More DNA−Aptamers for Small Drugs: A Capture−SELEX Coupled with Surface Plasmon Resonance and High-Throughput Sequencing

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S *Supporting Information*

ABSTRACT: To address limitations in the production of DNA aptamers against small molecules, we introduce a DNA-based capture-SELEX (systematic evolution of ligands by exponential enrichment) protocol with long and continuous randomized library for more flexibility, coupled with in-stream direct-specificity monitoring via SPR and high throughput sequencing (HTS). Applying this capture-SELEX on tobramycin shows that target-specificity arises at cycle number 8, which is confirmed by sequence convergence in HTS analysis. Interestingly, HTS also shows that the most enriched sequences are already visible after only two capture-SELEX cycles. The best aptamers displayed K_D of approximately 200 nM, similar to RNA and DNA-based aptamers previously selected for tobramycin. The lowest concentration of tobramycin detected on label-free SPR experiments with the selected aptamers is 20-fold smaller than the clinical range limit, demonstrating suitability for small-drug biosensing.

KEYWORDS: SELEX, aptamer, small molecules, surface plasmon resonance
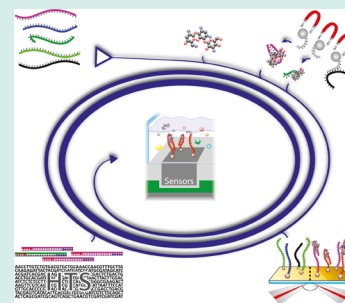
INTRODUCTION

Aptamers are single stranded oligonucleotides able to bind non-nucleotidic target molecules with high affinity. Aptamers are obtained through in vitro evolution-like protocols called SELEX (systematic evolution of ligands by exponential enrichment), and they were first selected more than 20 years ago on RNA[1,2] or DNA[3] backbones. When compared with antibodies, aptamers show several advantages for biosensing applications.[4,5] However, the availability of aptamer-ligands for small molecules is still insufficient.[4] This is mainly due to the need for target immobilization on a solid substrate during the SELEX, which can be challenging for small drugs while at the same time potentially decreasing the aptamer specificity.[7]

Aiming at circumventing this limitation, Nutiu and Li[10] developed a target-immobilization free SELEX protocol suitable for small molecules, later renamed capture-SELEX.[11] Briefly, the oligonucleotide library is designed with a docking sequence (of 15 nucleotides, nt) in between two randomized regions. The docking region of the library is used to load the ssDNA onto magnetic beads by hybridization with a complementary-biotinylated binding oligo (BO). When the target molecule binds to the recognizing ssDNA molecule, target-induced conformational switching[6] causes ssDNA elution from the bead and therefore its selection. The original DNA-based capture-SELEX strategy[10] and its developments[11,12] are based on libraries with randomized region of only 30−50 nt, split in two by a non variable region required for the capturing. Placing the immobilization region between two randomized sequences serves also to increase the chances that binding-induced conformational changes will result in aptamer release. However, longer randomized strings within the library sequence could allow for greater structural or contextual complexity and therefore could be preferable for targeting different molecules. Another DNA-based capture-SELEX has been developed for metal ion targets (Zn^{2+}) using a library with a continuous randomized strand of 50 nt,[14] but the library pool was generated by a cumbersome combination of chemical synthesis, PCR amplification, in vitro transcription and reverse transcription, and the whole protocols relies on fluorescence modification of the library. A different capture-SELEX protocol uses a RNA-based library with a longer (60 nt) continuous randomized strand,[15] but brings along the common problems of RNA-based probes like difficult/expensive synthesis (especially with modifications) and low resistance to biological fluids,[16,17] therefore showing minor appeal for biosensing.

In the present work, we introduce a DNA based capture-SELEX that combines the advantages of one single randomized region of 60nt with the stability of the DNA backbone, therefore merging high target versatility with stability of the selected aptamers in complex matrices (e.g., blood serum). The chosen target for the proof-of-concept SELEX is the amino-glycoside tobramycin (molecular weight: 467 Da), for which some RNA aptamers are available for comparison.[15,18] Moreover, tobramycin concentration in patients' blood needs to be routinely controlled due to high patient variability in tobramycin pharmacokinetics,[19] therefore prompting the development of a monitoring device addressed to this application.
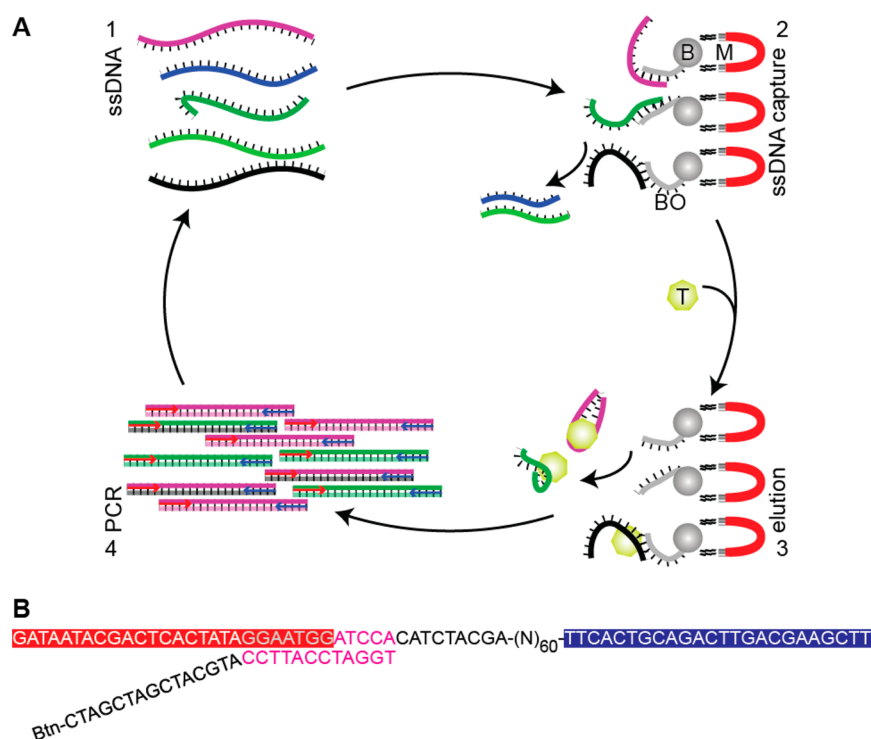
Figure 1. DNA-based Capture-SELEX strategy. (A) Schematic of each cycle of the Capture-SELEX, which consists of four steps: (1) ssDNA library, (2) binding of the ssDNA library to magnetic beads (B, separated via magnets, M) through a Binding Oligonucleotide (BO, in gray), (3) elution of the ssDNA in response to binding of the target (T, yellow) and the consequent conformational change (the black ssDNA binds but does not change conformation and it is therefore not eluted), (4) PCR amplification of the eluted ssDNA using the designed primers (red and blue arrow). The dsDNA amplified by PCR is then converted into the ssDNA library for the next cycle, therefore restarting at step 1. An aliquot of the eluted ssDNA for each cycle has been used as template for the further analyses shown in the paper, as well as for the high-throughput sequencing library preparation. (B) Structure and sequence of the ssDNA library (top) and of the biotinylated BO (bottom). The gray nucleotides show the annealing part between the library and the BO.

Another long-established limitation of the SELEX is the necessity of performing many cycles before identifying the selected aptamers, thus making the selection of aptamers a labor intensive and time-consuming process. Although some works show SELEX protocol with significantly reduced number of cycles using CE-SELEX,[8,20,21] high throughput sequencing (HTS) based-SELEX[22] or microfluidic/CHIP-based SELEX,[23−25] once again all the developed protocols are based on target immobilization or are poorly adaptable to low molecular weight targets. To investigate the progression of the capture-SELEX more deeply and thus to understand if the number of cycles can be drastically reduced, we coupled the capture-SELEX for the first time with a direct specificity evaluation performed on a surface plasmon resonance platform (SPR) and with HTS analysis of the selected cycle-libraries. In the present work we demonstrate that DNA-based capture-SELEX featuring a long and continuous randomized sequence yields to aptamers showing high affinity and that there is room for dramatically reducing the number of cycles required to select the best aptamer.

EXPERIMENTAL SECTION

Library Design and Oligonucleotides. The library was designed similarly to the work on RNA of Morse,[15] but on DNA (Figure 1). The library consists of a 101 base long oligonucleotide (Figure 1) with two constant regions of 21 nucleotides (nt) and 20 nt, on the 5′ and 3′, respectively, for PCR primers annealing, and a central randomized region of 60 nt (corresponding to almost 1.33×10^{36} possible sequences).

12 nt of the 5′-end serve as docking sequence to load the library onto the biotinylated superparamegnetic beads, while the following 9nt before the randomized region served as buffering region to be used in the library-loading optimization. The library sequence is the following: 5′-GGAATGGATCCA-CATCTACGA-$(N)_{60}$-TTCACTGCAGACTTGACGAA-3′. The primers used for amplification are 5′-GATAATACG-ACTCACTATAGGAATGGATCCACATCTACGA-3′ for the forward (with 5′-thiol when the downstream application is SPR instead of the SELEX), and 5′-phospho-AAGCTTCG-TCAAGTCTGCAGTGAA-3′ for the reverse. The Binding Oligonucleotide (BO) used to attach the library to the beads is 5′-TGGATCCATTCCATGCATCGATCGATC-3′-Biotin in which the first 12nt are annealing to the library. All oligonucleotides were purchased from Sigma-Aldrich.

Library Preparation. The first randomized ssDNA library is amplified by PCR using 2.37 nmoles of ssDNA template (about 1.43×10^{15} molecules out of 1.33×10^{36} possible sequences), 2 mM MgCl$_2$, 0.2 mM dNTPs, 1 μM of each primer forward (5′-GATAATACGACTCACTATAGGAAT-GGATCCACATCTACGA-3′) and reverse (5′-phopsho-AAGCTTCGTCAAGTCTGCAGTGAA-3′), and 0.0125 U/μL of Taq polymerase (Invitrogen and KAPA). Amplification is performed with the following cycle 2 min at 95 °C, 30 min at 95 °C to 30 min at 70 °C to 30 min at 72 °C for 25 times, 5 min at 72 °C, and it is verified by gel electrophoresis using standard techniques (2% agarose gel). An aliquot of 4 mL of the PCR product is then purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen) following the manufacturer's

instructions (300 μL of bead slurry). The purified dsDNA obtained is spectroscopically quantified using Nanodrop (Thermo Scientific). dsDNA is converted in the ssDNA library by taking advantage of the capability of the enzyme λ-Exonuclease (Fermentas) of selectively digest the strand that has a 5′ phosphorylation of a dsDNA. The reverse primer used in the PCR is 5′ phosphorylated, thus allowing digestion of the complementary strand. For dsDNA digestion roughly 100 μg (1.6 nmol) of dsDNA are digested according to the manufacturer instructions (150−210 U of enzyme) over a 4 h reaction. After enzyme heat-inactivation the ssDNA is purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen) accordingly to the manufacturer's protocol (300 μL of bead slurry). The purified ssDNA obtained is spectroscopically quantified using Nanodrop.

For the cycle-libraries (the pool at a given point in the SELEX process) obtained after each cycle approximatively 80% of the whole ssDNA recovered is used as PCR template with the same recipe as above (final volume 4 mL). The entire PCR is subsequently purified and treated as described above to obtain the ssDNA library for the next cycle (hereafter referred to as cycle-library).

Selection Step. Roughly 24 μg (0.8 nmol) of ssDNA were mixed with 4.8 μg (0.6 nmol) of biotinylated Binding Oligonucleotide (BO, 5′-TGGATCCATTCCATGCATCGA-TCGATC-3′-C$_6$−Biotin) in 1× bind and wash buffer (B&W, 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 M NaCl), and incubated at 95 °C for 10 min, 60 °C for 5 min, and slowly cooled down to 4 °C (ramp at about 0.05 °C/s) using a thermomix (Eppendorf). This mix was then incubated with 360 μL of streptavidin coated beads (Dynabeads M-280, Life-technology), previously washed 5 times in B&W buffer, overnight at 4 °C in a rotating wheel. The supernatant was then removed from the beads, and used to assess the binding efficiency of the library to the beads. The beads were subsequently washed twice with 10−15 beads volumes of 1× BB (50 mM Tris pH 7.4, 500 mM NaCl). Bound ssDNA was eluted by rotating for 1 h in 4 mL of BB containing tobramycin (Sigma) at the concentration of 200 μM for the first 4 cycles, and at 20 μM for the others. Two identical aliquots of beads, between 300 and 50 μL, were taken from the bead mix before elution, and used for negative and control elution by incubating them with 300 μL of either BB 1× (negative) or BB 1× with the same concentration of tobramycin as the big elution (positive). Eluted ssDNA was purified and concentrated using the ChargeSwitch PCR Clean-Up Kit (Invitrogen) following the manufacturer's indications, using 100 μL of bead slurry (15 μL for each control elution) and eluting the ssDNA in the same volume of the beads. While the main eluted ssDNA was amplified by PCR as mentioned above, the control aliquots were quantified by qPCR and semi-qPCR to assess the specificity of the tobramycin elution.

SELEX Evaluation by SPR. Eluted ssDNA from cycle-libraries of each even cycle of the SELEX was tested for affinity to the target tobramycin by performing a surface plasmon resonance (SPR) assay. Briefly, the purified ssDNA obtained from the cycle was amplified by PCR with the primers forward (thio-C6-5′-GATAATACGACTCACTATAGGAAT-GGATCCACATCTACGA-3′) and reverse (5′-phospho-AAGCTTCGTCAAGTCTGCAGTGAA-3′) in parallel with an aliquot of the initial library, and was then purified and converted to ssDNA as described above. Solutions of 50 μL at 0.6 μM of each ssDNA were used for overnight functionaliza-

tion of SPR bare gold chip (Au-chip, BIAcore, GE Healthcare), using the initial library for the reference flow-cell, and the tested cycle in the working flow-cell. After passivation with 1 mM 6-mercapto-hexanol for 30′, and several washes with TE 1X (10 mM Tris-HCl pH 7.4, 1 mM EDTA), the chip was used for kinetic analysis in the SPR machine BIAcore X100 (BIAcore, GE Healthcare). Running buffer and all the injections were based on TE 1×, and several serial dilution solutions of tobramycin (from 0.02 μM to 200 μM) were injected for 180 s at 20 μL/min, followed by a dissociation time of 240 s at 20 μL/min and a regeneration injection of NaCl 1 M with SDS 0.2% (120 s at 5 μL/min with 120 s of stabilization). The response was evaluated by fitting the curves, obtained by subtracting the signal from the reference flow-cell from the working flow-cell signal, with the software Scrubber 2.0 (Biologic software).

**Cycles-Library Sequencing and Sequence Analysis.** Aliquots of the even cycle-libraries from 2 to 12 were used as PCR templates to generate the sequencing libraries. PCR products were purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen) following the manufacturer's instructions using 13 μL of bead slurry, and yielding in more than 2.5 μg of dsDNA per each cycle-library. Library were then bound to the adaptors with one barcode per each cycle-library and sequenced with an Illumina HiSeq 2500 (Illumina) by the sequencing service of the University of Lausanne (Switzerland, unil.ch/dafl/home.html), generating about 4 M reads per each cycle-library. FastQ output files were reformatted to FASTA and low quality sequences were filtered out using a perl script (see Supporting Information). Sequence clustering was performed using a two steps strategy: Tallymer[26] software were used to recover the most frequent sequences per cluster (the full length of 101 nt as K-mer size cutoff); then for the best 50 sequences per cluster a MegaBlast search has been performed in order to allow a variability of 5% (∼3 nt considering only the variable region) in the retrieved results. This allowed to obtain the abundance of each cluster in every sequenced cycle-library, and thus to determine its SELEX-driven enrichment (see Supporting Information for further details on the analysis).

■ RESULTS

The SELEX Strategy. The selection strategy was designed to obtain DNA molecules that bind the target and undergo a major structural change causing their detachment from the supporting beads (Figure 1a). Similar strategies, recently referred to as capture-SELEX,[11] have been previously developed on DNA[10] and RNA molecules.[15] The DNA library in this work bears an uninterrupted randomized region of 60 nucleotides flanked by two fixed regions. The 5′-constant region is used for the primer annealing necessary for PCR amplification, and also as docking region for the capturing of the DNA on the beads via the binding oligo (BO). A BO with the docking sequence of 12 nt and a spacer of 14 nt was found to have the maximal capturing efficiency with the minimal docking region (data not shown).

Direct Evaluation of Aptamer Selection. Evaluating the aptamer selection during the SELEX is crucial both to confirm that a selection is taking place,[27] and to judge when to stop the SELEX to retrieve the aptamer sequences. Many methods have been proposed for this purpose in the SELEX protocols, however they usually need target immobilization on a support,[21,28−32] or they infer selection indirectly by looking at the decrease of complexity of the library.[33] Avoiding target

immobilization, capture-SELEX selection has so far been indirectly evaluated by comparing the amount of eluted ssDNA or RNA at each cycle with a blank control elution.[10−13,15,34] We therefore developed a direct protocol that allows for the first time in-stream direct quality control of the selected cycle-libraries of ssDNA via SPR. Briefly, an aliquot of each selected ssDNA cycle-library (even cycles) and of the initial library are used as template for a PCR with a thiolated 5′ primer and a phosphorylated 3′. PCR product can be thus readily converted in a thiolated-ssDNA library that is amenable for SPR-chip functionalization (see materials and methods). SPR binding analyses were then performed using the original library in the reference flow-cell, the current cycle-library in the working flow-cell, and injecting tobramycin at concentrations ranging from 0.02 to 200 μM (Figure 2A). The differential
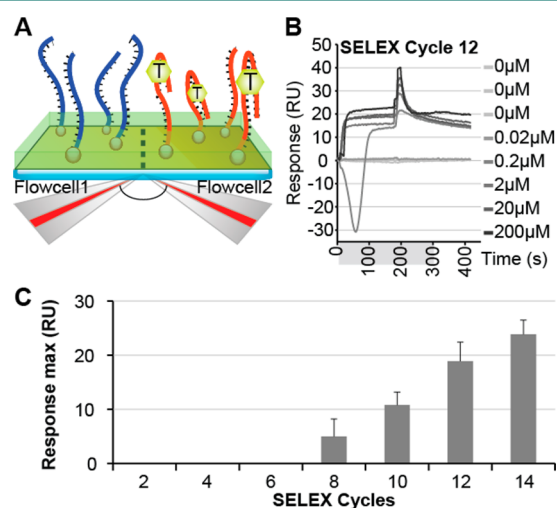
Figure 2. Direct analysis of aptamer selection. (A) Cycle-libraries of each even cycle of the SELEX were analyzed for direct binding to the target tobramycin via the SPR. The initial library (blue strings) was immobilized on the reference flow-cell (flow-cell1) of the bare gold SPR chip, while the working flow-cell (flow-cell2) was functionalized with the cycle-library tested (red strings). Both ssDNA cycle-libraries were attached via thiol-Au bond (spheres), and the whole chip was passivated with 6-mercapto-1-hexanol (green) to reduce unspecific binding. (B) Representative binding plot of the differential SPR signal obtained subtracting flow-cell1 to the signal of flow-cell2 after injections (gray box indicates injection time) of tobramycin solutions at different concentration (see legend on the right), RU: Response Units. (C) Maximum response (Rmax) of tobramycin per each tested cycle, calculated on the saturation point of each injection (see materials and methods). RU: Response Units. Note that, despite the low number of repetitions, the difference between cycle 12 and cycle 14 is not considered significant ($p = 0.19$), while the difference between cycle 10 and cycle 12 is ($p = 0.06$). Averages shown, error bars represent standard deviations ($n = 2$ for all except cycle 12 for which $n = 3$).

signal shows a clear binding difference between initial and selected cycle-libraries when using a late stage cycle-library (i.e., cycle number 12, Figure 2B). Kinetic analyses of the binding curves obtained, performed at the saturation stage (see materials and methods), allow to extract the maximum response (Rmax) for tobramycin for the cycle-libraries of each cycle (Figure 2C). From cycle number 8 on there is a clear binding response, which increases until cycle number 12−14. For the cycles earlier than number 8 it is not possible to detect a binding stronger than the initial library, although this can be

hidden by the limit of detection of the SPR or by the unspecific binding of tobramycin on both flow-cells.

These results show that SPR analysis of the cycle-libraries allows for direct target-binding evaluation of the capture-SELEX, and that in our capture-SELEX on tobramycin a visible enrichment starts at cycle 8 and goes toward saturation in cycle 12.

High-Throughput Sequencing of the Cycle-Libraries. To assess how the population of sequences is evolving in the cycle-libraries, we sequenced all the even cycle-libraries of our capture-SELEX from cycle 2 to cycle 12. HTS analysis (Illumina HiSeq), allows the simultaneous and quantitative sequencing of a high number of individual ssDNA from each SELEX cycle-library, hence giving full insights into the evolution of the sequence populations.[22,35,36] The six cycle-libraries of cycles 2−4−6−8−10−12 have been sequenced in parallel using 6 different barcodes, with bidirectional reads of 101nt (over 124nt, Figure 1B), generating about 4 M reads per cycle-library. After all the sequences with low quality and sequences not starting or ending with one of the primers were filtered out, the sequences were clustered with the freely available software Tallymer,[26] setting the K-mer to the full length (see materials and methods). Analyzing the number of reads for the 50 most represented clusters per each cycle-library shows that the complexity of the cycle-libraries decreases with the progression of the SELEX (Figure 3A). Indeed, cycle-library of cycle 2 only contains clusters with less than 50 sequences, of which 80% are below 25 sequences, showing a high degree of complexity. With the progression of the SELEX, the cycle-libraries progressively decrease the fraction of clusters with only 25 sequences while increasing the more populated clusters. Cycle-libraries of cycles 10 and 12 show a sharp increase of the clusters with high enrichment (i.e., with more than 200, 225, and 250 sequences). Comparing the size of each cluster throughout the different SELEX cycle-libraries clearly shows that the vast majority of them get enriched after cycle 6, although two clusters show an inconstant enrichment already in cycle-libraries of cycle 4/6 (Figure 3B). Interestingly, when comparing cluster frequencies of all cycles' cycle-libraries against cluster frequencies of the last cycle-library it emerges that many of the mostly enriched clusters of cycle 12 are already among the most abundant in the early cycles 2 and 4 (e.g., sequence ranked 2 in cycle 12 is also ranked 2 in cycle 2 and ranked 4 in cycles 4/8/10; Figure 3C). This indicates that the enrichment of the best clusters of the last cycles starts already at very early stages of the SELEX, although the number of sequences per cluster is still low.

The first HTS analysis performed on a capture-SELEX shows that there is sequence convergence during the SELEX, and that some clusters are clearly enriched from cycle 8, thus supporting the increase of response to the target tobramycin seen by SPR.

Candidate Aptamer Evaluation. Following the HTS of the last cycle (cycle 12), a list of the most populated 13 clusters was created in order to find the best aptamers. Aligning the representative sequences of each of these 13 clusters shows that there is no relevant homology between them (Figure 4A). Only few short stretches of nucleotides have a significant consensus. In position 14−15 of the randomized part two nucleotides are conserved in 9/13 clusters, while nucleotides 37 and 40 are conserved in all the clusters.

In the 3′ end of the randomized part, there are two conserved regions of 3 (47 to 49) and 4 nucleotides (56 to 59), which are shared by more than 12/13 clusters. Although the
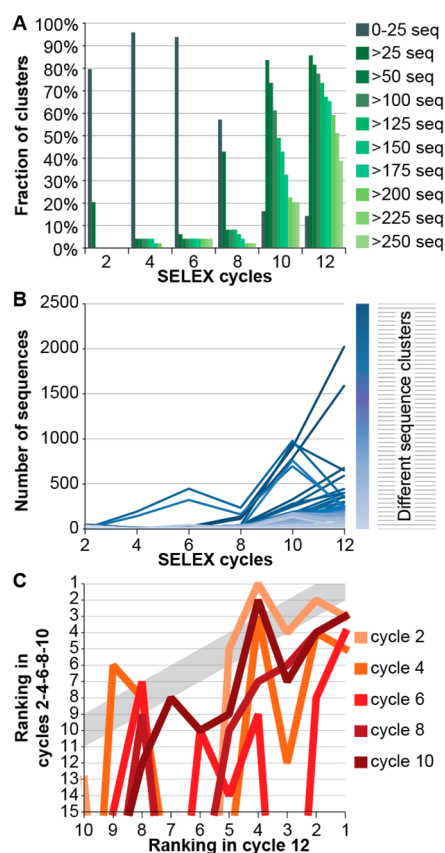
Figure 3. Sequence analysis of the cycle-libraries shows clusters convergence and enrichment. (A) Fraction of the most abundant 50 clusters with the specified number of sequences (see legend on the right) in each the SELEX-cycles. Note that with the progression of the SELEX the number of clusters with high number of sequences steadily increases. (B) Abundance (number of sequences) per each cluster in each SELEX cycle. Each line represents a single cluster. Clusters are then ordered according to their abundance in the cycle 12. Due to space restriction the cluster names are not shown in the figure legend. (C) Ranking based on the abundance of the 10 most populated clusters of SELEX cycle 12 in the libraries from SELEX cycle 2 to cycle 10 (see legend on the right). Note that the most abundant 5 clusters of SELEX cycle 12 and clusters 8 and 11 are also among the biggest clusters in almost all the earlier cycle-libraries. Gray shade shows the colinear enrichment area.

homology of these parts might be high, their short span seems to suggest that the clusters are different between them, at least in sequence. While identity higher than 70% is required to merge two clusters,[22] only less than 20% of the nucleotides in the randomized part show an identity higher than 70% between the 13 clusters. To further analyze possible structure similarities between the clusters, the secondary structure of the representative sequences were obtained by using the free-energy minimization algorithm Mfold[37] with salt correction at room temperature. Between the various structure we could not find a shared substructure such as an hairpin, loop or bulge (Supporting Information Figure 1) that is comparably long as the reported binding sites for tobramycin.[18,38] Indeed stem-loops of 26−27 nt have been showed to wrap around tobramycin, while we could find in 12/13 candidates only a stem sequence of 3 bp (5′-CCC-3′, or different GC rich stems of 3 bp) preceded and followed by different structures/sequences. Therefore, all the different clusters were considered as 13 new candidate DNA aptamers for tobramycin.

In order to evaluate the real specificity of the 13 new candidate DNA aptamers for the tobramycin molecule, we measured the binding via SPR. Similarly to the strategy used to evaluate the aptamer selection during the SELEX, each candidate was thiolated on the 5′ end and attached in ssDNA form onto the bare gold surface of the working flow-cell (flow-cell2, Figure 2A) of the SPR chip, while the reference flow-cell (flow-cell1) was functionalized with a reference oligonucleotide. The differential response clearly shows the binding of tobramycin on the candidate aptamers (an example is shown in Figure 4B) for different concentrations of the drug. Binding kinetic analysis of each candidate was then performed, and the binding parameters were extracted (K_D and Rmax, Table 1). Since each independent SPR experiment was performed identically (i.e., same oligonucleotide concentration for functionalization), these data can be compared and used to classify the candidate aptamers on their specificity for tobramycin. Overall, the candidate aptamers tested show affinity constants in the submicromolar range, with a maximum difference below 1 order of magnitude. Interestingly, the affinity does not perfectly copy the relative abundance, with 2 aptamers of the lowest half of the table showing a K_D similar to the first and second. Through sequence analysis and direct evaluation by SPR we found 13 sequence-independent candidate aptamers, of which 3 bind to tobramycin with a K_D around 200 nM. The best aptamer found is CLSE1215, which shows an affinity constant for tobramycin slightly below 200 nM.

Aptamer selectivity toward for concomitantly administered antibiotics (penicillin-like antibiotics like carbenicillin), and similar aminoglycosides (kanamycin A) has been measured via SPR with an identical protocol as described above. Carbenicillin does not produce any visible signal even at 200 μM (data not shown), while kanamycin gives a signal comparable to tobramycin in the association at 200 μM (Supporting Information Figure 2A). Interestingly, the dissociation of kanamycin is quicker than tobramycin, thus reducing the steady-state levels reached at lower concentrations. The K_D of the candidate aptamers for tobramycin is from 5 to 28 times lower than for kanamycin (Supporting Information Figure 2B), therefore showing a degree of selectivity between similar molecules despite the lack of any negative selection step in the capture-SELEX.

■ DISCUSSION

Aptamers are ideal ligands for small molecule detection in biosensors.[4−6] Despite thousands of papers on aptamers and an ever increasing demand for drug-specific biosensors, the availability of specific aptamers for small molecules is limited because the need of target immobilization typical of many SELEX protocols.[7] In this work, we present a new capture-SELEX method for developing DNA-based aptamers for small drugs with high target-flexibility because of the long and continuous randomized region. Although the docking region is placed outside the randomized sequence, therefore theoretically reducing the conformational-change driven elution, the capture-SELEX proposed succeeded to efficiently select binding ssDNA aptamers.

The candidate aptamers found were evaluated for their affinity to the target tobramycin via direct immobilization onto a SPR chip. The binding constants of the aptamers found are in the high nanomolar range, with the best binders showing a K_D around 200 nM. Starting from a library with very similar characteristic, aptamers for tobramycin developed by Morse on
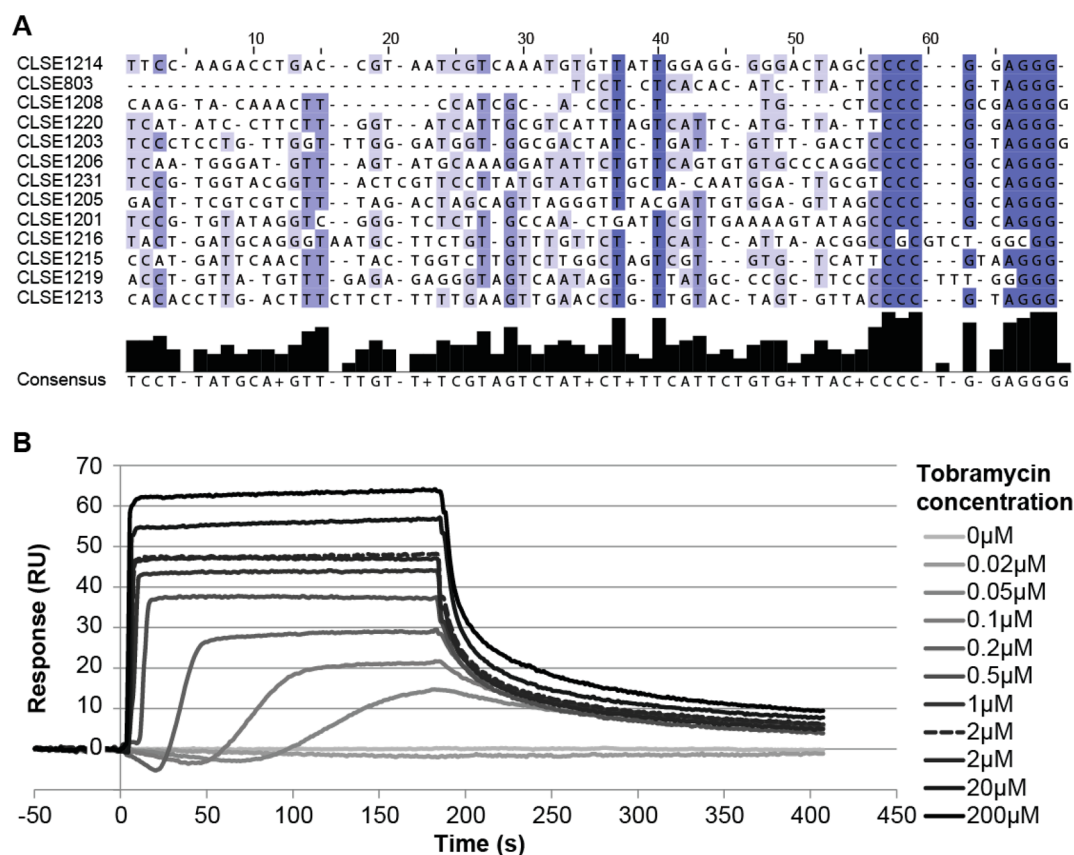
Figure 4. Candidate aptamers evaluation. (A) Sequence alignment of the most enriched 13 aptamers of cycle-library cycle 12. Light blue shading represents identity in 40−60% of the sequences, medium blue in 60−80% and dark blue in 80−100%. (B) Representative binding plot of the SPR analysis carried out on each candidate aptamer to evaluate the binding kinetic parameters with the target tobramycin. The plot shown is relative to the candidate CLSE1201 and it has been obtained using the whole sequence including the primer regions. Injection time is highlighted in gray in the x-axis.

Table 1. Validation of the Candidate Aptamers for Tobramycin Affinity and Maximum Response Calculated from the SPR Experiments (on Sequences Including the Primers)

candidate aptamer	no. of sequences in cycle 12	K_D (μM)	Rmax (RU[a])
CLSE1201[b]	1866	0.23	57.0
CLSE1203	1441	0.34	69.1
CLSE1205	676	0.65	54.0
CLSE1208	602	0.43	52.3
CLSE1206	590	0.66	65.9
CLSE1231	443	0.49	71.5
CLSE1215	375	0.30	67.1
CLSE1214	398	0.43	74.3
CLSE1220	344	0.26	31.7
CLSE1213	354	0.44	41.2
CLSE803	294	0.41	46.6
CLSE1216	302	0.54	60.2
CLSE1219	301	0.15	62.1

[a]Response units (arbitrary units from the SPR plots). [b]Short version without primers show a K_D of 0.52 μM

RNA[15] were reported with orders of magnitude higher binding constant of 16 and 500 μM, although this was measured by means of different techniques (fluorescence quenching). With the aim to investigate the mechanism of action of the antibiotic, shorter RNA-based aptamers specific for tobramycin were selected by Wang and Rando some 20 years ago[18] with K_D around 10 nM. Once again, the affinity was tested with a different assay (fluorescence-quenching competition). However, one of the same RNA-aptamers was later tested on SPR by Verhelst and co-workers,[39] showing the best K_D around 0.2 μM which has been confirmed in our lab (data not shown). Furthermore, in another recent paper short DNA-based aptamers were selected for kanamycin, but were also tested for tobramycin binding.[40] In fluorescence intensity analysis assays both kanamycin and tobramycin show dissociation constant around 0.1 μM, hence similar to the K_D of the aptamers selected in this work. Overall, comparing the affinities of our aptamers with the others available clearly indicates that RNA-based aptamers are not more avid for tobramycin than DNA-aptamers, and that the protocol developed in this work is able to select aptamers with similar low K_D than capture and "classic" SELEX.

SELEX progression has to be evaluated at each cycle, and it is usually performed by quantifying the radioactive/fluorescent oligonucleotides eluted (depending on the SELEX type this would be used to infer the discarded or selected oligonucleotides),[27] or as recently proposed by evaluating the library complexity through melting curve analysis.[33] Although important, the above-mentioned methods extrapolate the selection for target-specific oligonucleotides from indirect events like library complexity or increased elution, which could be severely impacted by unforeseen biases common in SELEX protocols. The presented direct evaluation of the SELEX progression via SPR-based measurement of the target-

affinity of the cycle-libraries overcomes this problem. We show that starting from cycle 8 the selected cycle-library shows a detectable, and since then increasing, binding of the target tobramycin, which is independent from any bias that might arise from other factors different from the target drug. Notably, the binding response peaks around cycle 12, therefore allowing to consciously stopping the SELEX to sequence the cycle-library (or to change parameters like stringency). This can play an important role in the quest of reducing the required number of cycles necessary to identify the target-specific aptamers. However, we do only see binding from cycle 8, which is clearly not a massive reduction of cycles number from 12. This could be due to the small molecular mass of tobramycin, which in a label-free mass-dependent assay like the SPR can hinder the detection of minor bindings. One alternative is to change for a signal-off SPR assay that exploits the oligonucleotide detachment (higher mass than tobramycin) from the surface used during the SELEX itself. A signal-off SPR assay has been tested for the similar antibiotics kanamycin by others,[41] but it seems to produce responses similar to our direct SPR assay for tobramycin. Another possibility is to improve the signal-to-noise ratio of the SPR by using more sensitive machines (like the BIAcore T100, GE healthcare, (their Web site), or by improving chip surface coverage.

On the other hand high-throughput sequencing has been reported to have the sensitivity required to drastically reduce the number of cycles in "classic" SELEX.[22,42] Performing HTS followed by rigorous sequence analysis on the presented capture-SELEX shows for the first time that the direct target-binding response of the cycle-libraries from cycle 8 is supported by an abrupt convergence of the libraries toward specific aptamers. More importantly, HTS analysis reveals that the most enriched aptamers of the last cycle-library are indeed already the most enriched in the early cycles, going down to cycle number 2 and hence showing potential to radically reduce the number of SELEX cycles required. However, the abundance ranking of cycle-library cycle 2 is only based on tens of sequences per cluster, and it is therefore prone to misleading ranking due to small biases. Nevertheless, with the continuous decrease of HTS costs and increase of their availability (reducing the waiting time for service-based HTS) it is not imprudent to foresee its use in capture-SELEX, even if only as SELEX evaluation tool, provided that the sequence analysis is thoroughly coupled with direct target-binding assays.

Tobramycin served in this work as proof-of-concept small drug target, but its frequent quantification in patients' blood, necessary to personalize the treatment,[19] spurs on the development of specific point-of-care biosensors.[43] Currently tobramycin in serum needs to be precisely measured in a concentrations range between 1 μM and 80 μM (Prof. T. Buclin and Prof. L. Decosterd, personal communication). The aptamers selected in the present work show a clear signal for tobramycin in buffer starting from 50 nM, while capture-SELEX obtained RNA-aptamers by Morse only sees tobramycin from around 20 μM.[15] Importantly, our aptamers do not show any binding with coadministered antibiotics from the penicillin group (e.g., carbenicillin, data not shown), while the exhibit more than 20-fold higher K_D with the similar aminoglycoside kanamycin. This leaves room for cushioning possibly required serum dilutions and hopefully for loss of signal due to the much stronger matrix effect of human serum.

CONCLUSIONS

We present here a new DNA-based capture-SELEX protocol for targeting small drugs (target-derivatization free) coupled with SPR in-stream evaluation and HTS analysis, which is flexible and bears potential for strongly reducing the number of required cycles. Using this protocol for other drugs will assess its true flexibility and speed of aptamer selection. Moreover, binding assays show that the selected aptamers are comparable with the available ones and that they might bear the capacity to detect tobramycin in label-free surface detection in the relevant blood concentration range.

ASSOCIATED CONTENT

Supporting Information
Supplementary Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions
The manuscript was written through contributions of all authors. F.M.S. performed the SELEX, SPR experiments, and DNA preparation for HTS, and P.M. performed the HTS sequence analysis.

ABBREVIATIONS

Au, gold; BO, binding oligo; bp, base pairs; HTS, high throughput sequencing; nt, nucleotides; Rmax, maximum response; RU, response units; SELEX, systematic evolution of ligands by exponential enrichment; SPR, surface plasmon resonance

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