Quantitative Comparison of Capture-SELEX, GO-SELEX and Gold-SELEX for Enrichment of Aptamers

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Abstract

Since 1990, numerous methods for aptamer selection have been developed, although a quantitative comparison of their sequence enrichment is lacking. In this study, we compared the enrichment factors of three library-immobilization SELEX methods (capture-SELEX, GO-SELEX, and gold-SELEX). We used a spiked library that contained multiple DNA aptamers with different affinities for adenosine. The aptamer separation efficiency was measured using qPCR and all of the three methods showed a very low DNA release (<1%) in the presence of 100 μM adenosine. Among these, barely any DNA was released from the gold nanoparticles. Deep sequencing was used to compare the enrichment of three aptamers: Ade1301, Ade1304, and the classical aptamer. Enrichment up to 30 to 50-fold was observed only for the capture-SELEX method, whereas the other two methods showed enrichment factors below 1. By blocking the primer binding regions of the library, GO-SELEX reached up to 14% enrichment. Finally, the enrichment of aptamers based on nonspecific release and target-induced release was discussed and the advantages of capture-SELEX were rationalized. Taken together, these results indicate that capture-SELEX is a much more efficient method for enriching aptamers.

Introduction

DNA aptamers have received tremendous interest in various fields due to their high stability, strong binding affinity and excellent selectivity.[1-5] In addition, aptamers have programmable structures and can be easily modified.[6, 7] Most DNA aptamers have been isolated using a combinatorial biology method called SELEX, in which a library with a large number of random DNA sequences is mixed with a target molecule.[8, 9] A critical step in SELEX is the separation of binding sequences from non-binding ones,[10, 11] and the separation efficiency determines the round of selection needed. Two main separation strategies have been developed for small-molecule targets. The field started in the early 1990s with covalent immobilization of target molecules on beads.[12] After incubation of the beads with a DNA library, binding sequences were retained, whereas non-binding sequences were discarded after extensive washing. However, not all target molecules can be properly immobilized without masking their binding sites (e.g., metal ions and some very small molecules). The other strategy is to immobilize the DNA library, which allows the use of free target molecules.[8, 13, 14] In this strategy, aptamer sequences are expected to be released from beads or other solid materials and enter the solution phase upon target binding. Since target molecules are not immobilized, essentially any molecule can be used as a target, and the library immobilization method has gained popularity in recent years.[3, 15-18]

The library immobilization method for small molecules was initially reported by Nutiu and Li in 2005, where the library was immobilized via hybridization to an immobilized DNA oligonucleotide.[13, 14] Later, this method was modified by the Stojanovic group to incorporate a base-paired region between the two primer binding regions of the library sequence.[8] This way, aptamer binding to its target inducing the formation of a hairpin structure, which effectively competes with DNA hybridization to release aptamer sequences. This method is also called capture-SELEX.[4, 19]

With the use of nanomaterials for DNA-based biosensor development, especially the pioneering work of adsorbing DNA aptamers onto graphene oxide (GO),[20] many adsorption-based SELEX methods have been reported. Two representative methods are GO-SELEX,[21] and gold-SELEX.[22] In these methods, a DNA library is immobilized via adsorption onto a nanomaterial. The assumption was that target binding can result in the desorption of aptamer sequences from nanomaterials,[20] or can inhibit the adsorption of aptamers,[23] whereas non-binding sequences are

adsorbed. These adsorption methods are popular since they are more cost-effective and simpler to perform compared to hybridization-based capture-SELEX.

Because the most important step in SELEX is to separate aptamers from non-aptamer sequences, a quantitative comparison of the separation efficiency in different methods is critical for the development of robust selection methods. We recently used capture-SELEX to obtain high affinity aptamers for adenosine and ATP, and the resulting libraries contained a few different aptamer sequences.[24] Herein, we spiked an enriched ATP selection library into a naïve library and systematically compared the separation efficiencies using quantitative PCR (qPCR) and deep sequencing. Based on our data, capture-SELEX allowed efficient aptamer enrichment, GO showed a much lower efficiency, while gold failed to enrich the spiked aptamer sequences.

Materials and Methods

Materials. The DNA samples used in this study were from Integrated DNA Technologies (Coralville, IA, USA, see Table S1 for sequences). Streptavidin agarose resin was from Fisher Scientific. Graphene oxide (GO) flakes were from ACS Material (Pasadena, CA, USA) and they were dispersed in water with the assistance of ultrasound. $HAuCl_4$ was from Sigma-Aldrich (St. Louis, MO). Sodium citrate was from Mandel Scientific (Guelph, ON, Canada). Adenosine, sodium hydroxide, hydrochloric acid, tris(hydroxymethyl)aminomethane (Tris), and Amicon Ultra-0.5 centrifugal filter unit (3k and 10k molecular weight cut off) were from Millipore-Sigma (Oakville, ON, Canada). Micro bio-spin chromatography columns and SsoFast EvaGreen Supermix were from Bio-Rad. dNTP mix, and Taq DNA polymerase with ThermoPol buffer were from New England Biolabs (Ipswich, MA, USA). All of the buffers and solutions were prepared with Milli-Q water. The SELEX buffer contained 50 mM Tris, pH 7.6, 500 mM NaCl and 20 mM $MgCl_2$.

Capture-SELEX. The spiked library was prepared by mixing 1 pmol of the round 9 library from our previous ATP selection with 99 pmol of the naïve library.[24] We then used this spiked library to do a one-round selection. The 100 pmol of the library DNA was dissolved in 500 µL SELEX buffer and annealed with a five-fold excess of biotinylated capture strand by heating the sample in boiling water for 1 min followed by gradual cooling to room temperature over 30 min.

Subsequently, 0.2 mL streptavidin-coated agarose beads were loaded into a micro chromatography column and washed five times using 500 μL SELEX buffer each time. The annealed DNA was then passed through the resin eight times to facilitate binding. Subsequently, the resin was washed 12 times to remove unbound library, and the eluted solutions from the 11th and 12th wash were collected as a reference for qPCR. Following the washing steps, 750 μL of a 100 μM adenosine dissolved in the SELEX buffer was added, and the eluted DNA was collected using gravity flow.

Gold-SELEX. Gold nanoparticles (AuNPs) of 13 nm diameter (10 nM) were prepared by the citrate reduction method,[25] and were concentrated 5-fold following a previous study.[22] The spiked library dissolved in the SELEX buffer was immediately cooled on ice after heating for 5 min in boiling water. Subsequently, the library (3.5 μM diluted in 28.6 μL SELEX buffer) was incubated with the concentrated AuNPs (1 mL, final 14.3 mM Na^+) for 30 min, and the resulting complex was centrifuged for 15 min and washed once by water to remove unbound library DNA. The target (100 μM adenosine in the SELEX buffer) was then added followed by incubation for 1 h with gentle shaking on a vertical rotator. The supernatant was then collected after centrifugation for 15 min. After each centrifugation, the AuNPs were dispersed with a brief ultrasonication treatment.

GO-SELEX. The annealed library (100 pmol) was incubated with 100 μM adenosine for 1 h in SELEX buffer, followed by incubation with 0.22 mg/mL GO for 1 h. The supernatant was then collected after centrifugation of the sample at 15000 rpm for 15 min.

Blocked GO-SELEX. The spiked library (100 pmol) was mixed with blocking DNA (the reverse primer and biotin capture strand, 200 pmol each) in SELEX buffer. The resulting mixture was annealed to ensure blocking. The subsequent steps were performed as outlined in the GO-SELEX described above.

qPCR test. For comparison purposes, all the samples, except for the capture-SELEX unbound library, were diluted to a final volume of 1 mL. The capture-SELEX unbound library was tested using a volume of 500 μL. To prepare qPCR reactions, the following components were mixed in a 20 μL system: 2× SsoFast EvaGreen Supermix, 8 μM forward primer, 8 μM reverse primer, 6 μL Milli-Q water and 2 μL template.

DNA sequencing. The libraries obtained after the one-round selection were PCR amplified using a combination of P5-506 and one of the P7-701, P7-702, or P7-703 sequencing primers (Table S1).

These primers were designed to include a unique index sequence for Illumina sequencing. The resulting PCR products were purified using small DNA fragment extraction kit (IBI Scientific). The concentration of the purified DNA was determined based on the absorbance at 260 nm (Tecan Spark), and the sequencing was performed at McMaster University. The sequencing data were analyzed using Geneious Prime software.

Results and Discussion

The three library-immobilization SELEX methods

Figure 1 illustrates the three library-immobilization SELEX methods studied in this work. In the capture-SELEX method (Figure 1A), the library was immobilized by hybridization to a biotinylated DNA attached to a streptavidin column via a 15-base pair region. After washing the unbound library, a target molecule (adenosine in this case) was added to elute aptamers. In library design, a 10-base pair (bp) stem is expected to form upon target binding.

For a fair comparison, the same library was used for the two adsorption-based SELEX methods. In gold-SELEX (Figure 1B), the library was incubated with AuNPs for 30 min and the resulting sample was centrifuged to remove unbound DNA. Adenosine was then incubated with the AuNP-library complex for 1 h. The supernatant containing the released sequences was collected.

Two GO-SELEX methods were reported in the literature. Method 1 just replaces AuNPs with GO as shown in Figure 1D. However, Wang and coworkers pointed out that this is not a reliable method,[26] and we were also concerned about nonspecific desorption of the DNA library.[27] In addition, when we searched the literature, most GO-SELEX papers (Table S2) used method 2 as shown in Figure 1C, where the library and target were mixed before adding GO. The assumption is that aptamer/target complexes are not adsorbed due to the formation of a rigid binding structure,[28] whereas non-aptamer sequences are adsorbed. For the development of biosensors, Fan et al. also articulated the advantage of this method.[23] Therefore, in this work, we used method 2 for GO-SELEX (Figure 1C).

It is interesting to note that no one used the method 2 in Figure 1C for AuNPs. In fact, the number of gold-SELEX papers was much lower than that of GO-SELEX. Based on our previous work to study aptamer-based label-free colorimetric biosensors, such a method is unlikely to work

for most targets due to strong and fast adsorption of target molecules to AuNPs, which can inhibit DNA adsorption in a sequence-independent manner.[29, 30]

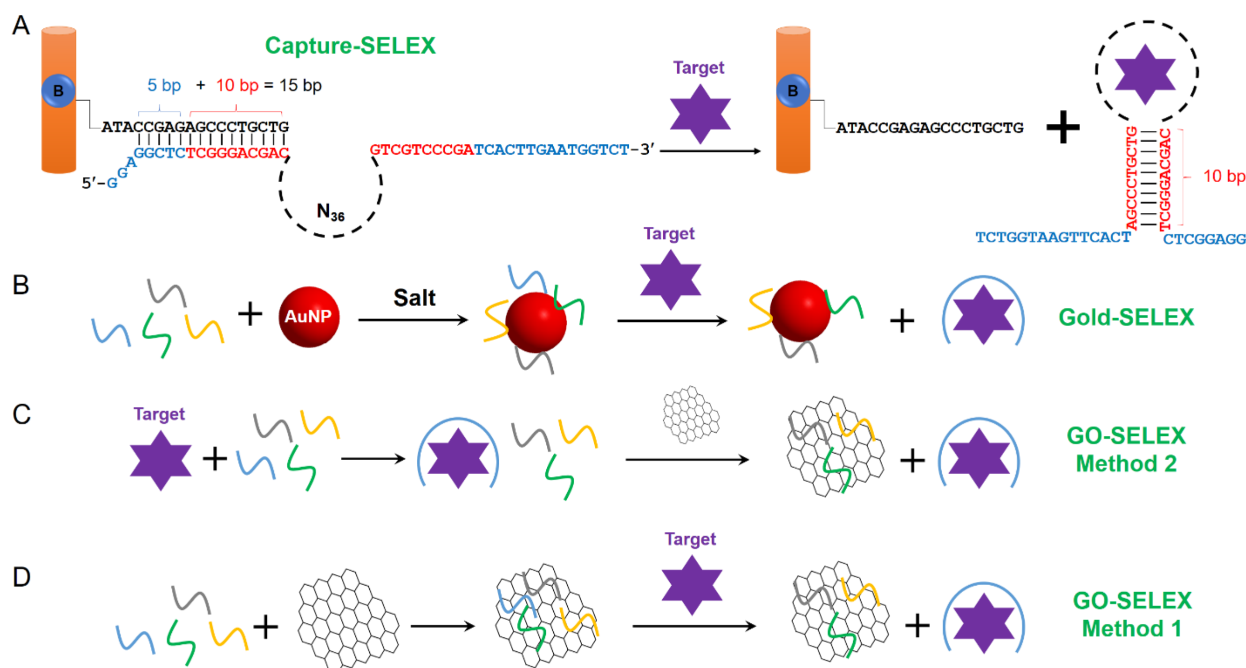

Figure 1. The three library-immobilization SELEX methods tested in this study: (A) capture-SELEX by hybridization, (B) gold-SELEX by adsorption on AuNPs, and (C) GO-SELEX by inhibited adsorption on GO (method 2). (D) Another GO-SELEX method (method 1), in which target binding is believed to induce aptamer desorption from GO, and this method was not tested in this work. The same library was used in all these studies, which contains an N_{36} random region flanked by two constant regions that can form a 10-bp stem.

Aptamer separation efficiency measured by qPCR

To compare the aptamer selection efficiency of the three methods, we spiked 1% of the round 9 library from our previous ATP selection[24] into a naïve library and performed one round of selection by adding 100 μM adenosine as a target. The partition of immobilized (mostly non-aptamer) and free DNA (potential aptamers) was first quantified using qPCR based on a standard curve (Figure S1). Figure 2A shows an example for the capture-SELEX method. The qPCR traces were normalized, and the PCR cycle number at a 50% fluorescence increase was compared. Each PCR cycle difference reflected a two-fold difference in the initial DNA concentration.[31, 32]

After immobilizing 100 pmol of the spiked library DNA via hybridization to streptavidin-coated agarose beads, the qPCR cycle of non-hybridized library DNA was is 11.4 (corresponding to an absolute initial DNA quantity of 0.85 pmol), and thus, the fraction of hybridized library was 99.1% ± 0.4%. Such a high hybridization efficiency was attributed to the use of excess biotinylated complementary DNA (5:1). The immobilized library was then subjected to extensive washing by buffer to remove unbound or weakly bound DNA. To quantify such nonspecific release, we washed the beads 12 times with SELEX buffer (each time 500 µL), and collected the last two washing eluents. They represented only 0.13% ± 0.01% of the immobilized library (Figure 2B, blue bar for the capture-SELEX set). Therefore, at this point, the majority of the library sequences were stably hybridized to the DNA on the beads.

After adding 100 µM adenosine, 0.41% ± 0.05% of the library was eluted (Figure 2B red bar in the capture-SELEX set). Therefore, most of the library was still immobilized after adding adenosine. This is reasonable since we only spiked 1% of the enriched library and the remaining 99% of the sequences were random and not expected to bind to adenosine. This extra 0.41% - 0.13% = 0.28% increase suggests selective aptamer enrichment, and at the beginning of the next round, 0.28%/0.41% = 68% of the eluted library could be aptamers. In other words, aptamer abundance increased from below 1% to around 68%, based on qPCR measurements.

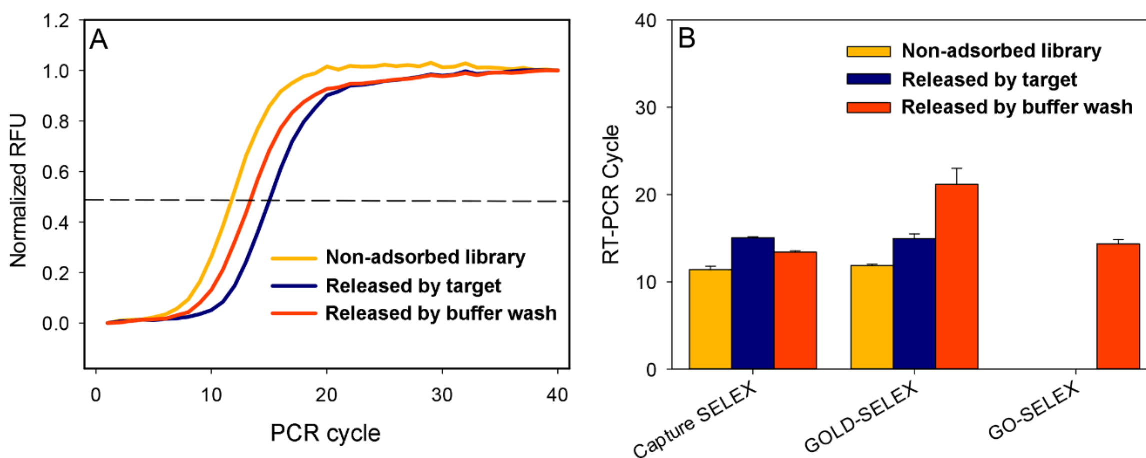

Figure 2. (A) Normalized qPCR fluorescence traces for the capture-SELEX sample. The target eluted trace has a lower PCR cycle number indicating it contained more initial DNA compared to the buffer wash sample. (B) The number of qPCR cycles for the three SELEX methods. The cycle is defined based on the 50% fluorescence increase and the error bar is from the standard deviation

of three independent measurements. For the GO-SELEX experiment, since method 2 was used, it only has one bar.

We then did a similar measurement for the gold-SELEX system, and 1.2% ± 0.2% of the library remained in the supernatant after adsorption by AuNPs (Figure 2, orange bar in the gold-SELEX set). Therefore, our 13 nm AuNPs (concentrated from 10 nM to 60 nM) absorbed most of the 100 nM library in the system (100 pmol library dissolved in 1 mL). Washing the library with water further decreased the DNA in the supernatant to 0.14% ± 0.06% (Figure 2, blue bar). After 1 h of incubation with 100 μM adenosine, only 0.003% ± 0.002% of the library was eluted released (Figure 2 red bar). Thus, most of the library remained adsorbed by AuNPs. In the capture-SELEX experiment, the blue bar is higher than the orange bar, which is expected for aptamer enrichment. However, in the gold-SELEX, the blue bar is even lower. The very low amount of DNA released from the AuNPs suggests that most spiked aptamers were still adsorbed onto the AuNPs. This result can be explained by the strong adsorption of DNA by AuNPs,[33] and adenosine could not compete with AuNPs for binding to DNA.

For GO-SELEX, we used a final of GO concentration of 221 μg/mL to adsorb 100 pmol of the library in 1 mL in the presence of 100 μM adenosine. After 1 h incubation, non-adsorbed DNA was separated by centrifugation and quantified using qPCR. Only 0.22% ± 0.07% of the library was in the supernatant, and the majority of the library was adsorbed onto GO (Figure 2, red bar). Since we used the inhibited adsorption method (method 2) for GO-SELEX, there were no buffer wash data. Nevertheless, since only 0.22% of the library was in the supernatant and we expected to have close to 1% aptamer in the library, most of the aptamer sequences in the library were adsorbed by GO, even in the presence of 100 μM adenosine.

For all three methods, the fraction of released library DNA in the presence of adenosine was quite low (< 1%). AuNPs had the highest DNA adsorption affinity, resulting in the lowest level of non-adsorbed DNA. Since the aptamer content in the library was below 1%, had the released DNA been significantly more than 1%, and the majority of the released DNA would be non-aptamers, which would likely lead to failed selections. Therefore, the low fractions of DNA released in our experiments suggested a reasonable condition for SELEX.

Separation efficiency measured by deep sequencing

Although qPCR can measure the concentration of released DNA, it provides no information on the sequence distribution. We then used deep sequencing to evaluate the enrichment of three adenosine-binding aptamers: Ade1301, Ade1304, and the classical aptamer. The classical aptamer reported by Huizenga and Szostak has a K_d of approximately 6 μM adenosine,[9] whereas the K_d of the Ade1301 and Ade1304 aptamers was approximately 200 nM.[24] We spiked 1% of the round 9 library from our previous ATP selection, which contained 48.5% Ade1301, 7.5% Ade1304, and 12.6% classical aptamers. We then sequenced the 1% spiked library, and the fractions of these aptamers were 0.7% Ade1301, 0.11% Ade1304, and 0.21% classical aptamers (Figure 3A, before SELEX), and the result was in agreement with the spiked amount.

We then sequenced the DNA in the supernatants for the three selection methods after adding 100 μM of adenosine. Approximately 42800 sequences were obtained for each sample, allowing statistical analysis. In the capture-SELEX method (Figure 3B, red bar), the Ade1301 aptamer was enriched 48.3-fold (from 0.7% to 35.9%). Similarly, the Ade1304 and classical aptamers were enriched 50.7-fold and 30.4-fold, respectively. The higher K_d value of the classical aptamer can explain its lower enrichment compared to the other two aptamers. The overall fold of enrichment was comparable to that estimated by qPCR (68-fold). After this round of selection, nearly half of the resulting library was aptamers (Figure 3A), indicating that capture-SELEX is an efficient method for aptamer selection.

In contrast, for gold-SELEX (Figure 3B, orange bar), the fractions of the Ade1301, Ade1304, and classical aptamers in the supernatant were only 0.3%, 0.08%, and 0.09%, respectively. Compared to the spiked library before adding adenosine, the abundance of the aptamers even decreased. This decrease is likely due to the aptamers being rich in purines, which have a relatively higher affinity to AuNPs.[34] Therefore, adenosine released very few DNA strands from the AuNPs, and the released DNA contained even fewer aptamer sequences than the initial library. Therefore, gold-SELEX cannot effectively enrich aptamers for adenosine.

For GO-SELEX (Figure 3B, yellow bar), the fractions of adenosine-released Ade1301, Ade1304, and classical aptamers were 0.8%, 0.08%, and 0.17%, respectively. The corresponding enrichment were 1.02, 0.82, and 0.82-fold (1-fold indicates no enrichment). Therefore, only Ade1301 was slightly enriched, while the overall aptamer content decreased. The decreased

relative abundance of aptamers could also be explained by the purine-rich aptamer sequences.[35] Compared to typical aptamers used in biosensors, which are truncated shorter sequences,[20] the library sequence is much longer with two additional primer binding regions that can interact with the GO surface regardless of aptamer binding. This may explain the poor efficiency of the aptamer enrichment. The error rate of Illumina sequencing is between 0.1 and 1%,[36] and our entire library length is below 80-nt. In addition, we examined three different sequences and they all gave consistent results. Therefore, our results are statistically significant.

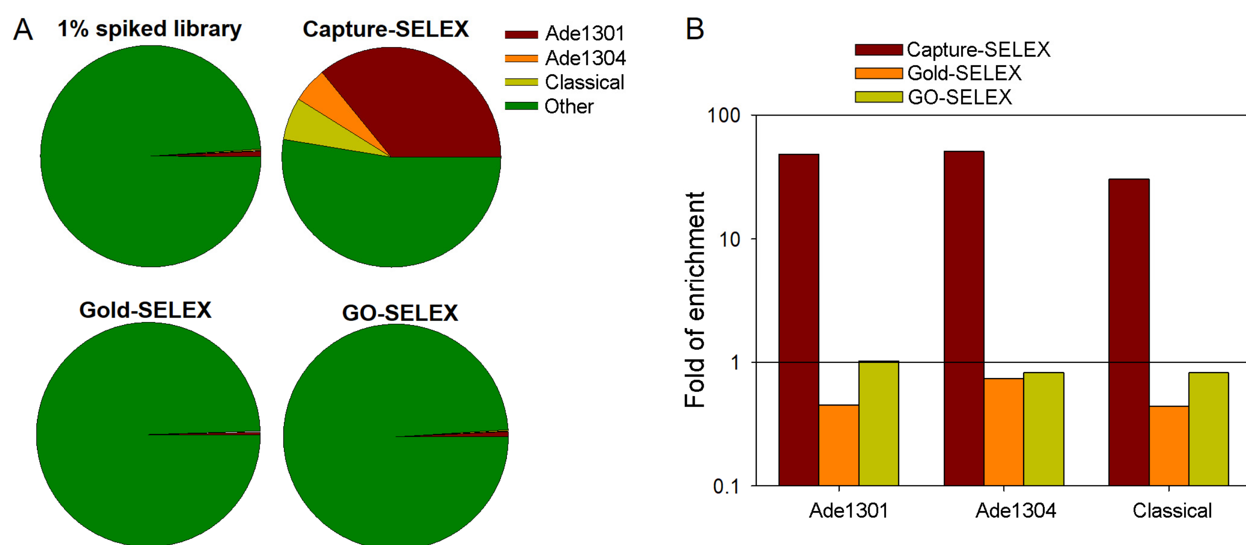

Figure 3. (A) Pie charts for the library compositions before and after the one-round selection using the 1% spiked library of the three SELEX methods. (B) The fold of aptamer enrichment after the one-round selection, where 1-fold means no enrichment of the aptamer sequence.

Blocked GO-SELEX

The poor enrichment of aptamers in the GO-SELEX was a bit surprising, since aptamer sensors based on the same principle have been repeatedly demonstrated by many labs.[37] We attributed the poor aptamer enrichment to the primer binding regions. The library has two constant primer binding regions, and its total length is approximately 40-nt. Despite the base paired region designed in our library, there were still 21 free nucleotides that could interact with GO.

To test this hypothesis, we blocked the primer binding regions by adding complementary DNA strands (Figure 4A). Because the adsorption of duplex DNA to GO is much weaker than the adsorption of single-stranded DNA,[27] we expected this system to have better enrichment. This blocked library was also subjected to one round of GO-SELEX. The DNA eluted collected in the presence of the target was 0.36% ± 0.19% (Figure S2), which was compared with the non-blocked experiment. Deep sequencing revealed that the three aptamers reached 1.14, 1.11, and 1.14-fold increase, respectively, which were all higher than the non-blocked (Figure 4B). This result indicated that GO-SELEX could, in principle, work when the primer binding regions are blocked. Nevertheless, compared to the ~50-fold enrichment in the capture-SELEX, the blocked GO-SELEX enriched less than 20%, which was over 200-fold less efficient than capture-SELEX.

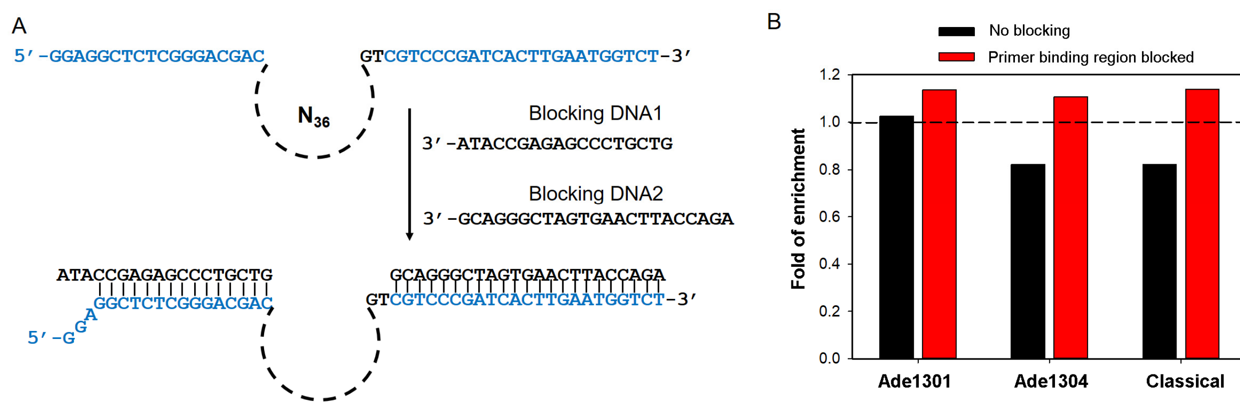

Figure 4. (A) A scheme of blocking the library by adding two complementary DNA strands to hybridize with the primer binding regions, which may decrease nonspecific adsorption to GO. (B) The fold of aptamer enrichment after the one-round blocked GO-SELEX, where 1-fold means no enrichment of aptamer sequences.

Thermodynamic aspect of library-immobilization SELEX

Many nanomaterials can adsorb DNA and have been used to develop aptamer-based biosensors.[38] Among them, GO was the first studied and the most used.[20, 39] These sensing studies have inspired researchers to use the same strategy for aptamer selection, and the hope was to specifically release aptamer containing sequences from nanomaterials. In this work, we studied GO and AuNPs as two representative nanomaterials, which have different adsorption strengths for DNA.[33] We also studied the hybridization-based capture-SELEX method. For any library-immobilized SELEX, the

assumption is that aptamer/target binding is stronger than the force for aptamer immobilization, and adding target molecules can shift the equilibrium to aptamer/target binding. Herein, we discuss the energy aspects of each of the selection methods.

For the capture-SELEX method, the library was immobilized via a 15 bp duplex. After binding to a target, the aptamer formed a hairpin structure with a 10 bp stem (Figure 5A). Considering that a hairpin has a higher melting temperature than two separate DNA strands of the same sequence,[40] the energy difference that aptamer binding requires is less than that of 5 bp. Based on the success of structure-switching (or strand displacement) design of many aptamers,[41, 42] aptamer binding can supply such energy. This aptamer release reaction is not dependent on the K_d of an aptamer, since even weakly binding aptamers can be released by using a sufficiently high target concentration (e.g., higher than the K_d of the aptamer). That's why aptamers with K_d values as high as 5 mM, such as the glucose aptamer, can still be isolated.[3] Recently, the Stojanovic group performed a systematic thermodynamic analysis of aptamer binding to target molecules containing different functional groups.[43] The ΔG of the displacement reaction (aptamer binding to a target molecule to displace the aptamer from the capture strand) was calculated to range from -7 to -40 kJ/mol for aptamers with K_d values ranging from low nM to mid-mM. Since target molecules can shift the equilibrium of aptamers but do not disrupt DNA hybridization (except when high concentrations of hydrogen bond disruptors, such as urea, or hydrophobic solvents are added), capture-SELEX showed good aptamer enrichment.

The above discussion is based on thermodynamics. Another note is that the 15 bp used to hybridize the library can be divided into two regions containing 5 and 10 bp, respectively (Figure 1A). Once an aptamer is bound to a target forming the 10 bp stem, the remaining 5 bp cannot stably hold the library strand, leading to its dissociation (Figure 5A). Therefore, this design lowers the kinetic barrier of the reaction. If aptamer binding needs to compete with the full 15 bp, the released of aptamer would be much lower, which was beautifully demonstrated by Nutiu and Li in their initial publication of structure-switching aptamers.[42] Therefore, a delicate balance between stable hybridization and aptamer release needs to be achieved.

GO can adsorb DNA with a range of affinities.[44, 45] In particular, based on the excellent performance of GO for the detection of adenosine/ATP,[37] one would expect GO to be a good surface for enrichment of adenosine aptamers. In the case of GO-SELEX, the assumption was that

aptamer/target complex cannot be adsorbed, but non-aptamer sequences are stably adsorbed. This assumption might be true for truncated aptamers, which were used in GO-based biosensors.[23] However, the library also contained primer binding regions that were of comparable length to the random region (Figure 5B). We have already demonstrated the adverse effect of the primer binding regions above.

For the gold-SELEX method, DNA adsorption was achieved via strong coordination interactions between DNA base and AuNPs, which is greater than 100 kJ/mol for each base.[46, 47] The major interactions between an aptamer and target molecules are much weaker π-π stacking, electrostatic interactions and hydrogen bonding.[48] The energy of hydrogen bonding is around 25 to 40 kJ/mol and the energy of π-π stacking is even lower (~10 kJ/mol).[49] Thus, the weaker binding affinity between aptamers and targets cannot compete with the stable adsorption of DNA to gold. This is why barely any aptamers were desorbed from AuNPs even for truncated aptamers.[33] For the DNA library with primer binding regions, the desorption of DNA it is even more difficult.

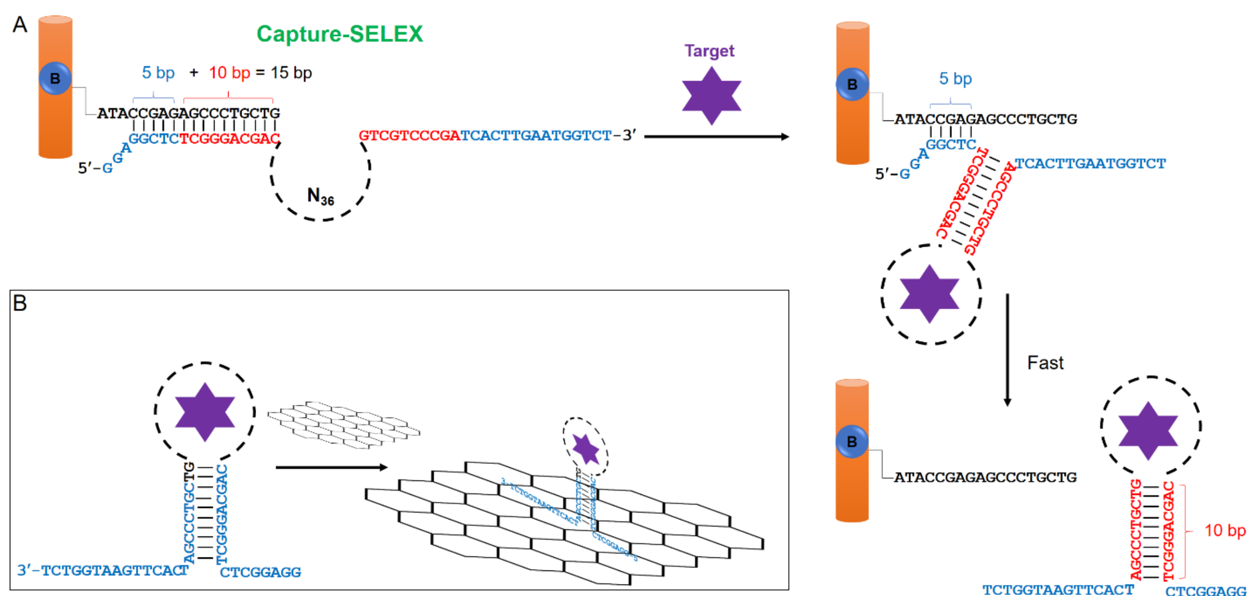

Figure 5. Schemes showing the immobilization of DNA in (A) capture-SELEX, and (B) GO-SELEX. In the capture-SELEX, the library was immobilized via a 15 bp region to the biotinylated capture strand. After binding with a target, the aptamer can form a hairpin structure with a 10-bp stem. In GO-SELEX, it is possible to have adsorption achieved via the primer binding regions even after binding to the target.

Enrichment factor and SELEX

Based on the above data, it was evident that different DNA immobilization methods resulted in significant differences in aptamer enrichment. Here, we mathematically describe the enrichment factor. Assuming the fraction of aptamer sequences in a library at round n is f_n, and at round n+1 is f_{n+1}, the enrichment factor is defined as f_{n+1}/f_n. After adding a target to the round n library, the round n+1 library is collected. Since more aptamers are expected to be released compared to non-aptamer sequences, we define a and b as the fraction of collected aptamers and non-aptamers, respectively. Therefore, in round n+1, the fraction of the aptamer is $f_{n+1}=\frac{af_n}{af_n+(1-f_n)b}$, and the enrichment factor is $f_{n+1}/f_n=\frac{a}{af_n+(1-f_n)b}$. It was estimated that for a naïve library, the chance of having aptamer sequences is about one in 10^{10} sequences ($f = 10^{-10}$).[12] 10^{10} is roughly equal to 4^{17}, suggesting that a typical aptamer has around 17 conserved nucleotides, which is true for many reported aptamers.[3, 24, 50] Therefore, for the initial rounds, $f_n \ll 1$, and $f_{n+1}/f_n \cong a/b$. Different target concentrations and different K_d values of aptamers can result in different enrichment factors. For the capture-SELEX using adenosine, the enrichment factor is around 50 for the 0.2 µM K_d aptamers using 100 µM adenosine. The enrichment factor of the classical aptamer is ~30. We plotted the theoretical enrichment curves as a function of the number of selection rounds with a/b ratios of 1, 2, and 10, respectively (Figure 6). After 11 rounds of selection, the a/b =2 sample would only be enriched slightly above 1 ppm (expected to see only 1 copy of aptamer if sequencing 1 million DNA). In blocked GO-SELEX, a/b was less than 1.2 in our system, and after 10 rounds, aptamers would still blow 1 ppb, whereas the a/b =10 sample is enriched to nearly 100% aptamer after 9 rounds. So, more rounds of selections are needed to select aptamers with a poor aptamer enrichment factor. For a/b =1, aptamers are barely enriched, regardless of the round of selection.

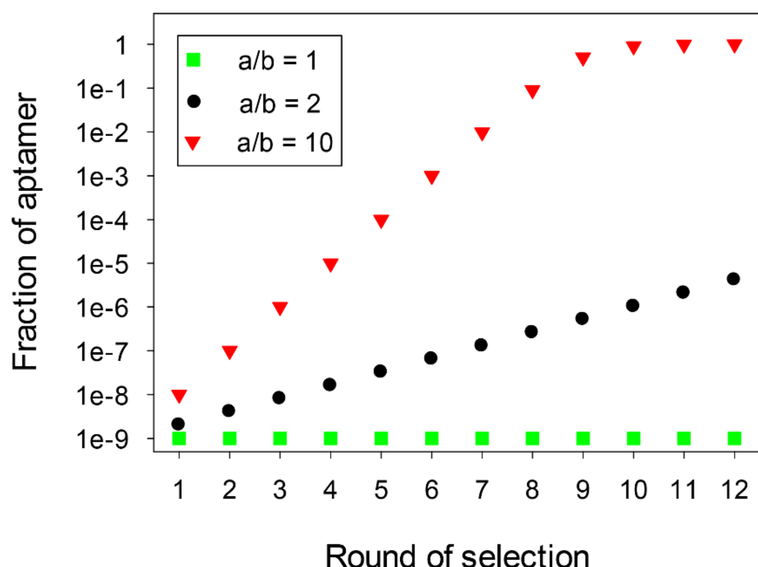

Figure 6. Faction of aptamers during different rounds of selection with three *a*/*b* ratios, which describes the ratio of specific aptamer release over nonspecific DNA dissociation.

For the two SELEX methods based on library adsorption, the enrichment factor was close to 1 for GO, but lower than 1 for AuNPs. Therefore, for adenosine, the chance of enriching aptamers is very low for either GO-SELEX or gold-SELEX. GO and AuNPs are two representative nanomaterials. GO has a lower DNA adsorption strength compared to AuNPs. For even weaker adsorbing materials, one may run into the problem of having large *b* values, and the enriched DNA could be those with poor adsorption affinity instead of good aptamers. For stronger adsorbing materials, the target may hardly induce DNA desorption and result in very low *a* and *b* values. Based on our understanding of nanomaterials, it is unlikely that other nanomaterials can do better than GO, such as carbon nanotubes and iron oxide magnetic nanoparticles.[51] This is because of two counter-acting factors. On one hand, it is desirable to have the library strongly adsorbed to the surface to avoid a high background release of DNA sequences. However, such strong adsorption can inhibit aptamer release. Surface adsorption is often nonspecific and each DNA base can contribute to the adsorption. It is difficult to ask aptamer binding to overcome all the adsorption forces at the same time.[52]

The hybridization-based capture-SELEX method showed a high enrichment factor (*a*/*b* ratio). Importantly, it allows precise tuning of *a* and *b* values by simply changing the number of

base pairs. For example, increasing the number of base pairs in the capture strand can decrease the *b* value.

In principle, both adsorption-based selection methods only considered target/aptamer binding and the adsorption of DNA, whereas the adsorption of target molecules is neglected. Therefore, in theory, our observations should be generalized to all small-molecule targets. In practice, however, the adsorption of the target molecules can affect the adsorption of DNA. In this study, we used adenosine as a model target molecule, which has strong interactions with both AuNPs[53] and GO.[35] For gold-SELEX, the released DNA might be even less if a non-adsorbing or weakly adsorbing target is used.

For GO-SELEX without blocking the primer binding regions, the majority of the DNA library was adsorbed in the presence of adenosine. Therefore, adenosine binding to aptamers in the library did not prevent the adsorption of adenosine/aptamer complexes. The likelihood of success by using even weaker adsorbing molecules is thus also low. For stronger adsorbing targets (e.g. in an extreme case if proteins are used as a target for GO-SELEX), proteins may serve as a blocker to inhibit the adsorption of DNA, regardless of sequence,[54] and selective enrichment of aptamer can hardly be achieved either.

Conclusions

Over the last few decades, numerous aptamer selection methods have been reported. However, a quantitative understanding in aptamer enrichment is lacking, which may be partially responsible for the report of some non-binding aptamers.[55, 56] In this study, we compared aptamer enrichment factors of three library-immobilization SELEX methods: capture-SELEX, GO-SELEX, and gold-SELEX. Instead of performing full selections, we utilized a spiked library containing multiple DNA aptamers with varying affinities to adenosine. To evaluate aptamer enrichment, we employed qPCR and observed that all three methods exhibited minimal DNA release, with less than 1% of the DNA released in the presence of 100 µM adenosine. Notably, the release of DNA from the AuNPs was particularly negligible. To further assess the enrichment of specific aptamers, namely Ade1301, Ade1304, and the classical aptamer, we employed deep sequencing. The results revealed that only the capture-SELEX method demonstrated significant enrichment, while the other two methods exhibited enrichment factors even below 1. The GO-SELEX can be slightly improved by blocking the primer binding regions, but the enrichment factor was still over 200-fold lower

compared to capture-SELEX. Our findings indicated that capture-SELEX is a more efficient approach for enriching aptamers. For a new SELEX method, using spiked library is a useful way to measure its enrichment factor and thus reliability of the method.

Supporting Information

DNA sequences used, experimental condition details of literature reported GO-SELEX and Gold-SELEX work, qPCR standard curve, qPCR of blocked GO-SELEX results.

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