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High-Affinity DNA Aptamer Generation Targeting von Willebrand Factor A1-Domain by Genetic Alphabet Expansion for Systematic Evolution of Ligands by Exponential Enrichment Using Two Types of Libraries Composed of Five Different Bases

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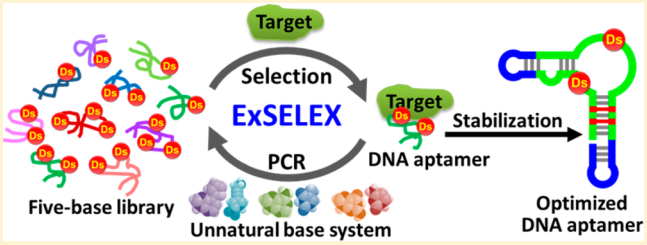
Supporting Information

ABSTRACT: The novel evolutionary engineering method ExSELEX (genetic alphabet expansion for systematic evolution of ligands by exponential enrichment) provides high-affinity DNA aptamers that specifically bind to target molecules, by introducing an artificial hydrophobic base analogue as a fifth component into DNA aptamers. Here, we present a newer version of ExSELEX, using a library with completely randomized sequences consisting of five components: four natural bases and one unnatural hydrophobic base, 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds). In contrast to the limited number of Ds-containing sequence combinations in our previous library, the increased complexity of the new randomized library could improve the success rates of high-affinity aptamer generation. To this end, we developed a sequencing method for each clone in the enriched library after several rounds of selection. Using the improved library, we generated a Ds-containing DNA aptamer targeting von Willebrand factor A1-domain (vWF) with significantly higher affinity (K_D = 75 pM), relative to those generated by the initial version of ExSELEX, as well as that of the known DNA aptamer consisting of only the natural bases. In addition, the Ds-containing DNA aptamer was stabilized by introducing a mini-hairpin DNA resistant to nucleases, without any loss of affinity (K_D = 61 pM). This new version is expected to consistently produce high-affinity DNA aptamers.



INTRODUCTION

DNA aptamers that bind to target molecules and cells are expected to function as an alternative to protein-based antibodies with several advantages: their systematic generation *in vitro*, high-purity, large-scale preparation, easy modification by chemical synthesis, high target specificity, and low immunogenicity in the body.[1−12] DNA aptamers are initially generated by an *in vitro* evolutionary engineering method, systematic evolution of ligands by exponential enrichment (SELEX),[13,14] using a DNA library with randomized base sequences, and the production of numerous conventional and modified DNA aptamers has been reported.[1−12,15−19] However, no DNA aptamer has been approved as a therapeutic yet, although several DNA aptamers are under evaluation in clinical trials. Only one modified RNA aptamer, pegaptanib (Macugen), targeting $VEGF_{165}$, has been used for the treatment of neovascular age-related macular degeneration.[20−22]

One of the major issues is the insufficient affinities of DNA aptamers to the targets for pharmaceutical purposes. This problem mainly arises from the fact that DNA molecules consist of only the four standard nucleotide components of A, G, C, and T, with similar chemical structures and physical properties. In contrast, proteins comprise 20 different amino acids with a variety of chemical and physical properties. Thus, the increased chemical diversity has the potential to improve both the affinity and specificity, by allowing the formation of aptamers with higher complexity than that obtained with the standard nucleobases. To this end, expansion of the genetic alphabet could address this intrinsic problem of DNA, by introducing artificial extra components as a third base pair with fifth and sixth bases.[23−30]

By developing genetic alphabet expansion, we have succeeded in generating high-affinity DNA aptamers by genetic alphabet expansion for SELEX (ExSELEX).[26] In the ExSELEX procedure, the highly hydrophobic unnatural base 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds) is introduced into DNA libraries, which can be accurately amplified by PCR involving the unnatural base pair between Ds and its pairing partner, 2-nitro-4-propynylpyrrole (Px) (Figure 1).[31−33] The Ds−Px pair exhibits high fidelity as a third base pair in PCR amplification and thus can be utilized in the SELEX procedure, in which PCR

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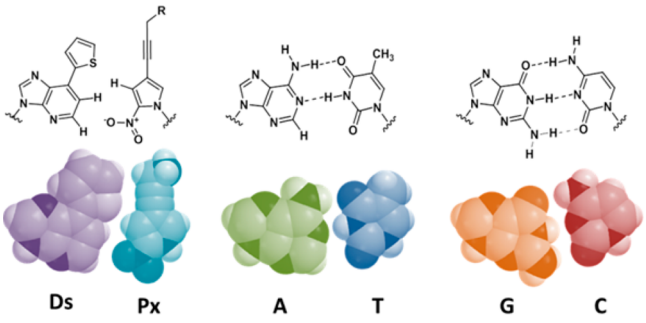

Figure 1. Chemical structures of the unnatural Ds–Px and natural A–T and G–C pairs.

is essential to amplify the isolated DNA library during the selection rounds. By using ExSELEX, we previously generated high-affinity Ds-containing DNA aptamers targeting $VEGF_{165}$ (K_D = ~1 pM) and interferon-γ (K_D = ~40 pM).[26] Only a few hydrophobic Ds bases significantly increased the aptamer affinities, which were much higher than those of the conventional DNA aptamers consisting of the natural bases.

The key issue of ExSELEX is the determination of the sequence of each Ds-containing clone in the enriched library, after the selection process. We previously addressed this issue by using a Ds-containing DNA library (Ds-predetermined library) comprising a limited number (~20) of sublibraries, in which one, two, or three Ds bases are embedded at predetermined positions in the natural-base randomized region (refer to Figure 2).[26] Each sublibrary included a unique barcode consisting of two or three natural bases to identify the Ds positions in each aptamer sequence obtained by next-generation sequencing, in which the Ds bases in the enriched library are replaced with natural bases by replacement PCR. The problem with this method is the low success rates of aptamer generation, because the limited number of sublibraries restricts the combinations of Ds positions in the library, resulting in lower complexity.

To expand the sequence space (complexity) of the library, we present a new version of ExSELEX using a DNA library with a completely randomized sequence including the Ds bases (Ds-randomized library). Each clone's sequence in the enriched library was determined by the following process: (1) IonPGM sequencing of the enriched library amplified by replacement PCR, (2) probe hybridization to isolate each clone from the Ds-containing enriched library, and (3) dye-terminator DNA sequencing involving the unnatural bases for each isolated clone[32,34] (refer to Figure 6).

Using two types of libraries, Ds-predetermined and Ds-randomized libraries, we performed ExSELEX targeting von Willebrand factor A1-domain (vWF). vWF is a multimeric glycoprotein that functions in platelet adhesion and aggregation, by interacting with glycoprotein Ib receptors on platelets.[35] Anti-vWF DNA and RNA aptamers, such as a natural-base DNA aptamer (ARC1172) and a 2′-OMe RNA/DNA aptamer (ARC1779), were previously developed as inhibitors of platelet activation and thrombosis.[36−42] By ExSELEX using the Ds-randomized library, we generated a Ds-containing anti-vWF DNA aptamer with higher affinity (K_D = 75 pM), relative to those of the ARC1172 DNA aptamer (K_D = 326 pM) and another Ds-containing DNA aptamer (K_D = 1.03 nM), obtained by the initial version of ExSELEX using the Ds-predetermined library. Furthermore, the Ds-containing anti-vWF and ARC1172 aptamers were stabilized by introducing

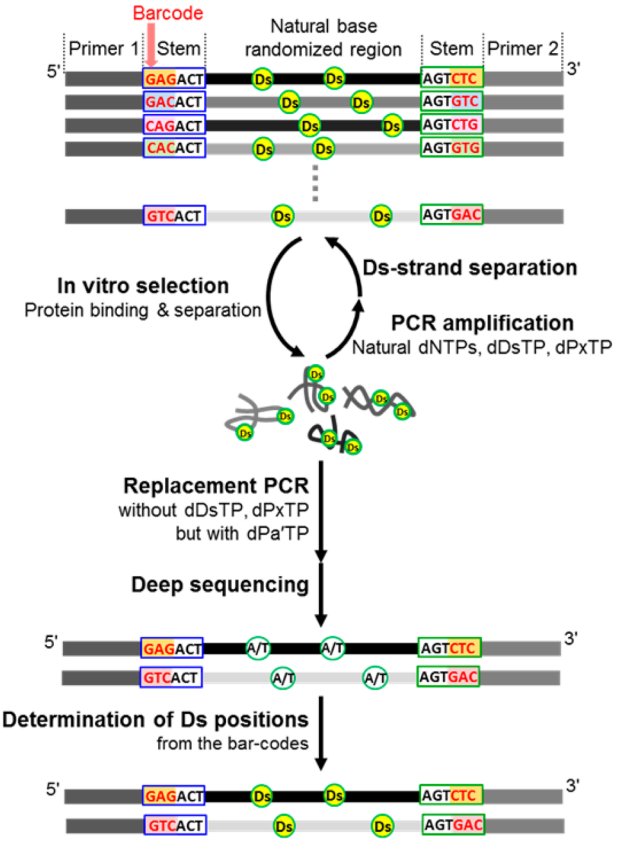

Figure 2. Scheme for the initial version of ExSELEX using the Ds-predetermined DNA library. The Ds-predetermined DNA library consists of a mixture of sublibraries, in which two Ds bases are embedded at predefined positions within the natural base randomized region. The Ds positions can be identified from the unique barcode in each sublibrary.

extraordinarily stable mini-hairpin DNA structures[43−46] without any loss of affinity (K_D = 61 pM and 128 pM, respectively). Notably, around 75% of the optimized Ds-containing DNA aptamer survived in human serum at 37 °C for 72 h.

RESULTS AND DISCUSSION

Initial ExSELEX Method Using the Ds-Predetermined Library. We first performed the previous ExSELEX method[26,47] with 28 newly synthesized sublibraries, containing two Ds bases at different defined positions in each 26-nucleotide (26-nt) region randomized with four natural base components (N26Ds2-01 to N26Ds2-28) (Figure 2 and SI, Table S1). In addition, we introduced 6-nt complementary sequences on both sides of the random region to form a stem structure, since all of the unnatural-base DNA aptamers that we have obtained so far form stem structures at both termini.[26] Furthermore, the 3-nt sequences in the stem were varied in each sublibrary, to serve as a barcode to define each Ds position in the sequencing process. The chemically synthesized sublibraries were purified by polyacrylamide-gel electrophoresis and mixed for use as the initial Ds-predetermined library.

ExSELEX was initiated with 1.8×10^{14} molecules with different sequences in the library (300 pmol). The conditions of each round are summarized in the SI, Table S2. We employed two methods for selection. In Method a, the protein–DNA complexes were treated with an activated biotin reagent, and the biotinylated complexes were captured with

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streptavidin beads to isolate the DNA fragments that bound to the target protein.[26] In Method b, the protein−DNA complexes were isolated from the free DNAs by a gel mobility shift assay. By combining these two methods, we performed eight rounds of selection with various DNA and protein concentrations. During each round, PCR was performed in the presence of dDsTP and dPxTP (diol derivative), using AccuPrime Pfx DNA polymerase (pol).[32] The isolated library was finally amplified by PCR (replacement PCR) in the absence of dDsTP and dPxTP, but in the presence of dPa′TP,[26,34] to facilitate the replacement of the unnatural bases with natural bases, in which the Ds bases in the library were finally replaced with A or T. We obtained 420 526 sequences. The sequences that appeared in >100 clones are listed in the SI, Table S3, and more than 90% of the sequences converged on a single family. From the barcodes in the sequences, we assigned two Ds base positions (positions 13 and 22) (SI, Table S3). However, besides these two positions in the main family, other positions, such as 12, 25, and 26, also showed some characteristic base variations (many A and T variants), which might originally correspond to the Ds base position. Thus, further examinations were performed using a sequencing method to confirm the Ds positions in the main family.

The sequencing method was actually developed for the new version of ExSELEX using a Ds-randomized library, as mentioned later. Based on the sequencing data obtained with IonPGM after replacement PCR, we designed a short DNA probe (5′-biotinylated 25-mer), in which T was chosen as the complementary base opposite Ds, to isolate the clone containing Ds bases from the enriched library (Figure 3 and SI, Table S3). The isolated clone was amplified by PCR in the presence of dDsTP and dPxTP for the following sequencing. Dye-terminator sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with a gel sequencer (ABI 377).[32,33] First, we used the Ds-containing strand of the amplified clone for sequencing in the presence or absence of dPa′TP or ddPa′TP (Pa′ = 4-propynylpyrrole-2-carbaldehyde). However, the cycle sequencing reaction using the Taq DNA pol mutant paused at the first Ds position (SI, Figure S1). Thus, we performed the cycle sequencing using the complementary strand, namely, the Px-containing strand, in the presence or absence of dDsTP or ddDsTP. We found that when using the Taq pol mutant provided in the sequencing kit, natural base substrates (mainly dATP) were misincorporated opposite Px, but the dideoxy-dye-terminators of the natural bases were not incorporated at the positions. Thus, in the absence of the unnatural base substrates, the sequencing reaction of Px-containing DNA proceeded by the incorporation of the natural base substrate opposite Px, and the unnatural base positions were recognized as a gap. Interestingly, the sequencing peak pattern in the absence of dDsTP was clearer than that in the presence of dDsTP (SI, Figure S1). By comparing the sequencing peak pattern with that of the clone after replacement PCR, three unnatural base positions (13, 22, and 26) were identified (Figure 3).

The two Ds bases at positions 13 and 22 were initially embedded into the sublibrary (N26Ds2-01, SI, Table S1), and the other one at position 26 might have been misincorporated into the DNA during PCR in the selection rounds. To examine the contributions of each Ds base in the aptamer to the binding, we chemically synthesized the aptamer (Pr-DsDsDs-40, 40-mer) and its variants, in which the Ds bases were replaced with A in various combinations (Figure 4 and SI, Table S4). Gel-

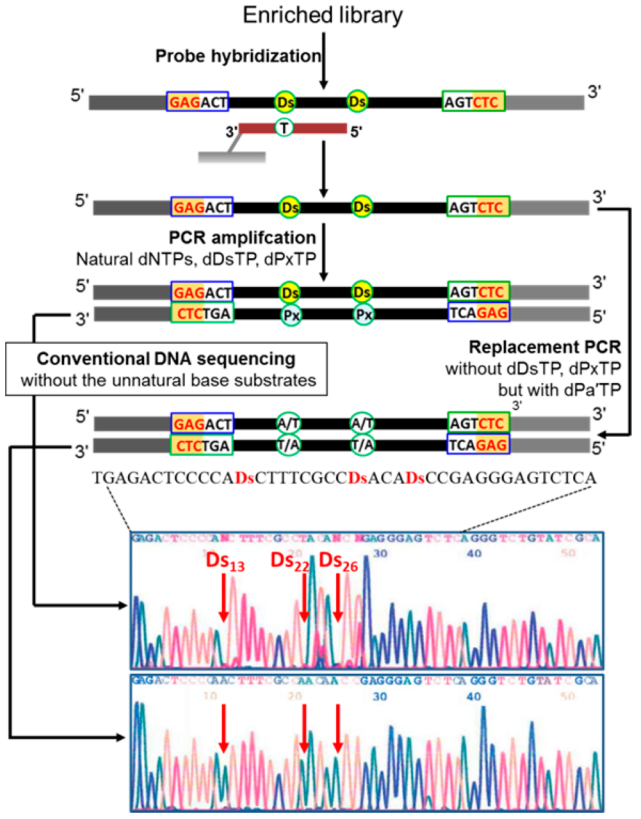

Figure 3. Identification of the Ds-base positions in the enriched DNA library in the initial version of ExSELEX. Through hybridization with a biotinylated probe, a specific clone was captured and subjected to PCR-amplification in either the presence of dDsTP and dPxTP or the absence of dDsTP and dPxTP (but with dPa′TP). The representative sequencing patterns are shown at the bottom and the others are shown in the SI, Figure S1.

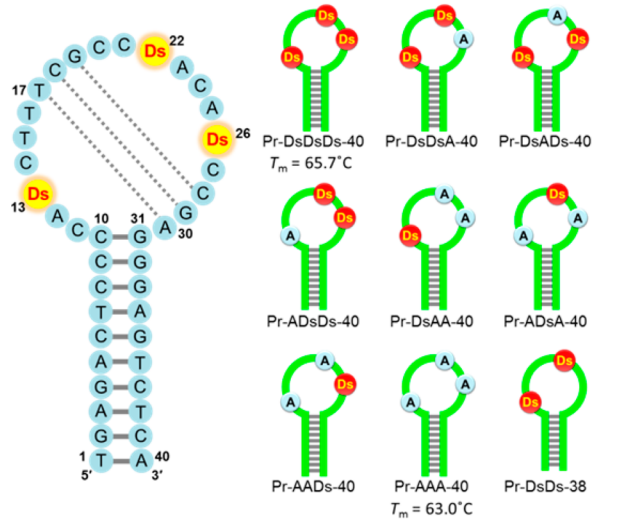

Figure 4. Schematic illustration of the secondary structures of the anti-vWF unnatural-base DNA aptamer, Pr-DsDsDs-40, and its variants. The sequence and presumed secondary structure of Pr-DsDsDs-40 are shown on the left, and each variant is schematically represented on the right, with the thermal stabilities of Pr-DsDsDs-40 and Pr-AAA-40. The possible base pairing within the loop region of Pr-DsDsDs-40 is indicated with dotted lines.

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shift assays of each aptamer or variant complex with vWF were performed by native-gel electrophoresis at 4 and 25 °C (Figure 5). The results revealed that all three of the Ds bases in the

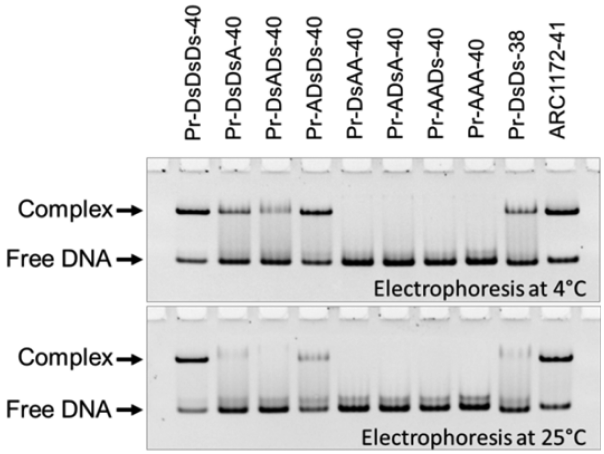

Figure 5. Binding analysis of each Pr-DsDsDs-40 aptamer variant by a gel mobility shift assay. Each aptamer variant (100 nM) was incubated with vWF (100 nM) at 37 °C for 30 min, and the complexes were separated from the free DNA on native 8% polyacrylamide gels with electrophoresis at either 4 °C (upper panel) or 25 °C (lower panel). The DNA bands on the gels were stained with SYBR Gold, and their band patterns were detected with a Bioimaging analyzer (Fuji Film LAS4000).

aptamer, especially the two Ds bases at positions 22 and 26, were essential for the tight binding, and the shifted bands corresponding to the complex of any two Ds → A variants with vWF were not detected on the gel at 25 °C. In this ExSELEX experiment, we serendipitously obtained the aptamer by the mutation of a natural to the Ds base during the selection process. These results suggested that the Ds-predetermined library containing two Ds bases was insufficient for generating high-affinity DNA aptamers, and increasing the complexity by using a Ds-randomized library should be necessary for ExSELEX.

New Version of ExSELEX Using the Randomized Library Containing Ds. We prepared the Ds-randomized library containing a 30-nt randomized region flanked by 6-nt complementary sequences, to include the stem structures, as well as the sequences of PCR primers (Figure 6 and SI, Table S1). The randomized region was synthesized using mixtures of phosphoramidite reagents consisting of Ds (10%) and the natural bases (22.5% each). In the N30 library synthesized using the ratio (10%) of the Ds amidite, 14%, 23%, 24%, and 18% of the DNA fragments would theoretically contain one, two, three, and four Ds bases, respectively. Our previous results indicated that only two or three Ds bases are sufficient to efficiently increase the affinity of DNA aptamers.

We performed seven rounds of selection using six sets (#1–#6) of the library (300 pmol each, total 1.08×10^{15} different molecules) for easier handling, as compared to that of one set of the 1.8 nmol library (Figure 6 and SI, Table S2). During the fourth to sixth rounds in the selection procedure, the known DNA aptamer, ARC1172 (41-mer, SI, Table S3),[39] was added as a competitor to accelerate the enrichment, because the complexity was higher than that obtained using the Ds-predetermined library. For the six sets, each enriched library was tested for binding ability by the gel-shift assay (SI, Figure

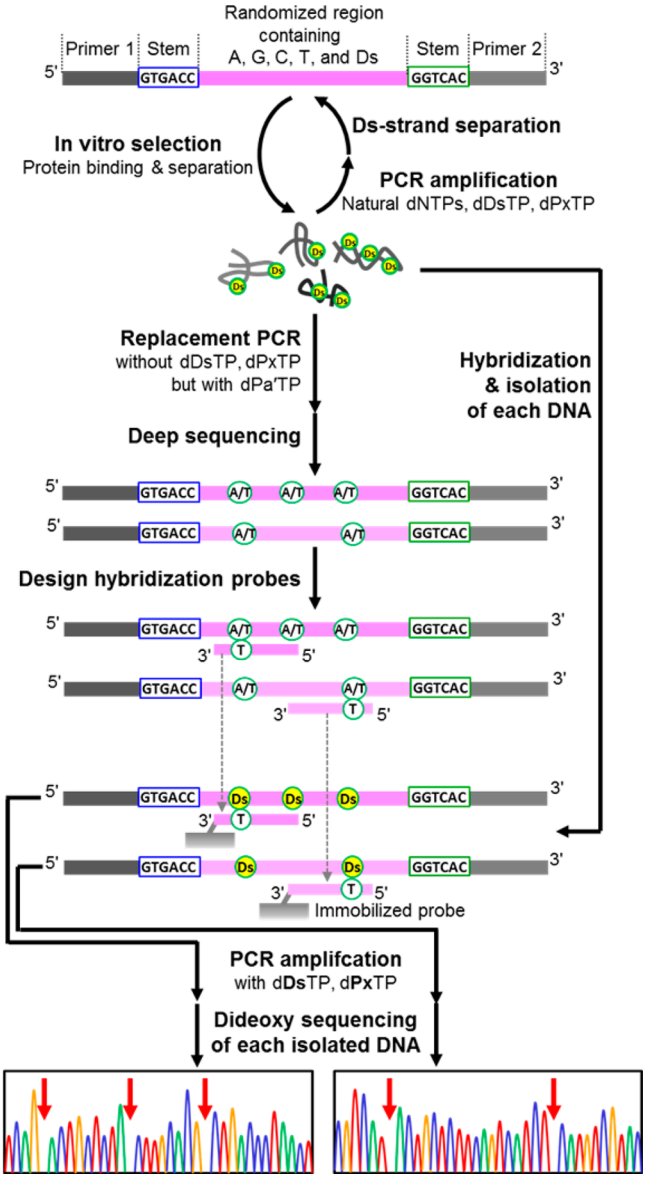

Figure 6. Scheme for a new version of ExSELEX using the Ds-randomized DNA library. After several rounds of selection, the enriched sequences were analyzed by deep sequencing after replacement PCR. The Ds positions in the enriched sequences were then identified through DNA sequencing analysis of PCR-amplified isolated DNA clones, as shown in Figure 3.

S2) under relatively harsh conditions at 37 °C, to select the sets containing the high-affinity DNA aptamers. All of the enriched libraries generated the shifted bands corresponding to the DNA–protein complex, and in two sets (#1 and #4), the shifted bands were quite clearly observed. Thus, we used these two enriched library sets for the following sequence analysis.

After replacement PCR of these two enriched libraries, the total sequences (#1, 151 495; #4, 180 152) were determined by IonPGM (SI, Tables S5 and S6). Theoretically, some percentage of the DNA in the initial library contained no Ds bases, but all of the enriched sequences seemed to have Ds bases. The natural-base DNA aptamers might be excluded by the higher-affinity Ds-containing DNA aptamers during the selection process.

Among the total sequences, more than 83% for #1 and 86% for #4 of the sequences were enriched to each single family, and

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the sequences of the main families of #1 and #4 were both quite similar, in which we predicted three Ds base positions (positions 10, 22, and 33) from the sequence variations. To confirm the sequences and the Ds positions of these families, each DNA fragment was isolated by probe hybridization from the Ds-containing enriched libraries of #1 and #4 (Figure 6), and the sequences were determined by dye-terminator cycle sequencing involving the unnatural base pair system using the complementary Px strands (Figure 7). The Ds bases at positions 22 (Ds in #1 and T in #4) and 33 (Ds in #1 and #4) were confirmed for both #1 and #4. In the selection process with seven rounds, the DNA library was amplified by 157 cycles of PCR in total, and this sequencing confirmed that the Ds bases still survived in the library, suggesting the high selectivity of the Ds−Px pair in replication. Although the Ds base at position 10 could not be confirmed due to disturbed sequencing patterns, the positions in both families should be assigned to Ds from the IonPGM data. The only difference between the two sequences of the main families #1 and #4 was Ds or T at position 22. Since the probability of two sequences existing with only one base difference in a 10^{15}-complexity library is very rare (a probability of $\sim 10^{-5}$%), we cannot exclude the possibility of contamination between the two libraries during the selection process. The predicted secondary structure of sequence #1 (Rn-DsDsDs-44, 44-mer) is shown in Figure 8.

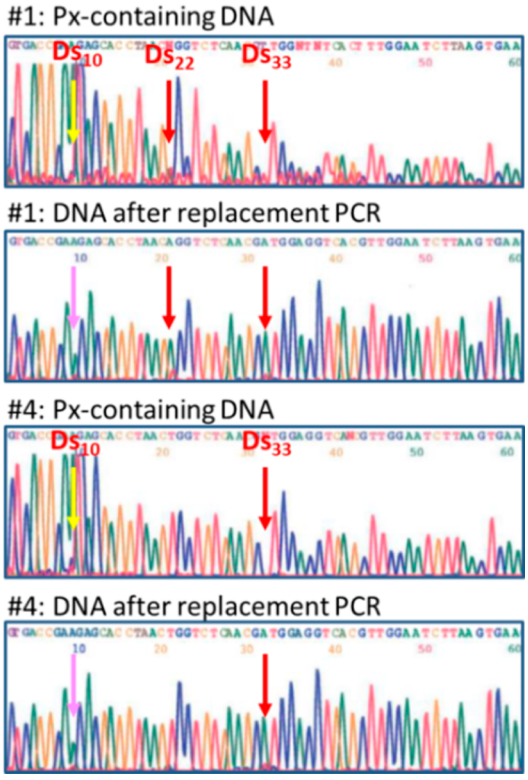

Figure 7. Sequencing analysis of the PCR-amplified products for the clone isolated from the seventh rounds of DNA libraries, set #1 and set #4, in the new version of ExSELEX. The red arrows indicate unnatural-base positions, Ds22 (#1) and Ds33 (#1 and #4), which were estimated from the sequencing peak patterns. The peak patterns around position 10, indicated with the yellow arrows, were disturbed when the Px-containing DNA was used as the sequencing template, but these patterns changed when the DNA obtained after replacement PCR was used as the sequencing template, as indicated with the pink arrows.

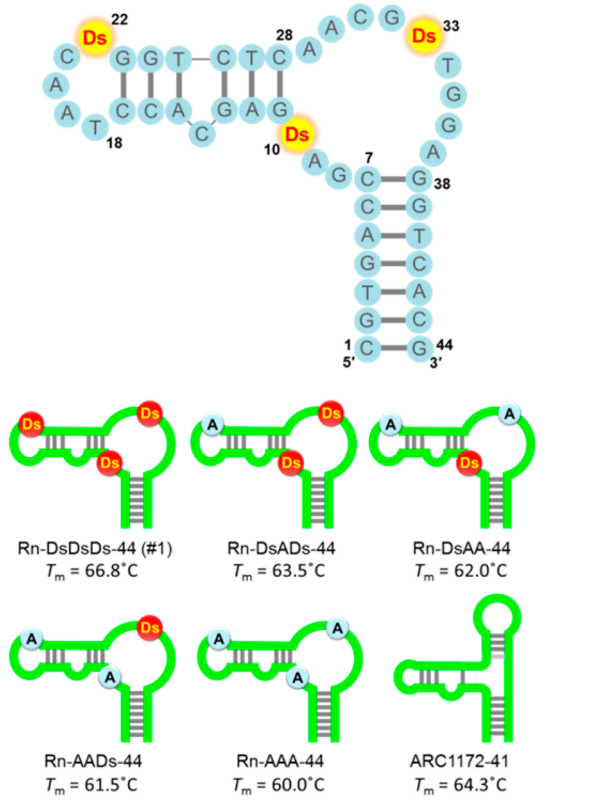

Figure 8. Schematic illustration of the secondary structures of the anti-vWF unnatural-base DNA aptamer, Rn-DsDsDs-44, and its variants. The sequence and presumed secondary structure of Rn-DsDsDs-44 are shown on the top, and each variant is schematically represented on the bottom with its thermal stability.

We chemically synthesized the Rn-DsDsDs-44 versions of #1 and its variants, in which the Ds bases were replaced with A in various combinations (Figure 8 and SI, Table S3). Gel-shift assays of each aptamer complex with vWF were performed by electrophoresis on gels containing 3 M urea at 4, 25, and 37 °C (Figure 9). In the gel-shift assay performed in the presence of 3 M urea, the complex of ARC1172-41 with vWF was not detected on the gel at 25 °C. However, Rn-DsDsDs-44 and Rn-DsADs-44 still bound to vWF even in the presence of 3 M urea at 37 °C, indicating their higher affinity as compared to that of ARC1172-41 and the importance of the two Ds bases at positions 10 and 33 in the aptamer. Interestingly, the presumed secondary structure of Rn-DsDsDs-44 shares some similarities with that of ARC1172. The Ds22 in the loop formed by bases 18−22 might not be necessary for the binding, and X-ray crystallography data of the complex of ARC1172 and vWF indicated that the similar loop region in ARC1172 also lacks a direct interaction with vWF.[39]

Only two Ds bases in Rn-DsDsDs-44 and Rn-DsADs-44 are essential for the tight binding to vWF. Nevertheless, these were not obtained from the Ds-predetermined library with the combinations of two Ds bases. Rn-DsADs-44 contains -N_2-Ds-N_{22}-Ds-N_4- (N = natural base) flanked by the terminal stem regions, but the Ds-predetermined library that we previously made did not contain this sequence pattern. Thus, to prepare

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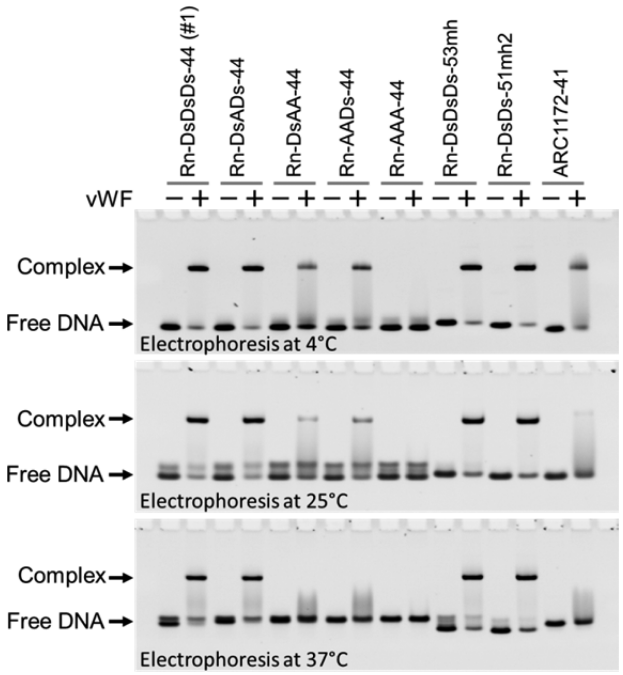

Figure 9. Binding analysis of each Rn-DsDsDs-44 aptamer variant by a gel mobility shift assay. Each aptamer variant (5′-biotinylated, 100 nM) was incubated with vWF (100 nM) at 37 °C for 30 min, and the complexes were separated from the free DNA on 8% polyacrylamide gels containing 3 M urea with electrophoresis at 4 (upper panel), 25 (middle panel), or 37 °C (lower panel). The DNA bands on the gels were stained with SYBR Gold, and their band patterns were detected with a Bioimaging analyzer (Fuji Film LAS4000).

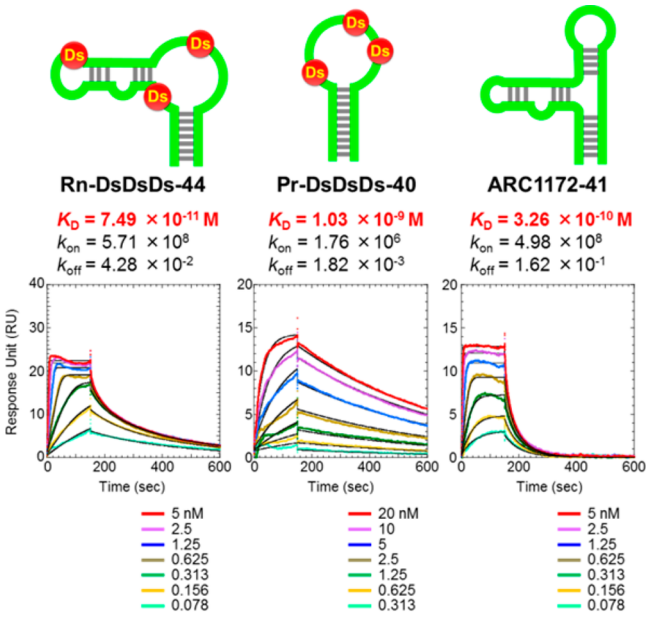

Figure 10. Binding analysis of anti-vWF DNA aptamers by a BIAcore T200 at 37 °C, using 0.078 to 5 nM vWF. The aptamers were biotinylated at their 5′-termini.

an ideal Ds-predetermined library, many more combinations and longer random regions might be required.

Characterization of Anti-vWF DNA Aptamers. The affinities of the aptamers to vWF were determined by surface plasmon resonance (SPR) using the 5′-biotinylated aptamers (Figure 10), in which a biotin-modified T residue was added to the 5′-terminus of each aptamer for immobilization on the sensor chip. Rn-DsDsDs-44 (K_D = 74.9 pM) exhibited the highest affinity, as compared to Pr-DsDsDs-40 (K_D = 1.03 nM) and ARC1172-41 (K_D = 326 pM). Although the K_D value of Pr-DsDsDs-40 was relatively high, its k_{off} value was the smallest among the three aptamers, indicating the slowest off-rate binding to the target. This binding property of Pr-DsDsDs-40 was confirmed by a gel-shift assay in the presence of 3 M urea at different temperatures (4, 25, and 37 °C), in which the complex with Pr-DsDsDs-40 exhibited higher stability than that with ARC1172-41 (Figure 11) on the gel at 25 °C. Among these three aptamers, the complex of Rn-DsDsDs-44 with vWF showed the highest stability, which was clearly observed even on a gel containing 3 M urea at 37 °C.

Since the thermal stabilities of the aptamers are also important for diagnostic and therapeutic applications, we measured the T_m values of the aptamers (SI, Figure S3 and Figures 4 and 8). All three aptamers (T_m = 65.7 °C for Pr-DsDsDs-40, 66.8 °C for Rn-DsDsDs-44, and 64.3 °C for ARC1172-41) exhibited high thermal stability. As we previously observed,[26] the Ds → A replacements of the Ds-containing DNA aptamers reduced their thermal stabilities. The high stacking ability of the hydrophobic Ds base might stabilize each aptamer's tertiary structure.

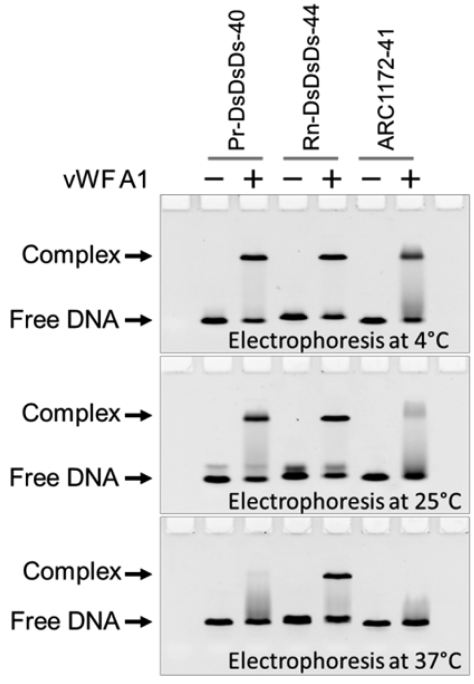

Figure 11. Comparison of the binding affinities of the anti-vWF aptamer, Pr-DsDsDs-40, Rn-DsDsDs-44, and the conventional aptamer ARC1172-41, in gel mobility shift assays. Each aptamer variant (5′-biotinylated, 100 nM) was incubated with vWF (100 nM) at 37 °C for 30 min, and the complexes were separated from the free DNA on 8% polyacrylamide gels containing 3 M urea, with electrophoresis at 4 °C (upper panel), 25 °C (middle panel), or 37 °C (lower panel). The DNA bands on the gels were stained with SYBR Gold, and their band patterns were detected with a Bioimaging analyzer (Fuji Film LAS4000).

Stabilization of Anti-vWF DNA Aptamers by the Introduction of Mini-hairpin DNAs. For pharmaceutical applications, DNA aptamers must be stable at 37 °C and resistant to degradation by nucleases. We previously developed

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a stabilization method by introducing extraordinarily stable mini-hairpin DNA sequences containing a GNA loop (N = A, G, C, or T)[43,44,48] into DNA aptamers.[45,46] Rn-DsDsDs-44 and ARC1172-41 were stabilized by this method (Figure 12). First,

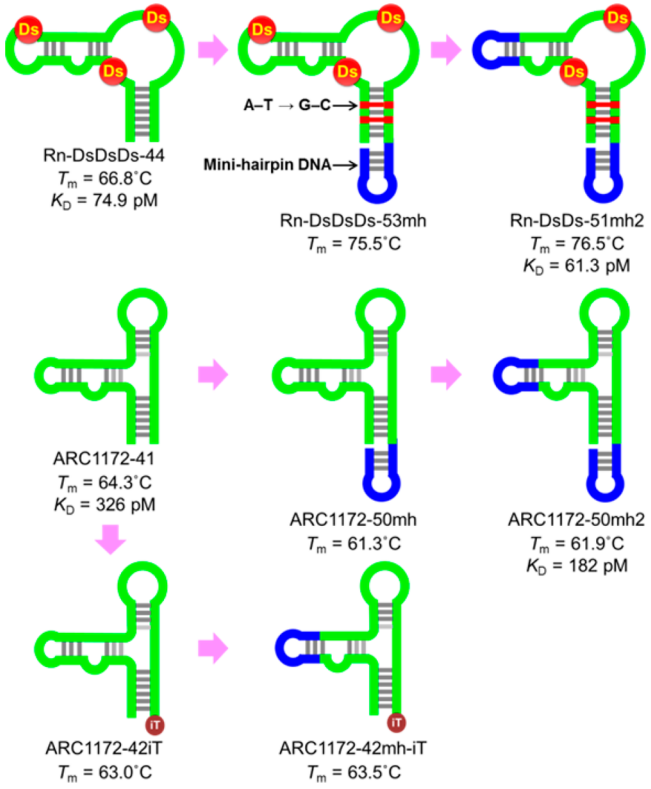

Figure 12. Stabilization of the anti-vWF aptamers, Rn-DsDsDs-44 and ARC1172-41, by introducing mini-hairpin DNAs. The modified regions are indicated by blue lines (mini-hairpin DNA), red lines (A–T replaced with G–C), and brown circles (iT: inverted-dT). Their sequences are shown in SI, Table S4. These variants were characterized by their thermal stabilities (SI, Figures S3 and S4), binding abilities (analyzed with a BIAcore T200, Figures 10 and 13), and nuclease resistances in 96% human serum at 37 °C (Figure 14).

the mini-hairpin DNA sequence was added to the 3′-termini of both aptamers (Rn-DsDsDs-53mh and ARC1172-50mh). In addition, two A–T pairs in the terminal stem region of Rn-DsDsDs-44 were replaced with G–C pairs, to thermally stabilize the stem structure (SI, Table S4). In contrast, the replacement of A–T with G–C pairs in the terminal stem region of ARC1172-41 reduced the affinity, and thus we left the A–T pairs within the stem (data not shown).

Next, the internal loops (TAACDs in Rn-DsDsDs-53mh and TTC in ARC1172-50mh) were replaced with the mini-hairpin loop (GAA). The X-ray crystallographic analysis of the ARC1172–vWF complex indicated no direct interaction between the TTC loop and vWF. As for Rn-DsDsDs-53mh, the gel-shift assay of Rn-DsADs-44 (Figure 9) revealed that the Ds base at position 22 in the loop of Rn-DsDsDs-44 did not affect the affinity. Thus, these regions could be replaced with the mini-hairpin DNA, and we finally designed Rn-DsDs-51mh2 and ARC1172-50mh2, in the same manner as that used for the stabilization of the anti-interferon-γ Ds-containing DNA aptamer.[45]

The thermal stabilities of these modified aptamers were measured (SI Figure S4 and Figure 12). By introduction of the mini-hairpin DNAs, the thermal stabilities of Rn-DsDsDs-53mh (T_m = 75.5 °C) and Rn-DsDs-51mh2 (T_m = 76.5 °C) significantly increased by around 10 °C. Unexpectedly, the thermal stability of ARC1172 (T_m = 61.3 °C for ARC1172-50mh and 61.9 °C for ARC1172-50mh2) was slightly reduced by introducing the mini-hairpin DNA to the 3′-terminus. These results suggested that the Ds-containing and natural-base DNA aptamers have different structural features.

The high-thermal stabilities of Rn-DsDsDs-53mh and Rn-DsDs-51mh2 were also confirmed by gel-shift assays at different temperatures (Figure 9). Both of the aptamers containing the mini-hairpin DNAs efficiently bound to vWF even at 37 °C, and the mobilities corresponding to the free DNAs, especially Rn-DsDs-51mh2, remained unaltered at 4, 25, and 37 °C. In contrast, the Ds-containing DNA aptamers without the mini-hairpin DNAs generated different band patterns corresponding to the free DNA on the gels at each temperature. At 4 °C, the mobilities of these aptamers without the mini-hairpin DNAs were faster than those of the aptamers with the mini-hairpin DNAs. At 25 °C, two bands appeared, and the upper bands may have resulted from the partially denatured structures. At 37 °C, they mostly gave only the upper bands. Thus, the introduced mini-hairpin DNAs stabilized the entire aptamer structures.

The stabilization method maintained the affinity of Rn-DsDsDs-53mh (K_D = 61.3 pM) and, interestingly, increased the affinity of Rn-DsDs-51mh2 (K_D = 182 pM) (Figure 13), as compared to the initial aptamers, Rn-DsDsDs-44 (K_D = 74.9 pM) and ARC1172-41 (K_D = 326 pM) (Figure 9). In addition, the specificities of both aptamers were also high, and other proteins (human interferon-γ, BSA, human TNF-α, human α-thrombin, and human NF-κB (p50)) clearly did not bind to each aptamer (Figure 14).

We confirmed that the stabilities of these aptamers, especially Rn-DsDs-51mh2, were increased in human serum; 75% of Rn-DsDs-51mh2 and 42% of ARC1172-50mh2 survived after an incubation at 37 °C for 72 h (Figure 15). Without the mini-hairpin DNA sequences, only 30% of Rn-DsDsDs-44 and 19% of ARC1172-41 remained in the human serum after 72 h. Based on the difference in the stabilities between Rn-DsDs-51mh2 and ARC1172-50mh2, the stabilization method is more effective for Ds-containing DNA aptamers than natural-base DNA aptamers.

We also examined the conventional stabilization method using an inverted T for ARC1172-41. One modification (ARC1172-42iT) was the attachment of the T to ARC1172-41 via the 3′–3′ linkage, and the other (ARC1172-42mh-iT) was designed by introducing the mini-hairpin DNA loop (GAA) into the internal loop of ARC1172-42iT (Figure 12). Although the inverted T method also stabilized the aptamer, it was less effective than the mini-hairpin DNA method (Figure 15). This is because the mini-hairpin DNA stabilizes the entire stem region, by stacking between the 3′-terminal base of the mini-hairpin DNA and the 5′-terminal base of the aptamer.

CONCLUSION

We developed a new version of ExSELEX using a completely randomized DNA library composed of A, G, C, T, and Ds, to efficiently generate high-affinity Ds-containing DNA aptamers. Benner and colleagues also reported the sequence variation analysis for the determination of their unnatural base positions in the aptamers.[27,49] To confirm the sequence of each aptamer, each isolated clone must be sequenced. Thus, we developed the

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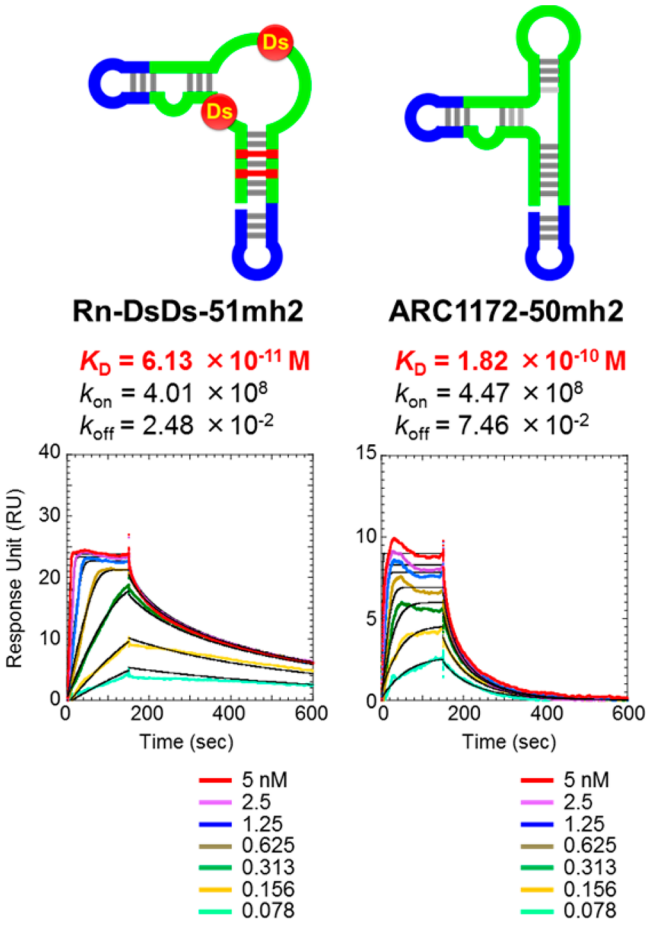

Figure 13. Binding abilities of the optimized anti-vWF DNA aptamers containing mini-hairpin DNAs, assessed using a BIAcore T200 at 37 °C with 0.078 to 5 nM vWF.

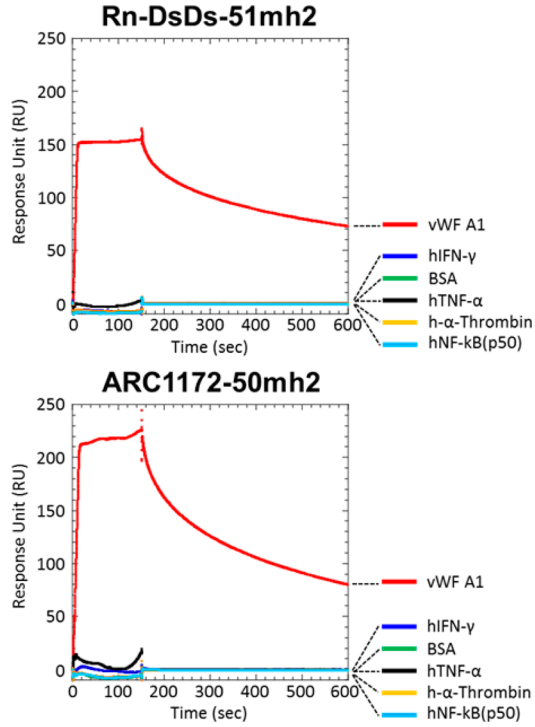

Figure 14. Binding specificities of the optimized anti-vWF DNA aptamers with mini-hairpin DNAs to various proteins, measured by SPR. Each DNA aptamer was immobilized on a Sensorchip SA by injecting a 25 nM DNA solution for 1 min, at a flow rate of 5 μL min^{-1}, instead of the 0.5 nM DNA solution for 160 s for the dissociation constant determinations (see Figures 10 and 13). Each injected protein was used at a 20 nM concentration (vWF A1 domain, human IFN-γ, BSA, human TNF-α, human-α-thrombin, and human NF-κB(p50)).

method to isolate each clone from the library. By probe hybridization, Ds-containing DNA aptamers can be specifically isolated from the enriched library after the selection process, and their sequences can be correctly determined. Using this method, we identified a DNA aptamer that binds to vWF with a K_D = 74.9 pM, indicating the effectiveness of the Ds-randomized library for ExSELEX.

The aptamer contains the terminal stem region, and thus it can be stabilized by the introduction of a mini-hairpin DNA for further applications.[45,46] By introducing two mini-hairpin DNA sequences into the internal and terminal regions of the aptamer, the optimized aptamer was significantly stabilized both thermally and enzymatically, without any loss of the affinity (K_D = 61.3 pM). Furthermore, we confirmed that the stabilization method can be used for natural-base DNA aptamers, as shown by the ARC1172 modifications. The optimized anti-vWF DNA aptamer could be tested for therapeutic purposes.

Since only a few Ds bases are required for tight binding to targets, ExSELEX using Ds-predetermined libraries is also quite promising. However, the complexity of the present Ds-predetermined library was insufficient, and many more sublibraries should be needed to increase the Ds-containing sequence combinations. In addition, our results indicated the possibility of Ds misincorporation within the library during PCR, and thus the sequencing method that we developed here is important to confirm the Ds positions in the generated DNA aptamers.

There is still some room to improve the ExSELEX method. For example, our sequencing method using the gel or capillary sequencer involving the unnatural base pairing is still complicated and time-consuming and often results in a disturbed peak pattern, as shown in Figure 7, which makes it difficult to determine the Ds positions correctly. In addition, the increased complexity with five different bases causes another technical problem in the ExSELEX scale limitation (~10^{15} molecules for selection). When using a library with a 30-nt randomized region, the total number of different sequence combinations is 9.31 × 10^{20} (=5^{30}) for five different bases, as compared to 1.15 × 10^{18} (=4^{30}) for four different standard bases, reducing the success rates of aptamer generation. Thus, further optimization of the Ds-randomized libraries is required in terms of the length and the unnatural-base content of the randomized region, as well as the Ds-containing sequencing methods. Efforts to achieve these improvements are now in progress.

METHODS

Reagents and Materials. The selection target, recombinant protein vWF A1 domain (human, amino acids 1238 to 1481), was purchased from U-Protein Express. The unnatural nucleotide substrates, dDsTP, dPxTP, dPa′TP, ddDsTP, and ddPa′TP, used for PCR were synthesized as described previously.[32,34,50] DNA fragments used in this study were either chemically synthesized with an Oligonucleotide Synthesizer nS-8 (Gene Design), using phosphor-

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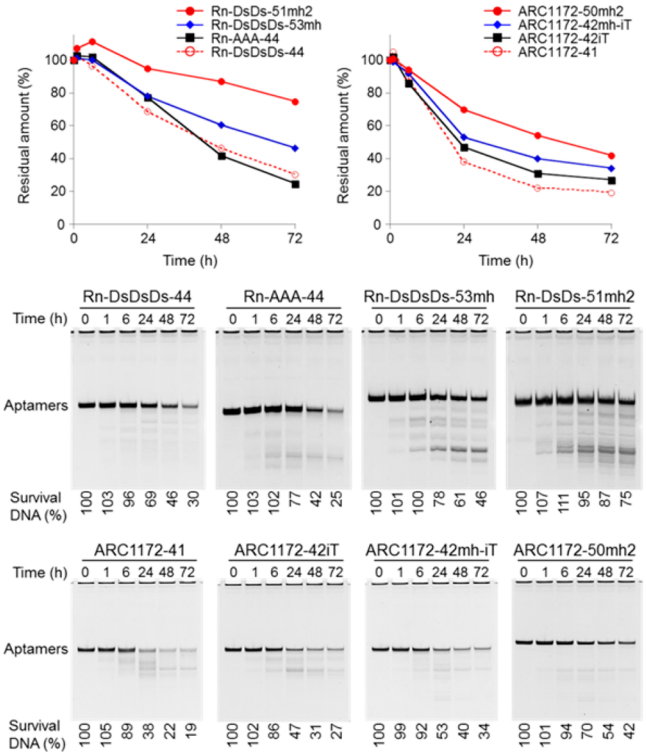

Figure 15. Nuclease resistance of anti-vWF DNA aptamer derivatives in 96% human serum. Each DNA aptamer (5 μL, Rn-DsDsDs-44, Rn-AAA-44, Rn-DsDsDs-53mh, Rn-DsDs-51mh2, ARC1172-41, ARC1172-42iT, ARC1172-42mh-iT, and ARC1172-50mh2; 50 μM) was mixed with human serum (120 μL), and the mixture (2 μM DNA in 96% serum) was incubated at 37 °C. Aliquots (10 μL) were removed at various time points from 0 to 72 h, and immediately mixed with 110 μL of denaturing solution (1× TBE containing 10 M urea). Each sample was analyzed by 15% denaturing polyacrylamide gel electrophoresis. DNA stained with SYBR Gold was detected with a bioimaging analyzer (Fuji Film LAS-4000). To determine the intact fraction, the band intensities were quantified with the Multi Gauge software. The residual amount (%) at each time point was plotted in the upper graphs. Each sample was analyzed twice, and one analysis is displayed.

amidite reagents for the natural, Ds, and Dss[50] bases and modifiers (Glen Research), or purchased from Invitrogen or Gene Design. The chemically synthesized DNA fragments were used after purification by denaturing gel electrophoresis, while the biotinylated probes were directly used after desalting purification.

SELEX Procedures. The Ds-predetermined library used in this study was prepared by mixing 28 different single-stranded DNA sublibraries (82-mer, SI, Table S1), each containing two Ds bases at defined positions in the 26-nt randomized region. Each sublibrary consisted of 5′-primer, 5′-stem (XXXACT), 26-nt randomized, 3′-stem (AGTYYY), and 3′-primer regions, where each combination of the XXX and YYY sequences functions as a barcode to identify the predetermined Ds positions.

The Ds-randomized DNA library, N30Ds-S6-006, was used for the new version of ExSELEX. Each ExSELEX procedure was performed as described previously,[26,47] with some modifications. The selection conditions for each round of ExSELEX are summarized in the SI, Table S2.

The binding of the DNA libraries to the target protein, vWF, was performed in Binding Buffer, 1× PBS supplemented with 0.005% (w/v) Nonidet P-40, at 25 °C. To isolate the target-bound DNA from the unbound DNA, the complexes were either captured on streptavidin-coated magnetic beads after the biotinylation of vWF, as described previously, or isolated by native PAGE (8% polyacrylamide gel in 0.5× TBE) in each round. In the separation by native PAGE, DNA fragments were eluted in water from the excised gel slices corresponding to the shifted band positions. The recovered DNA fragments were subjected to PCR in 1× AccuPrime Pfx Reaction mix (Life Technologies), supplemented with 0.1 mM each dNTP (final concn 0.4 mM each) and 0.5 mM $MgSO_4$ (final concn 1.5 mM), 0.05 mM each dDsTP and dPxTP, 1 μM each primer (Rev019-25 and Fow026-25-LT15 for the predetermined Ds DNA library; mkP25-006DssTs1 and mkP25-009Ts2-LT15 for the random-Ds DNA library; SI, Table S1), and 0.05 U/μL AccuPrime Pfx DNA polymerase (Life Technologies). PCR was performed as two-step cycling, 12−28 cycles of PCR amplification [94 °C for 15 s; 65 °C for 3 min 30 s], after 2 min at 94 °C for the initial denaturation step. We used the 5′-primer, mkP25-006DssTs1, in which the 5′-terminus was labeled with the fluorescent Dss base, and the 3′-primer, mkP25-009Ts2-LT15, in which the oligo dT15 was attached to the 5′-terminus via the C12 alkyl linker. Thus, the Ds-strands for the library were labeled with Dss and were shorter than the nonlabeled Px-strands, because the extension reaction of the Ds-strands was paused at the position opposite the C12 linker in the Px-strand template in the PCR amplification. The Ds-strands were then separated from the Px-strands on a denaturing 10% polyacrylamide gel for the next round of selection.

Deep DNA Sequencing. To determine the enriched sequences after the eighth round in the initial version of ExSELEX and the seventh round in the new version of ExSELEX (in Set #1 and Set #4), we performed deep sequencing with an IonPGM sequencer system (Life Technologies), after replacement PCR of the DNA fragments recovered from the shifted bands on a gel from the final round of each ExSELEX. To replace the unnatural bases with natural bases in the enriched DNA libraries, we performed PCR without dDsTP and dPxTP, but in the presence of dPa′TP (final concn 0.05 mM). Supplementation with dPa′TP facilitates the replacement of the unnatural base with any natural base (mainly A or T) via the Ds−Pa′ pairing followed by the A−Pa′ pairing.[47] The PCR products were purified with a silica-membrane PCR purification kit and then analyzed with the IonPGM sequencing system, according to the manufacturer's instructions.

Analysis of Sequence Data Sets Obtained by Deep DNA Sequencing. To analyze the sequence data obtained by deep sequencing, we extracted the specific sequences from the total data, by using the CLC Genomics Workbench software (CLC bio) with the following criteria: 5′-ACGACCGTTCTCTAATTTTGACGT-[38 bases]-AGGGTC-3′ or 5′-ACCAAATTATTGCGATACAGACCCT-[38 bases]-AACGT-3′ (extracted reads, 420 526 in total) for the Ds-predetermined library, and 5′-TATATCCGCCATACTTACGT-TGTCC[42 bases]-GTTGGA-3′ or 5′-GCGCGACTTCACTTAA-GATTCCAAC-[42 bases]-GGACAA-3′ (extracted reads, 151 495 reads for Set #1; 180 152 reads for Set #4) for the Ds-randomized library. The isolated sequences were processed and clustered with Microsoft Excel, using a custom-made program (SI, Tables S3, S5, and S6).

Determination of Ds Positions in Isolated DNA Fragments. To identify the Ds positions in each isolated clone from the enriched DNA libraries, we captured the targeted family sequences from each library, by using a specific hybridizing probe (5′-biotin-ACTCCCTC-GGTTGTTGGCGAAAGTTG-3′ for the Ds-predetermined library; 5′-biotin-CGTTGAGACCTGTTAGGTGCTCTTC-3′ for the Ds-randomized library). The DNA library (20 μL, 100 nM in Probing Buffer, 20 mM Tris-HCl, 0.5 M NaCl, 10 mM $MgCl_2$, pH 7.6) was annealed with a biotinylated DNA probe (1 μL, 5 μM in water), by heating at 90 °C for 3 min, followed by cooling down by −0.1 °C/s to 55 °C, and maintaining it at 55 °C for 15 min. The mixture was incubated with Hydrophilic Streptavidin Magnetic Beads (New England Biolabs) at 55 °C for 5 min, and the biotinylated probe and the DNA clones hybridized to the probe were captured. The collected magnetic beads were washed with 150 μL of the probing buffer (prewarmed at 55 °C) five times. The hybridized DNA clones were recovered from the beads by incubating them in 20 μL of water at 75 °C for 5 min. The recovered DNA was then subjected to 15-cycle PCR amplification in the presence of either dDsTP and dPxTP

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(0.05 mM each) or only dPa′TP (0.05 mM, replacement PCR) and purified by denaturing PAGE. DNA sequencing of these PCR-amplified DNAs was performed with BigDye terminator v1.1 (20 μL reactions, 0.15 pmol of DNA as template), in the presence of the unnatural substrates (dPa′TP, ddPa′TP, dDsTP, or ddDsTP), as described previously.[32,33] The sequencing peak patterns were analyzed on a gel sequencer, ABI377, using the Applied Biosystems Sequencing Analysis Software (ver. 3.2).

Gel Mobility Shift Assays. The binding abilities of the enriched libraries and each aptamer derivative to vWF were analyzed by gel electrophoresis. Each DNA (final concn 100 nM) was mixed with vWF (final concn 100 nM) in Binding Buffer (20 μL solution). After an incubation at 37 °C for 30 min, 5 μL of 25% glycerol supplemented with bromophenol blue was added, and the solutions were immediately subjected to PAGE (8% polyacrylamide gel containing 5% glycerol with or without 3 M urea in 0.5× TBE) at the indicated temperature. The DNA−vWF complexes were separated from the free DNAs. The band patterns on the gels were detected by using a bioimaging analyzer, LAS-4000 (Fuji Film), after staining with SYBR Gold.

SPR Measurements. The dissociation constants of the aptamers were determined by SPR, using a Biacore T200 (GE Healthcare) at 37 °C, as described previously.[46] The running buffer was 1× PBS supplemented with 0.05% (w/v) Nonidet P-40. Each biotinylated DNA was immobilized on a Sensor chip SA (GE Healthcare), and the interaction of the immobilized DNA aptamer with vWF was detected by monitoring injections of 0.078 to 5 nM vWF solutions (diluted with the running buffer) in the Kinetic Injection mode. The conditions were flow rate 100 μL min^{-1}, protein injection time 150 s, and dissociation time 450 s. Each curve fitting was done with a 1:1 binding model using the BIAevaluation T200 software, version 1.0 (GE Healthcare).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10767.

Sequence data obtained from ExSELEX, binding analysis by the gel-shift assay, thermal stability profiles of the aptamers, sequences of DNA fragments, ExSELEX conditions (PDF)

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Notes

The authors declare the following competing financial interest(s): A patent application describing ideas presented in this article has been filed by TagCyx Biotechnologies. M.K. and I.H. own stock in TagCyx Biotechnologies.

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