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RESEARCH PAPER

A general double library SELEX strategy for aptamer selection using unmodified nonimmobilized targets

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Abstract Aptamer discovery for unmodified nonimmobilized targets has been constantly presenting itself as a significant challenge to the research community. We demonstrate here a novel double library (DL) SELEX strategy and its usefulness and generality toward discovering both ssDNA- and RNA-based aptamers with nanomolar binding affinities toward unmodified targets of both small (e.g., doxycycline) and large (e.g., $VEGF_{165}$) sizes. The same selection strategy further allows for concurrent selection of an aptamer pair, recognizing discrete epitopes on the same protein, from the same selection cycles for the sandwich aptamer pair-based biosensor development (e.g., one aptamer for the recognition and the other for the signal transduction). These results establish the DL-SELEX method developed here as a valuable and highly accessible selection strategy for aptamer discovery, especially when chemical modifications of target molecules are not preferred or simply impossible.

Keywords Aptamer · SELEX · Nucleic acids · Nonimmobilization · Label-free

Introduction

As firstly reported in 1990 [1, 2], systematic evolution of ligands by exponential enrichment (SELEX) has proven as a powerful synthetic evolutionary process to rapidly identify, via iterative in vitro cycles of selection and amplification using a random nucleotide library, specific target-binding aptamers that offer an interesting alternative to antibodies [3–13]. Both conventional SELEX protocol and its many other variants rely on the use of either filtration membranes [1, 2] or chemical modifications of the target molecule [14] in order to facilitate partitioning of target-bound and target-unbound aptamer sequences. While filtration membranes often exhibit intrinsic binding toward, e.g., G-rich nucleic acids, with protein capture efficiency varying among proteins and between different experimental conditions, and do not work for small molecules and metal ions, chemical modifications of target molecule could produce drastic and unpredictable effects on their true structure and molecular properties for small molecules, and could lead to not only a possible disruption of proteins' conformation but also a restricted interaction surface accessible by aptamers for proteins that are immobilized on the bead. Similar to GO-SELEX [15], another possible benefit for SELEX selections using unmodified targets such as protein targets is that the same rounds of selection may enable a simultaneous discovery of two complementary aptamers that could recognize discrete binding sites on the same protein, allowing for construction of a sandwiched aptamer pair for protein recognition.

A significant part of recent scientific pursuits has been focused on developing new SELEX variants for aptamer selection against unmodified targets without using troublesome filtration membranes [14]. Some of the developed alternatives such as electrophoresis-based electrophoretic mobility shift assay (EMSA)-SELEX [16] and capillary electrophoresis (CE)-SELEX [17] are applicable only to large biologics, and those that can be or have been applied to small molecule targets include Capture-SELEX using capturing agents for immobilizing random aptamer library to the solid support

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[18, 19], instrument-intensive SELEXs relying on AFM [20], microarray [21] and fluorescence-activated cell sorting (FACS) [22], as well as those requiring specialized materials (e.g., graphene oxide-based GO-SELEX [15]) or matrix (e.g., SolGel SELEX [23]). Except for Capture-SELEX, all these new SELEX variants, despite having their own unique advantages, come with varying limitations, mainly including limited capacity in structural diversity of aptamer library [15–17, 20–23] (e.g., ~10^{16} diversity in conventional SELEX vs 10^{12} for CE-SELEX, 10^5 for microarray, reading speed of 10^7/h for FACS, nucleobase-dependent adsorption, and limited loading capacity in nucleic acids by graphene, etc.) and a need to use specialized or expensive instrument (capillary electrophoresis [17], AFM [20], microarray [21, 23], and FACS [22]) or material (e.g., graphene [15]).

In particular, for Capture-SELEX that was conceived originally by Li [18], a fixed-sequence domain of 15 nucleotides (nts), which is placed in the center of aptamer sequence and flanked by two random domains of 10 and 20 nts in length, was used to attach the aptamer library to beads via antisense oligonucleotides. In this way, any target-binding aptamer to be identified will contain a fixed 15-nt sequence in its center flanked by two target-binding sequences. In other words, only aptamers able to elicit sufficient target binding-induced conformational changes around the fixed domain will be released from the solid support for the next round of selection. This might be quite demanding and constitutes a possible reason why target-binding aptamers either come with low binding affinities or cannot be found. Although Worgall and Stojanovic recently moved the fixed sequence from the center to one of the two stem regions so that one continuous 30-nt random sequence domain, rather than two fragments, can be incorporated into the library design [19], this alternative location, however, still could not eliminate the same demanding need.

Aiming to overcome the limitations of Capture-SELEX via the use of a short random DNA library for capturing the random aptamer library to the solid support, we described here a new general selection strategy, termed double library SELEX (DL-SELEX), toward aptamer discovery using unmodified targets of both small and large sizes. Preserving the key features of conventional SELEX, our double library approach possesses the same capacity in library size, is generally applicable to both biological and small molecule targets, and does not require any specialized instrument or material, making it readily adaptable to routine use by laboratories with standard biological equipment and personnel. In addition, relative abundance and quick enrichment of both DNA and RNA aptamers with nanomolar binding affinities toward both small molecule (e.g., doxycycline) and protein (e.g., vascular endothelial growth factor 165—$VEGF_{165}$) targets can be achieved mostly with up to eight rounds of selection. Although our DL-SELEX requires target binding-induced conformational change for releasing active aptamers, a feature likely intrinsic to most of target-binding aptamers and useful for direct transformation into diagnosis and biosensor systems [24], it does advantageously offer new combined features unseen in both conventional SELEX and the abovementioned alternative screening methods for which target modifications are not needed, thereby pointing to a high potential for widespread use in biosensor development.

Materials and methods

Selection conditions and procedures for DL-SELEX

Oligonucleotides including random aptamer library, capturing library, and primers were purchased from Integrated DNA Technologies (USA) or Sangon Biotech (China). All procedures were carried out at room temperature. Briefly, the capture library containing a biotin group was incubated with streptavidin (SA) beads (high-performance streptavidin sepharose, GE Healthcare; binding capacity: biotin >300 nmol/mL). The beads (molar ratio of random library:binding capacity of the beads = 1:1.5) to capture random library were incubated with 500 μL of 0.15 M NaOH for 10 min after removing the supernatant, and then washed 3 times with 1 mL of 0.15 M NaOH and 5 times with 1 mL of binding buffer (20 mM Tris at pH 7.5 with 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl and 1 mM $CaCl_2$ for doxycycline, and 30 mM Tris at pH 7.5 with 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% bovine serum albumin for $VEGF_{165}$). The random 40-nt aptamer library (around 10^{16} diversity) was mixed with the capture library at a molar ratio of 1:1.5 for every cycle on beads, heated at 65 °C for 5 min and then incubated for 30 min at room temperature with gentle shaking (200 μL). After washing 5 times with binding buffer, an appropriate concentration of doxycycline (MP Biomedicals) or $VEGF_{165}$ (ProSpec, VEGF-A 165 amino acids from HEK293 cell line) was added and then the sample was incubated in binding buffer (200 μL) for 30 min with gentle shaking. The aptamer was eluted from flowthrough after G-25 microspin column purification (GE Healthcare) and subjected to phenol extraction and ethanol precipitation (2 volumes of 100% ethanol and 1/10 volume of sodium acetate at pH 5.2) with 20 μg of glycogen. After centrifugation, the recovered sequences were amplified by PCR using Taq polymerase system (2× PCR master mix, 1st Base) for DNA aptamer DL-SELEX or RT-PCR (RT using M-MuLV RTase, Invitrogen) for RNA aptamer DL-SELEX [95 °C 5 min, 10 to 20 cycles (95 °C 30 s, 55 °C 30 s, 72 °C 30 s), 72 °C 3 min] using forward F (CD1_CD3) and 5′-biotin containing R (CD2′_CD4′) primers or T7F (CD1_CD3) primer containing T7 promoter site instead of F (CD1_CD3) primer for RNA DL-SELEX. For the first round of DL-SELEX for DNA aptamers, PCR was

directly carried out after G-25 purification without ethanol precipitation, to minimize the loss of aptamer sequences and to preserve its maximum diversity after the first round. The PCR product was incubated with SA-coated beads. The beads were incubated with 0.15 M NaOH (300 μL) for 10 min and then washed with 0.15 M NaOH and binding buffer as described above. In this step, the first wash solution was collected and purified for the next cycle as the single-stranded DNA (ssDNA) aptamer pool for DNA DL-SELEX. As to DL-SELEX for RNA aptamer selection, the PCR product containing T7 promoter sequences was used for in vitro transcription reaction using T7 polymerase (Thermo Scientific) following the manufacturer's protocol, and then the supernatant containing RNAs was collected after incubation with streptavidin-coated beads (molar ratio of PCR template:binding capacity of the beads = 1:1.5) for 15 min at room temperature and purified by phenol extraction, ethanol precipitation, and/or PAGE gel extraction as the RNA aptamer pool for the next cycle. The other steps are exactly the same as DNA DL-SELEX. To recover and enrich the capture library, the remaining PCR template was incubated with SA-coated beads as well (for RNA DL-SELEX, SA-coated beads were incubated after in vitro transcription). Next, the complementary library (sRD′_CD4_CD5′) was added (molar ratio of PCR template:the complementary library = 1:1.5) and then heated at 65 °C for 5 min. After shaking incubation in binding buffer for 30 min, the unbound complementary library was washed with binding buffer 3 times. Then, bound complementary library sequences were eluted by incubation in 0.15 M NaOH (300 μL) for 10 min. The sequences were separated using G-25 column and subjected to ethanol precipitation with 20 μg of glycogen. Recovered complementary library was amplified by PCR using the capture library F (N16) and 5′-biotin-containing capture library R (CD5) primers [95 °C 5 min, 20 cycles (95 °C 30 s, 51 °C 30 s, 72 °C 30 s), 72 °C 3 min]. For the next cycle, the purified PCR products were incubated with SA-coated beads, which were then incubated with 0.15 M NaOH (500 μL) for 10 min and washed with 1 mL of 0.15 M NaOH and binding buffer. The enriched aptamer pool and capture library were used as described above for the next DL-SELEX cycle. After several rounds of DL-SELEX, enriched aptamers were cloned into pUC19 or pLUG-Prime TA-cloning vectors for the sequencing analysis (for sequence IDs, see ESM Table S1).

Fluorescence titration

Doxycycline or tetracycline (1 μM) was incubated with various concentrations of doxycycline (DOX) aptamers in binding buffer (total 200 μL). Fluorescence intensity was scanned from 400 to 700 nm using Cytation 3 multimode plate reader (BioTek) using a cuvette (10 mm path length) with excitation at 370 nm. Buffer and nucleic acid spectra were subtracted. Binding affinities (K_D values) were calculated as described previously [25] by using the fractional saturation of the fluorescence intensity. Specifically, the binding constant was calculated using one-site binding mode (F_i = [ligand]F_{max}/K_D + [ligand]), where F_i = the relative fluorescence intensity (fractional saturation) at a given aptamer concentration ([ligand]) and F_{max} = total fluorescence intensity at saturation of a given binding site.

Electrophoretic mobility shift assay

One hundred nanomolars of DNA aptamer was incubated with various concentrations of doxycycline at room temperature (total 10 μL). The sample was loaded and run in 10% native polyacrylamide gel at 100 V for 90 min in a cold room. The native gel was then stained with SYBR® Green EMSA nucleic acid gel stain dye using the manufacturer's protocol (Molecular Probes). Binding affinity (K_D value) was calculated as described previously [25] by nonlinear fitting. Briefly, binding of DOX to aptamers makes aptamers invisible in gels with the remaining visible bands corresponding to unbound DNAs. Therefore, bound DNA fractions can be calculated from the reduced band intensity compared to free DNA bands.

Determination of binding affinity using BLItz label-free interferometry assay

Aptamers (5′-biotinylated DNA or RNA) were purchased from Integrated DNA Technologies, and 3′-biotinylated RNA aptamers were prepared using Pierce™ RNA 3′ end biotinylation kit (Thermo Scientific). The BLItz label-free biosensor system (ForteBio, USA) was used for the determination of binding affinity using the manufacturer's protocol. Briefly, biotinylated aptamer (100 nM) was loaded onto SA sensor (300 s) for immobilization. Various concentrations of $VEGF_{165}$ protein were incubated with the aptamer-immobilized SA sensor or control SA sensor in binding buffer containing 0.1% BSA (the SA sensor binds to proteins non-specifically in the absence of 0.1% BSA in binding buffer). Both association and dissociation events were recorded for 300 s for the kinetic analysis after a waiting time of 60 s for baseline to stabilize. Binding kinetics data were analyzed using BLItz Pro 1.2 software.

Screening of aptamer pair for ELISA-like sandwich assay using BLItz label-free interferometry system

Biotinylated VA 22 (100 nM) was loaded onto SA sensor (300 s) for immobilization after a waiting time of 30 s for baseline to stabilize; 500 nM of $VEGF_{165}$ protein was then incubated with the aptamer-immobilized SA sensor for 300 s in binding buffer containing 0.1% BSA. After 60 s for baseline to stabilize, 1 μM of each RNA aptamer was loaded to

check if aptamer shows competitive binding or additional binding to the preformed VA 22-$VEGF_{165}$ complex. Both association and dissociation events were monitored for 300 s, respectively. Data were analyzed using BLItz Pro 1.2 software.

Results and discussion

As illustrated in Fig. 1a, our key idea is to introduce a biotin-labeled short 16-nt random domain (N16) as the capture library to immobilize onto the beads the aptamer library containing a random domain of 30–60 nts in length (e.g., N40 in our current study), rather than target molecules (for all the sequences used in DL-SELEX, see Electronic Supplementary Material (ESM) Table S1). To prevent the capture library from excessively binding to other 16-nt random regions of the aptamer library, a fixed G-rich 4-nt domain in green, which is complementary to the green region of the aptamer library, was designed into the capture library such that a more stable 20-nt duplex (4 fixed nucleotides and 16 random nucleotides) could be formed between the capture and aptamer libraries (Fig. 1b). In principle, this short 16-nt region (N16) could be as short as 8-nt in length since 12-nt duplexes are generally considered as being stable. Incubation of beads with target molecule releases from beads aptamers able to disrupt the short 20-nt duplex region via a target binding-induced conformational change. The released aptamers are then amplified for use in the next cycle of selection. Although there is the possibility that the target molecule can bind to 16-nt random region of the capture library instead of the aptamer library, this

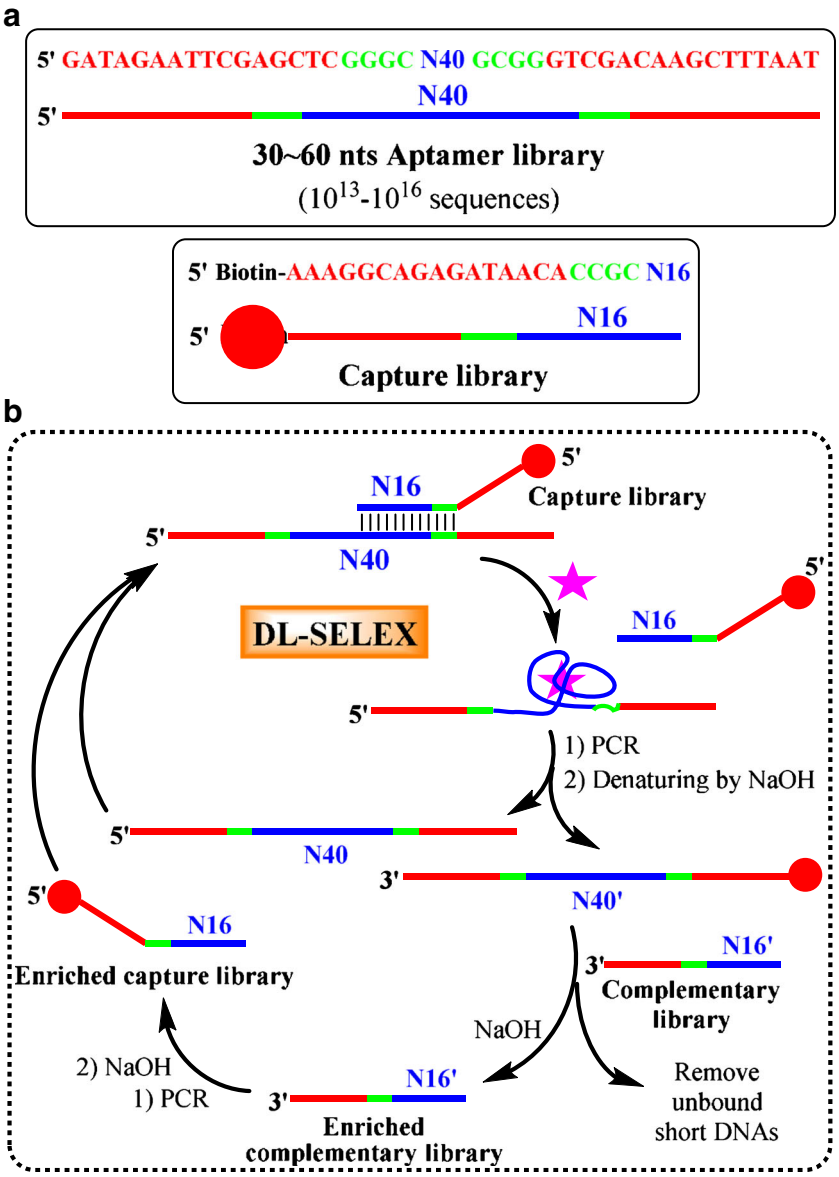

Fig. 1 **a** Schematic illustration of a double library (e.g., N40 and N16) approach for devising DL-SELEX for aptamer selection against unmodified nonimmobilized targets shown in **b**. For RNA DL-SELEX, RT-PCR is carried out instead of PCR, followed by subjecting the amplified PCR products containing T7 promoter to in vitro transcription using T7 polymerase for recovering the RNA library for the next cycle of selection (more details in the "Material and methods" section). The other steps are the same with DNA DL-SELEX

possibility is greatly reduced by introducing a constant 4-nt domain so as to form a more stable duplex of 20 bp (base pair) instead of 16 bp.

Similar to the aptamer library, the capture library can also be selectively enriched by recovering its active sequences that can capture the aptamers on beads and then release them upon incubation with targets. For this purpose, the amplified biotinylated sequence pools complementary to enriched and amplified aptamer library were employed to capture active sequences from the library complementary to the capture library. After eluting unbound sequences from the complementary library, these active sequences bound to the bead were released by denaturing using NaOH and then PCR-amplified to yield enriched and amplified capture library to pair with the amplified aptamer library for the next cycle. Compared to the conventional SELEX methods, every cycle of DL-SELEX takes roughly 6 more hours, an extra time needed to amplify and recover the capture library for the next round of selection. However, DL-SELEX does not require time-consuming modification of targets which is a highly desired feature particularly for small molecule targets. It might be worth pointing out that to shorten the selection time, amplification of capture library is not absolutely required. Instead, excess amounts of the capture library (e.g., biotin-CD5_CD4′_sRD) can be used for every cycle. This, however, may lead to some complications since some sequences from the capture library after selection may not bind enriched aptamers, possibly leading to nonspecific binding during the subsequent round of aptamer selections.

To demonstrate its effectiveness and generality in aptamer selection with unmodified nonimmobilized targets, DL-SELEX was carried out for both small molecule and protein target using both ssDNA and RNA random libraries.

As an antibiotic used to treat many different bacterial infections and with good fluorescence emission intensity around 510 nm, unmodified DOX (ESM Fig. S1) was chosen as small molecule target. Eight and 10 rounds of DL-SELEX were performed, respectively, to obtain DNA and RNA aptamers that could bind to DOX. During selection, the screening stringency was increased by systematically decreasing the target concentration from 10 μM to 1 nM for RNA aptamers and from 0.5 μM to 0.1 nM for DNA aptamers in Tris buffer at pH 7.5 (20 mM Tris with 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl and 1 mM $CaCl_2$). And the random aptamer library and the capture library that captures active aptamer sequences were successfully recovered, enriched, and amplified as expected from our design (ESM Fig. 2). More specifically, strong PCR bands, corresponding to the respective sequence lengths of 80 and 36 nts for the aptamer and capture libraries, were consistently found around and below 100 bp DNA fragment.

Enrichment of DOX-binding DNA aptamers was monitored using EMSA since we found that DNA aptamer pools of selected rounds did not cause any significant changes in fluorescence intensity of DOX and that DNAs bound to DOX are invisible in gel. Using EMSA to follow changes in DNA intensity in gel in the presence of increasing amounts of DOX, we observed clear enrichment at both rounds 5 and 8 (ESM Fig. S3). Interestingly, RNA aptamers seem to be capable of enhancing DOX's fluorescence intensity. This enhancement increases from 1.5% at round 0 for the initial random library to 8.8% at round 5 and all the way up to 26.8% at round 10 with respect to the fluorescence intensity of DOX in the absence of RNAs (ESM Fig. S4).

For protein target, 7 rounds of DL-SELEX for both DNA and RNA aptamer selections were carried out with increasingly reduced amounts of $VEGF_{165}$ starting from round 6 in Tris buffer at pH 7.5 (30 mM Tris with 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) (ESM Fig. S2); 0.1% bovine serum albumin (~15 μM of BSA) was used to prevent nonspecific binding of oligonucleotides to $VEGF_{165}$. Enrichment and binding affinity of both DNA and RNA aptamer pools at round 7 were monitored using label-free biosensor assays (BLItz system, ForteBio, USA) via immobilization of 5′-biotinylated aptamer pools onto streptavidin sensor. The binding biolayer interferometry (BLI) signals obtained reveal significant binding of both DNA and RNA aptamer pools to $VEGF_{165}$ even in the presence of 0.1% of BSA and quick enrichment of both aptamer libraries after just 7 rounds of DL-SELEX cycles (ESM Fig. S5).

The enriched aptamer pools at final rounds were cloned using specific restriction enzyme sites designed into the two constant 20-nt domains at two ends of the aptamer library and sequenced (ESM Table S2). The M-fold program [26] was used to predict the secondary structures for the best four binders within their own categories and for truncation into various lengths (ESM Fig. S6–S9). They were presented in Fig. 2 as DOX 6 (39-nt DNA), DOX 7 (46-nt RNA), VA ($VEGF_{165}$ aptamer) 6 (45-nt DNA), and VA 22 (46-nt RNA).

Using the EMSA method, the binding affinity (K_D) of DOX 6 to DOX was determined to be 150 ± 25 nM (Fig. 3a). In our effort to examine the binding specificity of DOX 6, we surprisingly found that DOX 6, which lacks the ability to modulate the intrinsic fluorescence of DOX, could significantly increase the fluorescence intensity of tetracycline (TET), which differs from DOX by the relative positioning involving just one hydroxyl group (ESM Fig. S1). Intrigued by this finding, fluorescence titrations of TET at 1 μM with DOX 6 were carried out at emission wavelength of 530 nm, and the nonlinear curve fitting yielded a K_D value of 54 ± 9 nM for TET (Fig. 3b and ESM Fig. S10a) much lower than that for DOX (150 ± 25 nM). This K_D value is even lower than both DNA (63–483 nM) [27] and RNA (1 μM) [28] aptamers previously selected against TET by the conventional SELEX. This is interesting since TET is not our intended target, but it binds to DOX 6 stronger than DOX does.

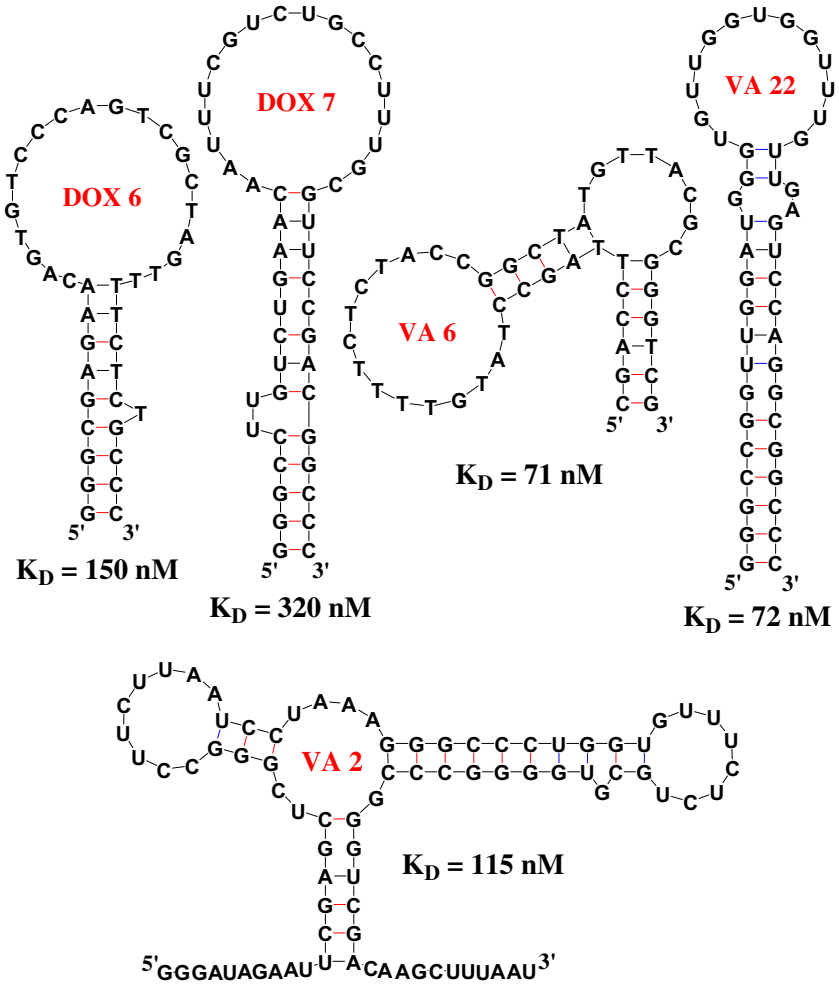

Fig. 2 Structures of truncated DOX 6, DOX 7, VA 6, VA 22 and VA 2

Similarly, using the conventional SELEX against TET, others found that the selected aptamers can also recognize DOX [27]. This suggests a difficulty to differentiate between DOX and TET by either our or the conventional SELEX methods. Although we are not exactly sure about the reason why TET binding to our aptamer is tighter than DOX, this might be attributed to the relative location and orientation of hydroxyl groups in TET, which differ slightly from DOX and might allow DOX 6 to bind TET more tightly than DOX. It remains to be seen if the binding specificity obtained using DOX could be increased or not when negative selections involving TET were incorporated into selection cycles or whether or not tighter binders to TET can be identified with TET used as the target for selection. Similarly, DOX 7 can enhance the fluorescence intensity of both DOX and TET. Monitoring the changes in fluorescence intensity at 530 nm gave rise to K_D values of 320 ± 130 and 180 ± 25 nM for DOX 7 to bind DOX and TET (ESM Fig. S10b, c), respectively. Again, DOX 7 binds TET tighter than DOX, and such a binding is 4 times tighter than the 1-μM binding affinity exhibited by the previously discovered RNA aptamer toward TET [28]. All these comparisons using TET suggest that the DL-SELEX approach is at least as good as the conventional SELEX in terms of binding affinity for small molecule targets while offering benefits (e.g., target modifications are not needed) impossible to attain using the conventional SELEX protocol.

For VA 6 and VA 22, their binding affinities toward $VEGF_{165}$ were determined to be 71 ± 3 and 72 ± 3 nM (ESM Fig. S11), respectively, by using the BLItz system in Tris buffer containing 0.1% BSA. For comparison, the previously discovered VEGF aptamer, possessing the highest binding affinity of 0.37 nM [29] among aptamers containing only natural nucleotides, was determined to be about 7.1 nM under our conditions containing 0.1% BSA (ESM Fig. S12). This suggests that our selected aptamers exhibit very high binding affinities that are only 10 times weaker than the best binder in the relatively harsh conditions. For additional comparisons, K_D values for all the previously selected aptamers containing natural nucleotides range from 0.37 nM to several hundred nanomolars in less stringent conditions containing no BSA [29, 30]. Furthermore, both VA 6 and VA 22 aptamers showed insignificant binding to structurally unrelated nontarget proteins including interferon-γ and human thrombin proteins, which were routinely used for checking nonspecific binding

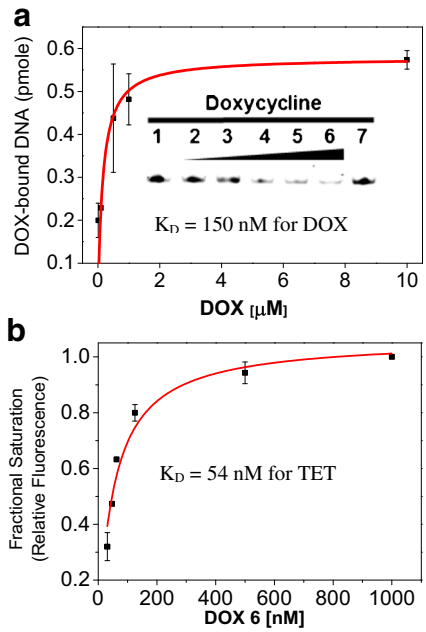

Fig. 3 Determination of binding affinities of DOX 6 toward **a** DOX using EMSA and **b** TET using fluorescence titrations. In **a**, 100 nM of DOX 6 was incubated with various concentrations of DOX (*lanes 1* and *7* contain no DOX; *lanes 2–6* correspond to 10, 100, 0.5, 1, and 10 μM of DOX 6, respectively) and electrophoresed in 10% native polyacrylamide gel. In EMSA using our set of conditions, the DOX-DOX 6 complex could not be detected, but the signal from unbound DOX 6 is observed to decrease with increasing aptamer concentration. From EMSA results, the calculated K_D value of DOX 6 for DOX was 150 ± 25 nM. In **b**, the concentration for TET was set to 1 μM with those of DOX 6 varied from 31.3 nM to 1 μM. Fluorescence intensity was measured at 530 nm with subtraction of background signals from buffer and nucleic acids. The calculated K_D value for DOX 6 toward TET was 54 ± 9 nM

of the aptamer [31], under the identical conditions in the presence of 0.1% BSA (ESM Fig. S13). This suggests that the interactions between the two aptamers and $VEGF_{165}$ proceed in a specific manner.

The above comparative binding values related to TET substrate suggest that the selection using DL-SELEX strategy, enabling small molecule target to retain its true molecular property and to fully expose its potential binding sites recognizable by aptamers, is a useful alternative screening method for aptamer discovery, especially for small molecules that carry no suitable functional groups or that are difficult for chemical modification.

Similarly, for unmodified and nonimmobilized protein target that is supposed to expose all its possible binding sites in solution for recognition by aptamers, we further looked into the possibility of concurrently selecting aptamers with binding sites different from, e.g., VA 6 and VA 22, on the same target protein as recently demonstrated by GO-SELEX strategy [32]. Such aptamer pairs are generally difficult to obtain using the conventional SELEX methods. The ability to simultaneously discover an aptamer pair recognizing discrete epitopes on the same protein certainly is important given the huge potential of ELISA-like aptamer-based sandwich assay or biosensors to compete or even replace antibody-based ELISA assay and biosensors for medical diagnosis [12, 33]. This issue becomes even somewhat urgent further given that only limited numbers of such aptamer pairs for sandwich-type recognition have been discovered so far [32, 34–38] since the formulation of SELEX protocol in 1990.

As an initial demonstration along this line, we worked on RNA samples and immobilized 3′-biotinylated VA 22 (100 nM) onto streptavidin (SA)-coated biosensor using BLItz label-free biosensor system (ForteBio, USA). After loading 500 nM $VEGF_{165}$ to preform the protein-RNA (e.g., $VEGF_{165}$-VA 22) complex for 300 s on the sensor and waiting for 60 s for baseline to stabilize, Tris buffers containing no RNAs, 1 μM of VA 22, and 1 μM of RNA to be examined were loaded, respectively, to generate sensorgrams similar to those presented in ESM Fig. S14 for comparison. In this way, all the other 14 sequences listed in ESM Table S2 were tested. Among a few RNA aptamers that could elicit a sensorgram different from both blank buffer and buffer containing VA 22 (data not shown), VA 2 (#2 RNA aptamer, ESM Table S2) emerged with a unique binding pattern. In comparison with the blue BLI signal obtained with the use of buffer alone (Fig. 4), VA 22 displays a competitive binding pattern (e.g., BLI signal decreased significantly more than the control buffer during the association step as a result of competitive release of $VEGF_{165}$ from the preformed $VEGF_{165}$-VA 22 complex by the newly added VA 22) toward the preformed $VEGF_{165}$-VA 22 complex in the way it should act (blue BLI signal) and VA 2 desirably exhibits additional but noncompetitive binding toward the same complex (red BLI signal increased during the association step as VA 2 binds to a different binding site). These results confirm noninterference between VA 22 and VA

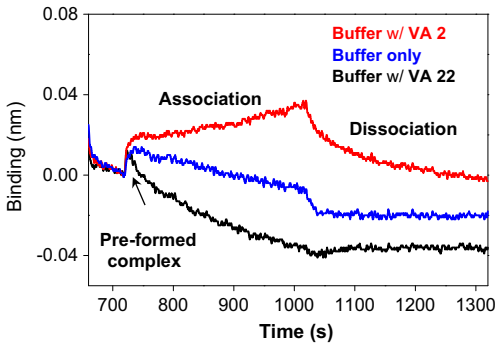

Fig. 4 VA 2 exhibits a complementary binding to $VEGF_{165}$ with respect to that of VA 22 using a label-free biosensor assay in the BLItz system. In the assay, the VA 22-$VEGF_{165}$ complex was preformed in streptavidin sensor using 3′-biotinylated VA 22 (100 nM), followed by loading buffers containing no RNA (as the control), 1 μM of VA 22, and 1 μM of VA 2, respectively. Both the association and dissociation events for aptamers to bind or dissociate the preformed complex were monitored for 300 s. The corresponding association and dissociation signals from Fig. S14 (see ESM) were normalized and presented here. Binding (nm) refers to changes in optical interference (e.g., shift of the wavelength)

2 in binding to the same protein target. In addition, the binding affinity of VA 2 toward $VEGF_{165}$ was determined to be 115 ± 45 nM (ESM Fig. S15), a reasonably good value that suggests a possible use of VA 22 and VA 2 in aptamer-based sandwich assay [12]. Successful discovery of VA 2 demonstrates that DL-SELEX renders unmodified protein with the ability to expose all its possible epitopes to possible binders during selection. It might be worth pointing out that our effort put to discover VA 2 in the last round appears to be much less than the previous approaches using competitor [30], aptamer-target complex [37], or decoy [38], all of which require many additional rounds of selection for finding aptamer pairs.

Conclusions

In summary, we established here a new aptamer selection technology, DL-SELEX, which employs a 20-nt capture library containing a 16-nt random domain to efficiently separate target-bound and target-unbound aptamer sequences without a need to modify target molecules for immobilization onto solid support. Using this selection strategy, high-quality target-binding aptamers of both DNA and RNA types, which are comparable to or even better than those selected using the conventional SELEX, were successfully discovered for unmodified targets, both small and large.

Compared to the conventional SELEX methods, the biggest advantage of the DL-SELEX is that unmodified targets can be used. For small molecule targets, this eliminates a tedious and troublesome need for covalent modifications, a useful and friendly feature for biologists who are interested in small molecules. For large molecular targets such as proteins, this suggests the ability of DL-SELEX to retain the true structure and molecular properties of unmodified protein targets while enabling all their possible binding sites to be accessible for recognition by aptamers. Consequently, using $VEGF_{165}$ protein as the example, our very minimal effort, when compared to other previously developed tedious approaches [30, 37, 38], quickly led to the discovery of two aptamers that likely could recognize discrete binding sites on the same protein. To the best of our knowledge, this is the first example of selecting a sandwich aptamer pair by structure-switching mechanism, thereby suggesting great applicability of the strategy in concurrently finding pairs of aptamers for the same target for biosensor development, together with GO-SELEX strategy [32]. Since our DL-SELEX method mostly resembles the conventional SELEX that can be routinely carried out in an ordinary laboratory setting, we believe DL-SELEX, which does not require targets to be modified and immobilized in any way, offers a highly accessible selection strategy to efficiently and reproducibly generate high-quality aptamers against molecular targets of varying sizes for which chemical modifications are undesired or impossible. Further incorporation of unnatural base technology [31] into DL-SELEX might enable rapid identification of aptamer binders with extraordinarily high binding affinities toward unmodified targets.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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