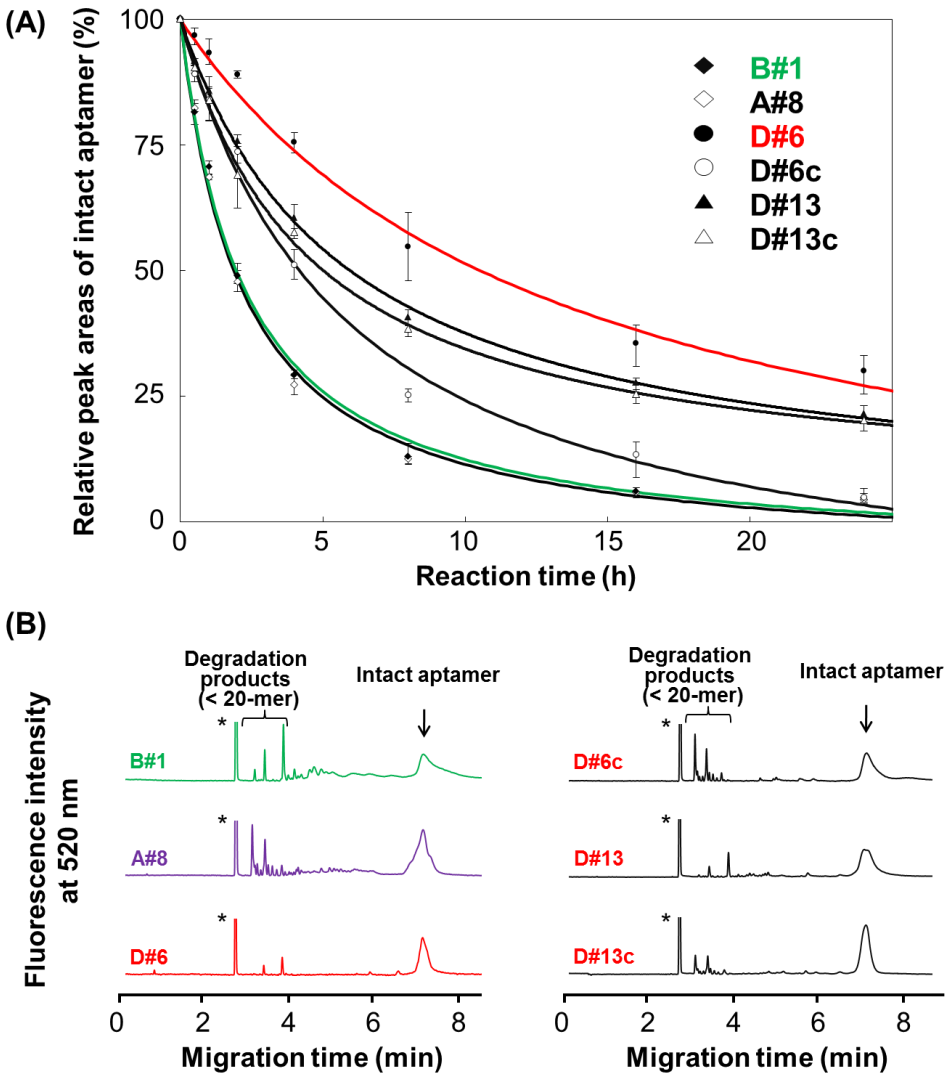


Figure 4-10. Biostability of TBAs. (A) Degradation of TBAs over time in 80% human serum at 37°C. (B) Representative capillary electrograms for reaction mixtures after 2 h-incubation; asterisks indicate the internal standard (fluorescein). All samples were resolved by CE with denaturing 7M urea ssDNA 100-R Gel, and degradation products were migrated in the order of increasing length prior to intact aptamers.

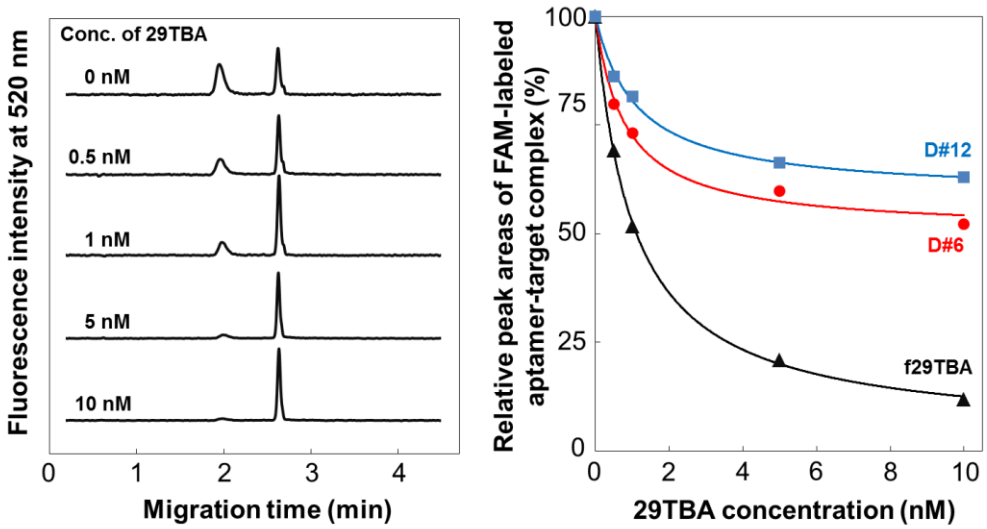


Figure 4-11. Competitive binding assays with 29TBA as a competitor. (A) Representative capillary electrograms of the assays using f29TBA (1 nM) and human thrombin (1 nM) in addition to 0–10-fold equivalents of 29TBA (0, 0.5, 1, 5 and 10 nM). The peak of the aptamer-target complex was observed prior to that of the free aptamer in NECEEM. (B) Effects of 29TBA on the target-binding ability of D#6 (closed circles), D#12 (open circles) and f29TBA (closed triangles). Ratio of the peak area of the aptamer–target complex to the total peak area in the absence of 29TBA was set at 100%.

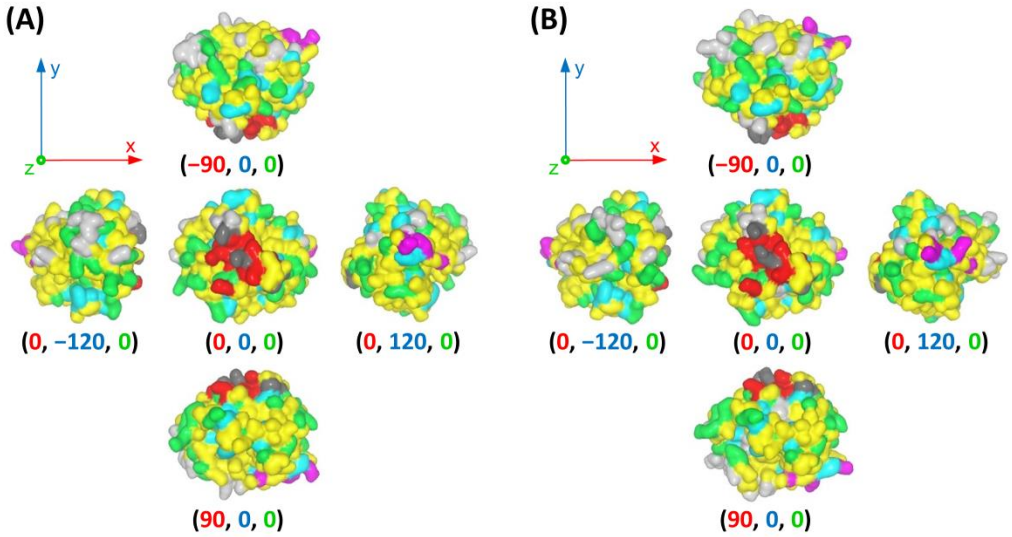


Figure 4-12. Steric structures of human thrombin (A) and rat thrombin (B) obtained from 2C8Y and 3HK3 in Protein Data Bank, respectively. Non-consensus residues are shown in grey, while those in exosite 2 are shown in black. Consensus residues in the exosites 1 and 2 are shown in pink and red, and those of cationic (His, Lys, Arg), aromatic (Phe, Trp, Tyr) and the other amino acids are shown in green, blue and yellow, respectively. Numbers in parentheses indicate rotation angles around the x, y and z axes. Display convention states that the x-axis increases from left to right, y-axis increases from bottom to top and z-axis increases from back to front pointing at viewer as shown. A positive rotation is defined as anti-clockwise for the coordinate system when viewed with the axis of rotation pointing at viewer as shown. For example, a rotation from the x-axis to the y-axis about the z-axis is positive. Note that the object will rotate clockwise when the coordinate system rotates anti-clockwise.

Table 4-1. Sequences and affinities of representative TBAs recovered from libraries A–D

Aptamer[a]	Sequence[b]	K_d (nM)[c]
A#1,2,3,4,6	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	3.1
A#5,14	TCGCCTTGCCGGATCGCAGATTGTCAGGTGGCTTCGTGGTTCGGTTGGTGTGGTCCGTGAGCCTGACACC	1.9
A#8,9,11,12,13,16	TCGCCTTGCCGGATCGCAGATCGTGGCAGGATCCGTTGGTTTTGGTTGGGTGGTCCGTGAGCCTGACACC	7.9
B#1,8	TCGCCTTGCCGGATCGCAGATCGTGGCAGGATCCGTTGGTTTTGGTTGGGTGGTCCGTGAGCCTGACACC	3.7
B#2,15	TCGCCTTGCCGGATCGCAGAGGGCACTTGGCTGGTTGGTGGGTTTTGCCCTGGTCCGTGAGCCTGACACC	5.0
B#3,5,6,10,12,14	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	3.3
B#4	TCGCCTTGCCGGATCGCAGAGTGTGGTGGGTTGGCTCTGGGTGACCTCTGTGGTCCGTGAGCCTGACACC	1.9
B#11	TCGCCTTGCCGGATCGCAGAAGTGGCCGGCTCCGCGTGGGGGGTTGGGTTTGGTCCGTGAGCCTGACACC	2.1
C#1	TCGCCTTGCCGGATCGCAGAAAGCAGC**t**CGCG**t**ACGAA**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	0.57
C#2,11	TCGCCTTGCCGGATCGCAGAG**t**GGGGA**t**C**t**C**t**G**ttt**A**t**CCAC**tt**CAG**t**GC**t**GG**t**CCG**t**GAGCC**t**GACACC	2.6
C#4	TCGCCTTGCCGGATCGCAGAGGGAGGCACGCGA**t**GCGAG**tttA**CCC**t**ACG**t**GG**t**CCG**t**GAGCC**t**GACACC	2.9
C#5,14,20	TCGCCTTGCCGGATCGCAGA**tt**CGGGGGCGC**t**CCCC**t**CA**t**G**tttA**CCC**t**AG**t**GG**t**CCG**t**GAGCC**t**GACACC	10
C#7	TCGCCTTGCCGGATCGCAGAGGGAGGGCAGCGGCAGA**tt**GGCGG**tt**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	34
C#8	TCGCCTTGCCGGATCGCAGAC**tt**G**tt**A**t**ACGG**t**C**t**C**tt**GGCCCGG**tt**GGC**t**GG**t**CCG**t**GAGCC**t**GACACC	4.3
C#9	TCGCCTTGCCGGATCGCAGA**t**GGCCCCCCG**ttt**GCGG**tttttt**CG**t**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	7.5
C#10	TCGCCTTGCCGGATCGCAGA**tt**C**t**AGGGAAAGGC**t**CGC**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	1.7
D#1,10	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CAAAGG**t**CCGACGAG**tttt**GC**t**AGC**t**GG**t**CCG**t**GAGCC**t**GACACC	2.3
D#2,3	TCGCCTTGCCGGATCGCAGAGCCG**t**GAGGACGCGC**tt**G**tt**GGGGGG**tt**G**tt**GG**t**CCG**t**GAGCC**t**GACACC	4.0
D#6,9	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGGGG**t**CC**tt**AG**t**GG**tt**GG**t**CCG**t**GAGCC**t**GACACC	0.26
D#7	TCGCCTTGCCGGATCGCAGA**t**GCGGAG**t**GGCCAA**t**CAC**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	0.79
D#11	TCGCCTTGCCGGATCGCAGAACGGGCG**t**GGC**tt**CA**t**AA**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	0.50
D#12	TCGCCTTGCCGGATCGCAGA**tt**AG**t**CGAAGG**tt**CC**t**GGCCAC**tt**A**t**A**t**CG**t**GG**t**CCG**t**GAGCC**t**GACACC	0.093
D#13	TCGCCTTGCCGGATCGCAGACGGCCGAGG**t**GGCCAACG**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	0.91

[a]The name of each aptamer indicates the type of library used and the clone number. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in squares (A, G, C, and T). The C5-modified thymidine is shown in bold letters (**t**). Regions of potential G-quadruplex are marked with gray boxes. Identified sequence motifs $\mathbf{t}_2G_2\mathbf{t}C(A/G)A_2G_2\mathbf{t}$, $\mathbf{t}_2AG\mathbf{t}CGA_2G_2\mathbf{t}$, $\mathbf{t}_5G\mathbf{t}_2G_2$, and $G\mathbf{t}_3AC_3\mathbf{t}$ are marked as blue, green, yellow, and orange boxes, respectively. [c]K_d values were determined by non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured.

Table 4-2. Sequences and affinities of TBAs recovered from library A

Aptamer[a]	Sequence[b]	K_d (nM)[c]	G (%)[d]
A#1,2,3,4,6	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	3.1	33
A#5,14	TCGCCTTGCCGGATCGCAGATTGTCAGGTGGCTTCGTGGTTCGGTTGGTGTGGTCCGTGAGCCTGACACC	1.9	43
A#7,10,15	TCGCCTTGCCGGATCGCAGACAGCCGGCTCACGTTGGCACGGTTGGTTATTGGTCCGTGAGCCTGACACC	6.9	33
A#8,9,11,12,13,16	TCGCCTTGCCGGATCGCAGATCGTGGCAGGATCCGTTGGTTTTGGTTGGGTGGTCCGTGAGCCTGACACC	7.9	43
A#17	TCGCCTTGCCGGATCGCAGACAGGCTCTGTGTGGATTTAGGGTTGGTAGTTGGTCCGTGAGCCTGACACC	n.d.	40
A#18	TCGCCTTGCCGGATCGCAGAAGTGGCTGGGGTTTGGTGGGGGGTTGGGTATGGTCCGTGAGCCTGACACC	n.d.	60
A#19	TCGCCTTGCCGGATCGCAGAACGAATGGCGCACGTTGGTTGTGGTTGGATTGGTCCGTGAGCCTGACACC	n.d.	40
A#20	TCGCCTTGCCGGATCGCAGATTGTCAGGTGGCTTCGTGGTTCGGTTGGTCTGGTCCGTGAGCCTGACACC	n.d.	40
A#1c	TCGCCTTGCCGGATCGCAGAGAA**t**CAGG**tt**CACG**tt**GG**tt**CGG**tt**GG**t**Att**GGt**CCG**t**GAGCC**t**GACACC	NB	n/a
A#5b	TCGCCTTGCCGGATCGCAGATTGTCAGGTGGCTTCGTGGTTCGGTTGGTGTGGTCCGTGAGCCTGACACC	24	n/a
A#5c	TCGCCTTGCCGGATCGCAGA**tt**G**t**CAGG**t**GGC**tt**CG**t**GG**tt**CGG**tt**GG**t**G**t**GG**t**CCG**t**GAGCC**t**GACACC	NB	n/a

[a]The name of each aptamer indicates the type of library used and the clone number. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in square (A, G, C and T). The C5-modified thymidine is shown in bold letters (**t**). Regions of potential G-quadruplex are marked with gray boxes. [c]K_d values were determined by non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. NB, no binding; n.d., not determined. [d]G-content (%) in the random region. n/a, not applicable.

Table 4-3. Sequences and affinities of TBAs recovered from library B

Aptamer[a]	Sequence[b]	K_d (nM)[c]	G (%)[d]
B#1,8	TCGCCTTGCCGGATCGCAGATCGTGGCAGGATCCGTTGGTTTTGGTTGGGTGGTCCGTGAGCCTGACACC	3.7	43
B#2,15	TCGCCTTGCCGGATCGCAGAGGGCACTTGGCTGGTTGGTGGGTTTTGCCCTGGTCCGTGAGCCTGACACC	5.0	43
B#3,5,6,10,12,14	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	3.3	33
B#4	TCGCCTTGCCGGATCGCAGAGTGTGGTGGGTTGGCTCTGGGTGACCTCTGTGGTCCGTGAGCCTGACACC	1.9	47
B#7,9,13	TCGCCTTGCCGGATCGCAGAACGTGGCAGGATCCGTTGGTTTTGGTTGGGTGGTCCGTGAGCCTGACACC	2.1	43
B#11	TCGCCTTGCCGGATCGCAGAAGTGGCCGGCTCCGCGTGGGGGGTTGGGTTTGGTCCGTGAGCCTGACACC	2.1	53
B#16	TCGCCTTGCCGGATCGCAGAGTGTGGGGGGTTGGCTCTGGGTGACCTCTGTGGTCCGTGAGCCTGACACC	n.d.	50
B#17	TCGCCTTGCCGGATCGCAGAATGTCAGGCGGCTTCGTGGTTCGGTTGGTGTGGTCCGTGAGCCTGACACC	n.d.	43
B#18	TCGCCTTGCCGGATCGCAGAATGTCAGGTGGCATCGTGGTTCGGTTGGTCTGGTCCGTGAGCCTGACACC	n.d.	40
B#19	TCGCCTTGCCGGATCGCAGAATGTCAGGTGGCTTCGTGGTTAGGTTGGTGTGGTCCGTGAGCCTGACACC	n.d.	43
B#20	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTGGGTTGGTATTGGTCCGTGAGCCTGACACC	n.d.	37
B#2a	TCGCCTTGCCGGATCGCAGAGGGCACTTGGCTGGTTGGTGGGTTTTGCCCTGGTCCGTGAGCCTGACACC	10	n/a
B#4a	TCGCCTTGCCGGATCGCAGAGTGTGGTGGGTTGGCTCTGGGTGACCTCTGTGGTCCGTGAGCCTGACACC	23	n/a
B#11a	TCGCCTTGCCGGATCGCAGAAGTGGCCGGCTCCGCGTGGGGGGTTGGGTTTGGTCCGTGAGCCTGACACC	5.9	n/a

[a]The name of each aptamer indicates the type of library used and the clone number. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in square (A, G, C and T). Regions of potential G-quadruplex are marked with gray boxes. [c]K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. n.d., not determined. [d]G-content (%) in the random region. n/a, not applicable.

Table 4-4. Sequences and affinities of TBAs recovered from library C

Aptamer[a]	Sequence[b]	K_d (nM)[c]	G (%)[d]
C#1	TCGCCTTGCCGGATCGCAGAAAGCAGC**t**CGCG**t**ACGAA**ttttt**G**tt**GG**t**AT**GG**t**CCG**t**GAGCC**t**GACACC	0.57	27
C#2,11	TCGCCTTGCCGGATCGCAGA**G**TGGGGA**t**C**t**C**t**G**ttt**A**t**CCAC**tt**CAG**t**GC**t**GG**t**CCG**t**GAGCC**t**GACACC	2.6	27
C#3,6	TCGCCTTGCCGGATCGCAGA**t**GCGGGGGCCA**t**CGCGCG**ttt**ACCC**t**A**t**GC**t**GG**t**CCG**t**GAGCC**t**GACACC	3.0	33
C#4	TCGCCTTGCCGGATCGCAGAGGGAGGCACGCGA**t**GCGAG**tttt**ACCC**t**ACG**t**GG**t**CCG**t**GAGCC**t**GACACC	2.9	37
C#5,14,20	TCGCCTTGCCGGATCGCAGA**tt**CGGGGGCGC**t**CCCC**t**CA**t**G**tttt**ACCC**t**AG**t**GG**t**CCG**t**GAGCC**t**GACACC	10	26
C#7	TCGCCTTGCCGGATCGCAGAGGGAGGGCAGCGGCAGA**tt**GGCGG**tt**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	34	53
C#8	TCGCCTTGCCGGATCGCAGAC**tt**G**tt**A**t**ACGG**t**C**t**C**tt**GGCCCGG**tt**GGC**t**GG**t**CCG**t**GAGCC**t**GACACC	4.3	30
C#9	TCGCCTTGCCGGATCGCAGA**t**GGCCCCCCG**ttt**GCGG**tttttt**CG**t**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	7.5	30
C#10	TCGCCTTGCCGGATCGCAGA**tt**C**t**AGGGAAAGGC**t**CGC**ttttt**G**tt**GG**t**AT**GG**t**CCG**t**GAGCC**t**GACACC	1.7	30
C#12	TCGCCTTGCCGGATCGCAGA**t**GCGGGGGCCA**t**CGCG**t**G**tttt**ACCC**t**A**t**GC**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	33
C#13	TCGCCTTGCCGGATCGCAGA**t**GGAGGCACGCGA**t**GCGAG**tttt**ACCC**t**ACG**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	33
C#15	TCGCCTTGCCGGATCGCAGA**ttt**AGGCGGCAAG**tt**CA**t**C**t**GCGG**tt**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	33
C#16	TCGCCTTGCCGGATCGCAGA**tt**CGGG**t**GGCA**tt**GGCCCG**tttt**ACCC**t**CGC**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	30
C#17	TCGCCTTGCCGGATCGCAGAAACGGC**t**ACC**t**GGC**t**C**t**A**t**AG**t**A**t**G**t**AGGC**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	27
C#18	TCGCCTTGCCGGATCGCAGA**tt**GCC**t**C**t**A**tt**CAGGGC**t**AAG**tt**GG**t**AGG**tt**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	30
C#19	TCGCCTTGCCGGATCGCAGAC**t**GGCACGCA**t**G**tt**GACG**t**G**tttt**ACCC**t**AG**t**GG**t**CCG**t**GAGCC**t**GACACC	n.d.	27
C#1a	TCGCCTTGCCGGATCGCAGAAAGCAGCTCGCGTACGAATTTTTGTTGGTATGGTCCGTGAGCCTGACACC	NB	n/a
C#1d	TCGCCTTGCCGGATCGCAGAAAGCAGC**t**CGCG**t**ACGAA**ttttt**G**tt**GG**t**AT**GG**t**CCG**t**GAGCC**t**GACACC	0.54	n/a
C#2a	TCGCCTTGCCGGATCGCAGAGTGGGGATCTCTGTTTATCCACTTCAGTGCTGGTCCGTGAGCCTGACACC	NB	n/a
C#4a	TCGCCTTGCCGGATCGCAGAGGGAGGCACGCGATGCGAGTTTACCCTACGTGGTCCGTGAGCCTGACACC	NB	n/a
C#5d	TCGCCTTGCCGGATCGCAGA**tt**CGGGGGCGC**t**CCCC**t**CA**t**G**tttt**ACCC**t**AG**t**GG**t**CCG**t**GAGCC**t**GACACC	14	n/a
C#7a	TCGCCTTGCCGGATCGCAGAGGGAGGGCAGCGGCAGATTGGCGGTTAGGCTGGTCCGTGAGCCTGACACC	NB	n/a
C#8a	TCGCCTTGCCGGATCGCAGACTTGTTATACGGTCTCTTGGCCCGGTTGGCTGGTCCGTGAGCCTGACACC	NB	n/a
C#8d	TCGCCTTGCCGGATCGCAGAC**tt**G**tt**A**t**ACGG**t**C**t**C**tt**GGCCCGG**tt**GGC**t**GG**t**CCG**t**GAGCC**t**GACACC	4.6	n/a
C#9a	TCGCCTTGCCGGATCGCAGATGGCCCCCCGTTTGCGGTTTTTTCGTAGGCTGGTCCGTGAGCCTGACACC	NB	n/a

[a]The name of each aptamer indicates the type of library used and the clone number. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in square (A, G, C and T). The C5-modified thymidine is shown in bold letters (**t**). Regions of potential G-quadruplex are marked with gray boxes. Identified sequence motifs $\mathbf{t}_5G\mathbf{t}_2G_2$ and $G\mathbf{t}_3AC_3\mathbf{t}$ are marked as yellow and orange boxes, respectively. [c]K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. NB, no binding; n.d., not determined. [d]G-content (%) in the random region. n/a, not applicable.

Table 4-5. Sequences and affinities of TBAs recovered from library D

Aptamer[a]	Sequence[b]	K_d (nM)[c]	G (%)[d]
D#1,10	TCGCCTTGCCGGATCGCAGATTGGTCAAAGGTCCGACGAGTTTTGCTAGCTGGTCCGTGAGCCTGACACC	2.3	30
D#2,3	TCGCCTTGCCGGATCGCAGAGCCGTGAGGACGCGCTTGTTGGGGGGTTGTTGGTCCGTGAGCCTGACACC	4.0	50
D#4	TCGCCTTGCCGGATCGCAGAGCTGTGAGTCCGATGTTCTATGTAGGTTGATGGTCCGTGAGCCTGACACC	2.8	33
D#5	TCGCCTTGCCGGATCGCAGATGAAGGCAAACGTCCCCACTGTTGTTTTGCTGGTCCGTGAGCCTGACACC	7.0	23
D#6,9	TCGCCTTGCCGGATCGCAGATTGGTCGAAGGTTCCGGGGTCCTTAGTGGTTGGTCCGTGAGCCTGACACC	0.26	40
D#7	TCGCCTTGCCGGATCGCAGATGCGGAGTGGCCAATCACTTTTTGTTGGCATGGTCCGTGAGCCTGACACC	0.79	30
D#8	TCGCCTTGCCGGATCGCAGATTCGCGCAGTCCCAACCTCGTTCGTTTGTATGGTCCGTGAGCCTGACACC	15	20
D#11	TCGCCTTGCCGGATCGCAGAACGGGCGTGGCTTCATAATTTTTGTTGGTATGGTCCGTGAGCCTGACACC	0.50	30
D#12	TCGCCTTGCCGGATCGCAGATTAGTCGAAGGTTCCTGGCCACTTATATCGTGGTCCGTGAGCCTGACACC	0.093	23
D#13	TCGCCTTGCCGGATCGCAGACGGCCGAGGTGGCCAACGTTTTTGTTGGTATGGTCCGTGAGCCTGACACC	0.91	37
D#14	TCGCCTTGCCGGATCGCAGATGGCAGTACCACCTAAGTTTCATTATGCGTTGGTCCGTGAGCCTGACACC	n.d.	20
D#15	TCGCCTTGCCGGATCGCAGATTGACTCTGCCGTCTTGGCAACAGTTCACCTGGTCCGTGAGCCTGACACC	n.d.	20
D#16	TCGCCTTGCCGGATCGCAGATGCGGACAGGCCGTCGCATGTTGTTTTGAGTGGTCCGTGAGCCTGACACC	n.d.	37
D#17	TCGCCTTGCCGGATCGCAGATTGCACGCGGCTCTATTCGTATTCTTAGGCTGGTCCGTGAGCCTGACACC	n.d.	23
D#18	TCGCCTTGCCGGATCGCAGATGTGGCCTATAGGCAAAACGCGGCGTTGACTGGTCCGTGAGCCTGACACC	n.d.	33
D#19	TCGCCTTGCCGGATCGCAGATTCTGGAGTACATGTTGGTTCCCCGTGCACTGGTCCGTGAGCCTGACACC	n.d.	27
D#20	TCGCCTTGCCGGATCGCAGATTGGTCGAAGGTTCCGGGGTCTTTAGTGGTTGGTCCGTGAGCCTGACACC	n.d.	40
D#1a	TCGCCTTGCCGGATCGCAGATTGGTCAAAGGTCCGACGAGTTTTGCTAGCTGGTCCGTGAGCCTGACACC	NB	n/a
D#1b	TCGCCTTGCCGGATCGCAGATTGGTCAAAGGTCCGACGAGTTTTGCTAGCTGGTCCGTGAGCCTGACACC	NB	n/a
D#1c	TCGCCTTGCCGGATCGCAGATTGGTCAAAGGTCCGACGAGTTTTGCTAGCTGGTCCGTGAGCCTGACACC	NB	n/a
D#2a	TCGCCTTGCCGGATCGCAGAGCCGTGAGGACGCGCTTGTTGGGGGGTTGTTGGTCCGTGAGCCTGACACC	NB	n/a
D#2b	TCGCCTTGCCGGATCGCAGAGCCGTGAGGACGCGCTTGTTGGGGGGTTGTTGGTCCGTGAGCCTGACACC	NB	n/a
D#2c	TCGCCTTGCCGGATCGCAGAGCCGTGAGGACGCGCTTGTTGGGGGGTTGTTGGTCCGTGAGCCTGACACC	6.2	n/a
D#6a	TCGCCTTGCCGGATCGCAGATTGGTCGAAGGTTCCGGGGTCCTTAGTGGTTGGTCCGTGAGCCTGACACC	NB	n/a
D#6b	TCGCCTTGCCGGATCGCAGATTGGTCGAAGGTTCCGGGGTCCTTAGTGGTTGGTCCGTGAGCCTGACACC	NB	n/a
D#6c	TCGCCTTGCCGGATCGCAGATTGGTCGAAGGTTCCGGGGTCCTTAGTGGTTGGTCCGTGAGCCTGACACC	NB	n/a
D#7a	TCGCCTTGCCGGATCGCAGATGCGGAGTGGCCAATCACTTTTTGTTGGCATGGTCCGTGAGCCTGACACC	NB	n/a
D#7b	TCGCCTTGCCGGATCGCAGATGCGGAGTGGCCAATCACTTTTTGTTGGCATGGTCCGTGAGCCTGACACC	NB	n/a
D#7c	TCGCCTTGCCGGATCGCAGATGCGGAGTGGCCAATCACTTTTTGTTGGCATGGTCCGTGAGCCTGACACC	5.2	n/a
D#11a	TCGCCTTGCCGGATCGCAGAACGGGCGTGGCTTCATAATTTTTGTTGGTATGGTCCGTGAGCCTGACACC	NB	n/a
D#11b	TCGCCTTGCCGGATCGCAGAACGGGCGTGGCTTCATAATTTTTGTTGGTATGGTCCGTGAGCCTGACACC	NB	n/a
D#11c	TCGCCTTGCCGGATCGCAGAACGGGCGTGGCTTCATAATTTTTGTTGGTATGGTCCGTGAGCCTGACACC	0.48	n/a
D#12a	TCGCCTTGCCGGATCGCAGATTAGTCGAAGGTTCCTGGCCACTTATATCGTGGTCCGTGAGCCTGACACC	NB	n/a
D#12b	TCGCCTTGCCGGATCGCAGATTAGTCGAAGGTTCCTGGCCACTTATATCGTGGTCCGTGAGCCTGACACC	NB	n/a
D#12c	TCGCCTTGCCGGATCGCAGATTAGTCGAAGGTTCCTGGCCACTTATATCGTGGTCCGTGAGCCTGACACC	87	n/a
D#13a	TCGCCTTGCCGGATCGCAGACGGCCGAGGTGGCCAACGTTTTTGTTGGTATGGTCCGTGAGCCTGACACC	NB	n/a
D#13b	TCGCCTTGCCGGATCGCAGACGGCCGAGGTGGCCAACGTTTTTGTTGGTATGGTCCGTGAGCCTGACACC	NB	n/a
D#13c	TCGCCTTGCCGGATCGCAGACGGCCGAGGTGGCCAACGTTTTTGTTGGTATGGTCCGTGAGCCTGACACC	0.73	n/a

[a]The name of each aptamer indicates the type of library used and the clone number. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in square (A, G, C and T). The C5-modified thymidine is shown in bold letters (**t**). Identified sequence motifs $\mathbf{t}_2G_2\mathbf{t}C(A/G)A_2G_2\mathbf{t}$, $\mathbf{t}_2AG\mathbf{t}CGA_2G_2\mathbf{t}$ and $\mathbf{t}_5G\mathbf{t}_2G_2$ are

marked as blue, green and yellow boxes, respectively. [c]K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. NB, no binding; n.d., not determined. [d]G-content (%) in the random region. n/a, not applicable.

Table 4-6. Sequences and affinities of A#1, C#1, D#6 and their fragments.

Aptamer[a]	Sequence[b]	K_d (nM)[c]
A#1	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	3.1
A#1_1-66	TCGCCTTGCCGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGA	3.2
A#1_10-70	CGGATCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	4.3
A#1_14-70	TCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	10
A#1_14-66	TCGCAGAGAATCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGA	NB
A#1_24-70	TCAGGTTCACGTTGGTTCGGTTGGTATTGGTCCGTGAGCCTGACACC	NB
A#1_1-23	TCGCCTTGCCGGATCGCAGAGAA	NB
A#1_36-53	TGGTTCGGTTGGTATTGG	NB
C#1	TCGCCTTGCCGGATCGCAGAAAGCAGC**t**CGCG**t**ACGAA**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GACACC	0.57
C#1_26′-67	*GTCAGGC***t**CGCG**t**ACGAA**ttttt**G**tt**GG**t**A**t**GG**t**CCG**t**GAGCC**t**GAC	NB
D#6	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGGGG**t**CC**tt**AG**t**GG**tt**GG**t**CCG**t**GAGCC**t**GACACC	0.26
D#6_1-65	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGGGG**t**CC**tt**AG**t**GG**tt**GG**t**CCG**t**GAGCC**t**G	0.35
D#6_1-58	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGGGG**t**CC**tt**AG**t**GG**tt**GG**t**CCG**t**	0.82
D#6_1-49	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGGGG**t**CC**tt**AG**t**GG	9.8
D#6_1-37	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**tt**CCGG	NB
D#6_1-32	TCGCCTTGCCGGATCGCAGA**tt**GG**t**CGAAGG**t**	NB
D#6_35′-70	*GGTGT*CGGGG**t**CC**tt**AG**t**GG**tt**GG**t**CCG**t**GAGCC**t**GACACC	NB

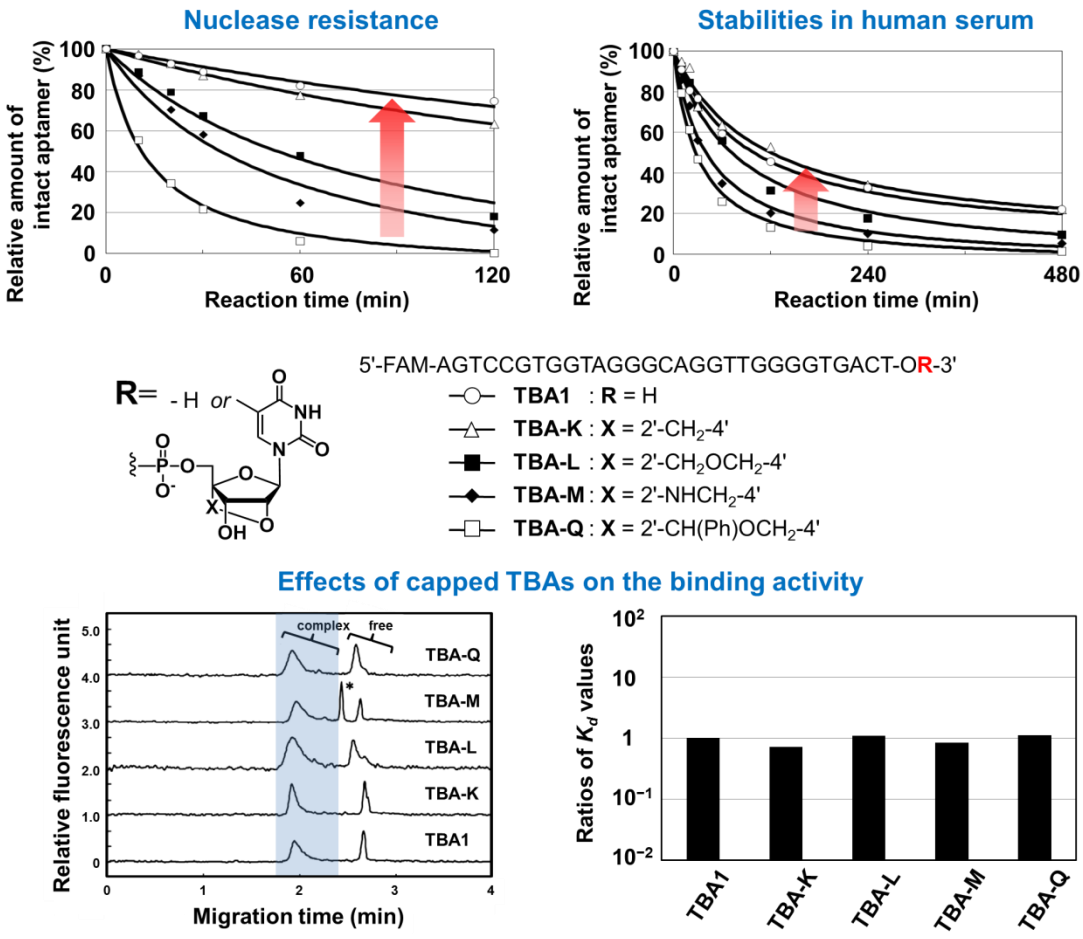
[a]The name of each aptamer indicates the type of library used, clone number followed by an underscore and numbers of base positions cut out. [b]Sequences are aligned in the 5' to 3' direction. Underlined regions are derived from the primer or primer-binding regions. The B/L nucleotides are enclosed in square (A, G, C and T). The C5-modified thymidine and bases introduced so as to form stem structures are shown in bold letters (**t**) and italic capitals, respectively. Regions of potential G-quadruplex are marked with gray boxes. Identified sequence motifs $\mathbf{t}_2G_2\mathbf{t}CGA_2G_2\mathbf{t}$ and $\mathbf{t}_5G\mathbf{t}_2G_2$ are marked as blue and yellow boxes, respectively. [c]K_d values were determined by NECEEM. Precision of determination of K_d values (% relative standard deviation) was <10% for all analytes measured. NB, no binding.

Chapter 5

Concluding Remarks

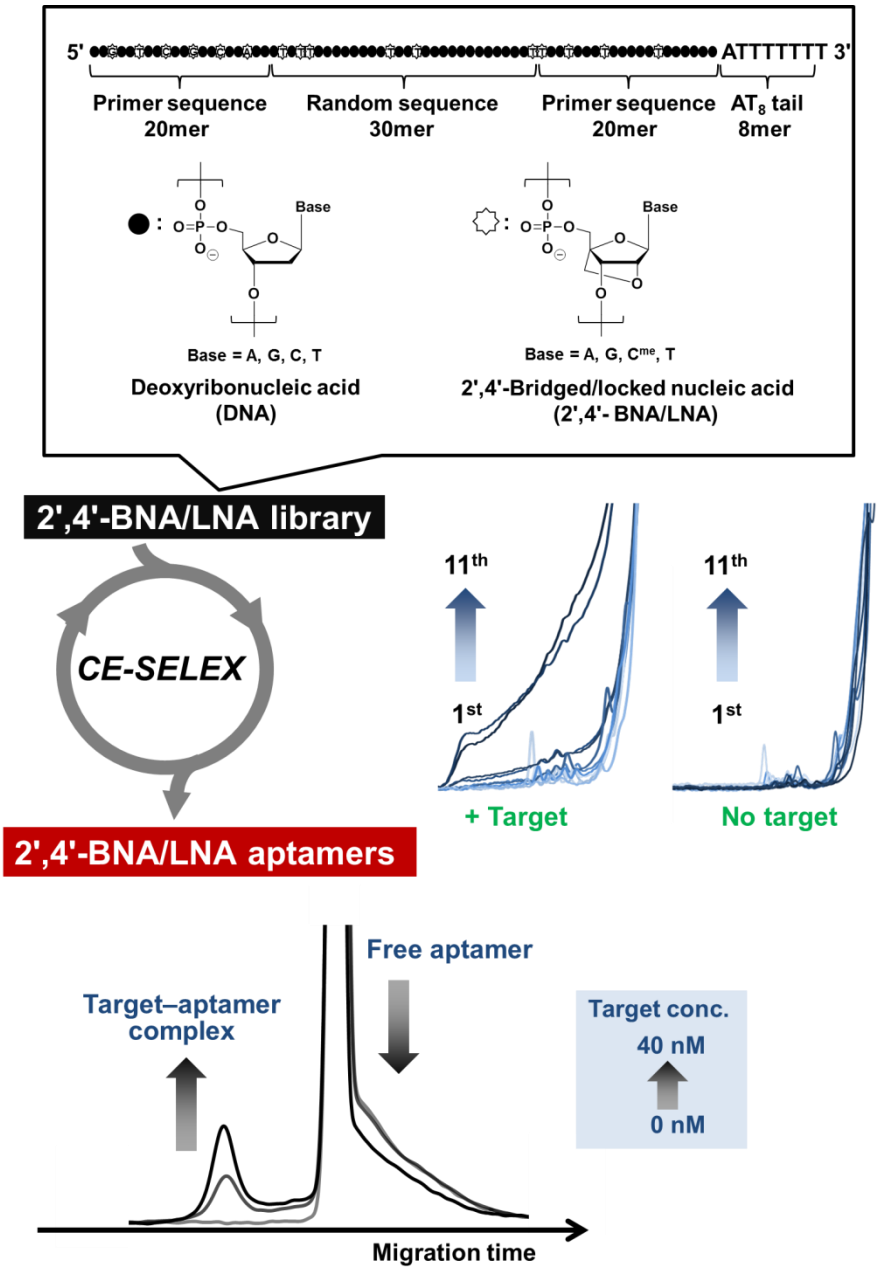
- **I successfully demonstrated enhancements of the nuclease resistances and the stabilities in human serum by capping the 3'-ends of TBAs with bridged nucleotides.**

  The binding abilities of the aptamers were not affected by the capping. The capping could be simply executed *via* a one-step enzymatic process using 2',4'-bridged nucleoside 5'-triphosphate and terminal deoxynucleotidyl transferase (Chapter 2).



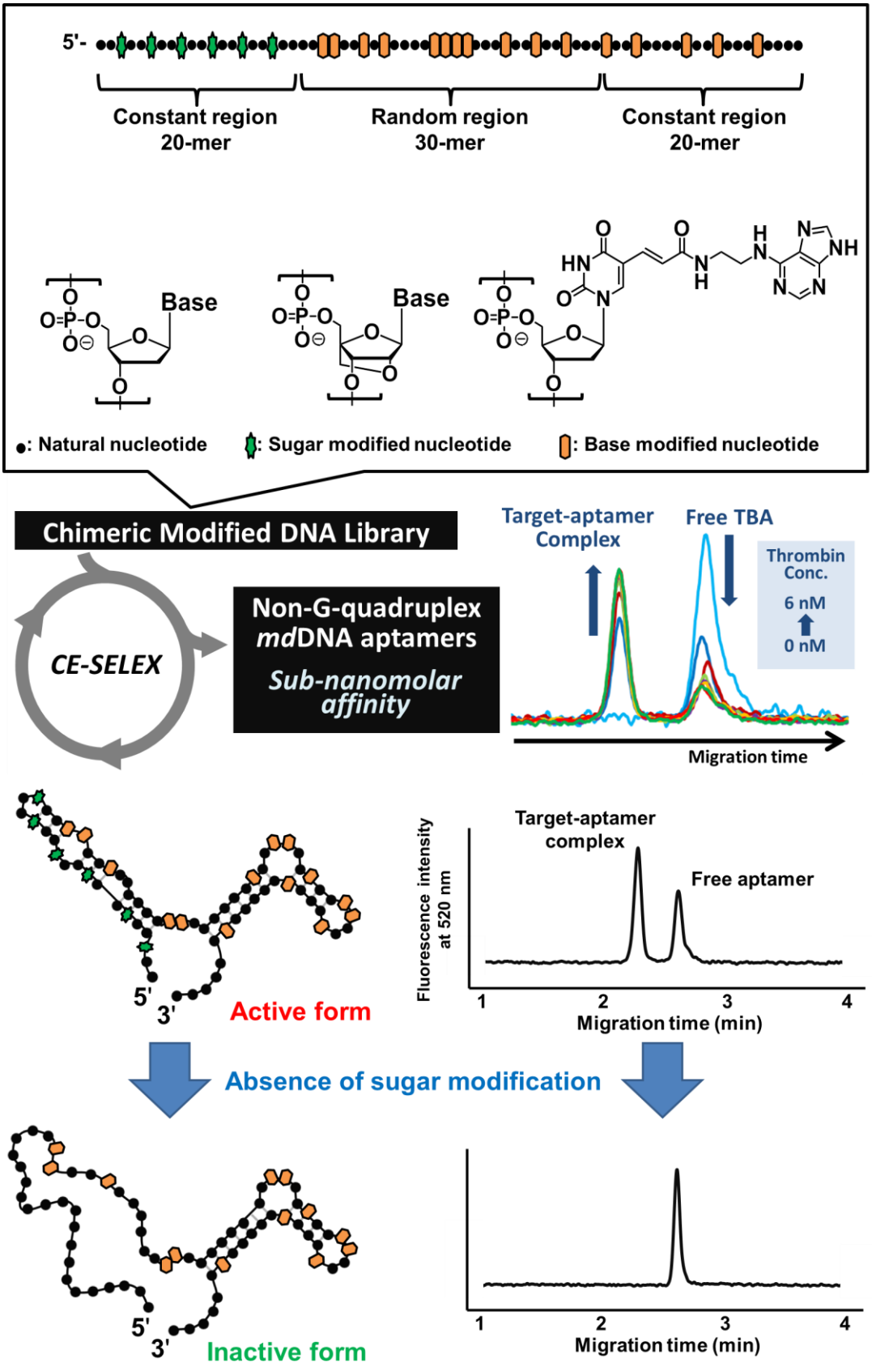
- **I successfully obtained DNA-based aptamers that contain B/L nucleotides over the entire length using a CE-SELEX method.**

  A modified DNA library was prepared with an enzyme mix of *KOD* Dash and *KOD* mutant DNA polymerases. Forty 2',4'-BNA/LNA aptamers were isolated from an enriched pool and classified into six groups according to their sequence. 2',4'-BNA/LNA aptamers of groups V and VI bound human thrombin with K_d values in the range of several 10 nanomolar levels (Chapter 3).



- **I successfully obtained high affinity chemically modified DNA aptamers for human α-thrombin from ODN libraries by using a CE–SELEX method.**

  The libraries contained B/L nucleotides in the primer region and/or C5-modified thymidine bearing N^6-ethyladenine (**t**) in the nonprimer region. Modified DNA aptamers showed high binding affinities to the target, with K_d values in the range of subnanomolar to several ten nanomolar levels. The introduction of base modification significantly suppressed the frequency of G-quadruplex motifs, which are often seen in thrombin-binding DNA aptamers. The resulting alternatives contained the 10-mer consensus sequence $\mathbf{t}_5G\mathbf{t}_2G_2$, which is frequently found in modified DNA aptamers with subnanomolar protein binding affinities. Furthermore, some base- and sugar-modified DNA aptamers with the 12-mer consensus sequence $\mathbf{t}_2G_2\mathbf{t}C(A/G)A_2G_2\mathbf{t}$ displayed binding activities that were dependent on the presence of B/L nucleotides in the primer region. Such aptamers were interestingly not recovered from a natural DNA library or from DNA libraries modified with either B/L nucleotides or **t**'s. This emerging characteristic binding property will enable the creation of a direct selection methodology for DNA-based molecular switches that are triggered by chemical conversion of B/L nucleotides introduced to constant sequence regions in ODN libraries (Chapter 4).


5'-
Constant region
20-mer
Random region
30-mer
Constant region
20-mer
Base
Base
●: Natural nucleotide
: Sugar modified nucleotide
: Base modified nucleotide
Chimeric Modified DNA Library
CE-SELEX
Non-G-quadruplex
mdDNA aptamers
Sub-nanomolar
affinity
Target-aptamer
Complex
Free TBA
Thrombin
Conc.
6 nM
0 nM
Migration time
5'
3'
Active form
Target-aptamer
complex
Free aptamer
Fluorescence intensity
at 520 nm
1
2
3
4
Migration time (min)
Absence of sugar modification
5'
3'
Inactive form
1
2
3
4
Migration time (min)

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Publication List

Related Publications

Chapter 2

Kasahara Y, Kitadume S, Morihiro K, Kuwahara M, Ozaki H, Sawai H, Imanishi T, Obika S. Effect of 3'-end capping of aptamer with various 2',4'-bridged nucleotides: Enzymatic post-modification toward a practical use of polyclonal aptamers. *Bioorg Med Chem Lett.* 2010; 20(5): 1626–1629.

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Chapter 4

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Other Publications*

(1) Fujita H, Imaizumi Y, **Kasahara Y**, Kitadume S, Ozaki H, Kuwahara M, Sugimoto N. Structural and Affinity Analyses of G-Quadruplex DNA Aptamers for Camptothecin Derivatives. *Pharmaceuticals (Basel)*. 2013; 6(9): 1082–1093.

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**Publications to which the author has contributed but which are not related to the doctoral thesis.*

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