Review

SELEX-based DNA aptamer selection: a perspective from the advancement of separation techniques

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Abstract

DNA aptamers, which are short, single-stranded DNA sequences that selectively bind to target substances (proteins, cells, small molecules, metal ions) can be acquired by means of the systematic evolution of ligands by exponential enrichment (SELEX) methodology. In the SELEX procedure, one of the keys for the effective acquisition of high-affinity and functional aptamer sequences is the separation stage to isolate target-bound DNA from unbound DNA in a randomized DNA library. In this review, various remarkable advancements in separation techniques for SELEX-based aptamer selection developed in this decade, are described and discussed, including CE-, microfluidic chip-, solid phase-, and FACS-based SELEX, along with other methods.

Keywords:
DNA aptamer, SELEX, separation techniques
1. DNA aptamer and in vitro selection

1-1. Nucleic acid aptamer

The nucleic acid aptamer is a short, single-stranded (ss) DNA or RNA sequence that selectively binds with a target molecule such as an antibody. (The term “aptamer” was coined from the Latin “aptus,” meaning fit, and the Greek “meros,” meaning part). Compared with antibodies, which are immunoglobulin proteins composed of 20 amino acids, aptamers are composed of only four types of nucleic acids (A, G, C, and T [or U] for DNA [or RNA]; where A = adenine, C = cytosine, G = guanine, T = thymine, and U = uracil). While the diversity of sequence patterns and chemical interactions between the aptamer and the target molecule is poorer than that of the antibody, the feasibility in terms of synthesis, chemical modification, chemical and thermal stability, and experimental handling makes nucleic acid aptamers a unique and useful molecular system.

Aptamers generated by systematic evolution of ligands by exponential enrichment (SELEX or in vitro selection) were first reported individually by Szostak and Gold in 1990.\textsuperscript{1,2} The aptamer recognizes a target by the formation of unique and various higher order structures (three-dimensional shapes) via intramolecular interaction (hairpin loop, bulge, G-quadruplex [G4], double helix, triplex, base-phosphate zipper).\textsuperscript{3,4} It then binds to the target molecule via hydrogen bonding and hydrophobic interactions originating from their nucleobases and sugar groups as well as electrostatic interactions by the phosphate groups. It has been proven that aptamers can bind with various molecules such as proteins,\textsuperscript{5} cells,\textsuperscript{6} metal ions,\textsuperscript{7} and small organic molecules\textsuperscript{8} with high selectivity and stability (with a dissociation equilibrium constant, $K_d$, in the micromolar to picomolar range). Since aptamers provide attractive chemical properties, a number of studies have reported on versatile applications of nucleic aptamers,\textsuperscript{9} such as aptasensors,\textsuperscript{10}
pharmaceuticals,\textsuperscript{11,12} drug delivery systems,\textsuperscript{13} enzyme-linked oligonucleotide assays (ELONA), or enzyme-linked apta-sorbent assays (ELASA).\textsuperscript{14}

\section*{1-2. DNA aptamer selection (SELEX)}

Nucleic aptamers can generally be acquired by the SELEX methodology. In SELEX, the aptamer sequences are selected from the initial randomized ssDNA library (or RNA library after transcription) (see Figure 1 for the processes). An almost completely randomized library can be purchased from reagent suppliers. The randomized library sequence is composed of two primer regions (around 20 bases each) located at the 3' and 5' ends and an $N$-base randomized region between two primer regions (around 20–60 bases each), which are employed in polymerization chain reaction (PCR) amplification. Although a library with an $N$-base randomized region theoretically has $4^N$ sequences, the number of sequences served in the selection process is actually $10^{11}$–$10^{15}$ owing to the limitation of sample handling.

The library is incubated with target substances, followed by separation of the target–DNA (or target–RNA) complexes from free (unbound) DNA (or RNA) molecules. The recovered (partitioned or fractionated) DNA, including target-bound sequences (called the selection pool), is amplified by PCR, and then ssDNA from the pool is prepared for the next selection “round” (for RNA aptamers, reverse transcription is necessary). The series of binding, separation, fractionation, PCR amplification, and single stranding in the selection process is called a “round.”

While the population of DNA sequences in the selection pool is at a trace level in the first round, the aptamer sequences strongly binding to the target substance are enriched in the pool by repeating rounds (including some rounds of counter [negative]
selection in which ssDNA unbound to a non-target substance is partitioned) for aptamer selectivity. The enriched sequences are determined by cloning with electrophoretic sequencing or next-generation sequencing (NGS). The affinity of the sequences obtained is evaluated based on the dissociation equilibrium constant ($K_d$) and dissociation rate constant ($k_{off}$) measured by a binding assay (for example, surface plasmon resonance [SPR], capillary electrophoresis, or bio-layer interferometry).

In the past decade, more research on DNA aptamers than on RNA aptamers has been reported because the selection process is more rapid, simple, and reliable compared with that for RNA. In this paper, research on DNA aptamer selection is exclusively reviewed.

1-3. Subject of this review
To date, various improvements to obtain high-affinity DNA aptamers have been reported. The strategies can be classified as follows: 1) initial library, 2) separation techniques, 3) sequencing, 4) post-sequencing treatment and modification, and 5) a combination of 1–4. There are many reviews of DNA aptamers with respect to the progress of discovery and functionalization of aptamers (for example, ref. 5, 9, 13). In this review, we mainly focus on new separation (and partitioning) principles and technologies and those coupled with other techniques reported in the past decade. The separation stage of the process is one of the most important factors in obtaining aptamer sequences, because the separation properties and efficiency are directly related to the success of aptamer acquisition for various targets as well as binding affinity and function of the aptamer obtained. Thus, choosing the type of separation technique is frequently a key to accessing the desired aptamers.

Conventional separation of target–DNA complexes and free DNA is conducted.
using magnetic bead-based technology for proteins, which are the targets of DNA aptamers in most cases. The target proteins are immobilized onto microbeads through covalent bonding, and the beads containing target–DNA complexes are isolated from a mixture of beads and ssDNA libraries by a magnetic field. This method usually requires 5–10 rounds for acquisition because the resolution efficiency is not very high. In addition, the negative effect of immobilizing the target is of concern. When live cells are the target substance, the cell–DNA complexes are separated by centrifugation for floating cells or by washing from adherent cells (called cell-SELEX). In these cases, 10–30 rounds are necessary for enrichment of aptamer sequences. While there have been fewer reports on DNA aptamers for small molecules than for proteins and cells, due to the difficulty of binding/free separation, efficient separation techniques suitable for small molecules have been developed (vide infra). The separation techniques targeting proteins, cells, and small molecules have been improved and are reviewed in this paper. In addition, the suitability of each separation technique for structure-switching aptamers, unnatural DNA aptamers, and slow off-rate aptamers is also reviewed.

Improvements in the separation stage of the round process can be categorized as follows: 1) decrease in the number of rounds by higher separation efficiency (ex. use of capillary electrophoresis [CE], microfluidic chip, and fluorescence-activated cell sorting [FACS]), 2) development of separation methods suitable for targets (ex. cells, small molecules), 3) development of separation methods suitable for unique libraries, and 4) development of separation techniques that enable access to aptamer functions (ex. slow off-rate modified aptamer (SOMAmer), xeno nucleic acids [XNA]); combinations of 1–4 are also used. Each approach is mapped in Figure 1.

It should be noted that only remarkable advancements in separation in the field
of DNA aptamer selection, to the best of our knowledge, are followed in this paper. This article reviews the advanced separation technologies applied to DNA aptamer selection, and the parameters indicating separation efficiency and affinity of aptamers obtained are stated as much as possible: the number of bases in the randomized library, $N_{\text{lib}}$; the number of rounds, $N_{\text{round}}$; and the dissociation equilibrium constant, $K_d$.

2. Selection methodologies from the perspective of separation

2-1. Capillary electrophoresis-based selection

Since the pioneering work in CE application to SELEX by Bowser et al. in 2004 (for human IgE, $N_{\text{lib}} = 40$, $N_{\text{round}} = 4$, $K_d = 23–30$ nM; and for neuropeptide Y, $N_{\text{lib}} = 40$, $N_{\text{round}} = 4$, $K_d = 300$ nM–1 μM), it has been well known that the high resolution of CE is very effective for aptamer selection. In CE-SELEX, the sample (a mixture of target DNA and the DNA library, including the target–DNA complex) is separated, and the order of migration is generally protein, complex, and free ssDNA according to their unique electrophoretic mobility, $\mu$, ($\mu = q/6 \pi r \eta$; here, $q$, $r$, $\eta$ represent the charge, radius of the ion, and viscosity, respectively) in the presence of electroosmotic flow (EOF). Since ultratrace complexes are undetectable in CE even with laser-induced fluorescence detection, partitioning is conducted from the protein peak to the rising point of the ssDNA peak.

Aside from the very high resolution of CE, another advantage of CE-SELEX is that the complex fraction migrates before free DNA does, which is effective in avoiding contamination from the free DNA fraction. Thus, CE can decrease $N_{\text{round}}$ to $< 4$ rounds. It should be emphasized that no species to be separated are immobilized in a free aqueous solution in CE. While CE separation is almost ideal for aptamer selection, there are two
drawbacks: fewer sequence patterns ($10^{11}$–$10^{12}$) are made available for selection due to its low injection volume (some nL to some hundreds of nL), and also, the necessity for the target-DNA complexes to demonstrate an electrophoretic mobility shift in order to be separated (that is, the ratio of $q$ to $r$ should be changed as a result of complexation). For protein targets (relatively large molecules), the $\mu$ of the complex (with polyanionic DNA) significantly differs from that of the native protein in most instances, thus allowing for effective partitioning. However, it is difficult to obtain aptamers for small molecules by CE-SELEX because a mobility shift rarely occurs through complexation without changes in $q$ and $r$ of the DNA during complexation with the small molecule target. While DNA aptamers for $N$-methyl mesoporphyrin (molecular weight (MW) 580) were found by CE-SELEX ($N_{\text{lib}} = 40$, $N_{\text{round}} = 3$, $K_d = 0.88$–$43$ μM), and the observed aptamers catalyzed the metal insertion reaction of mesoporphyrin with two-fold rate enhancement, there have been few other reports of small molecules found by CE-SELEX. Krylov et al. proposed an approach similar to CE-SELEX, called non-SELEX, in which partitioned samples containing complexes are successively served for separation without PCR amplification ($N_{\text{lib}} = 39$, $N_{\text{round}} = 3$ for h-Ras protein). Since then, many DNA aptamers binding to proteins have been found by means of CE-SELEX and non-SELEX (see ref. 5, for example). There seem fewer reports on non-SELEX than on CE-SELEX, since PCR amplification in CE-SELEX provides a higher possibility of finding aptamer candidates.

A study reported the improvement of non-SELEX combined with nitrocellulose membrane filtering to reduce nonbinding sequences prior to CE-based partitioning (dual-partitioning). The advantage of this approach compared to the usual CE-based selection is the large diversity of the initial library (over $10^{15}$ sequences). This approach reduces the selection process to two rounds of CE partitioning and has resulted in the
identification of an aptamer for cholesterol esterase ($N_{lib} = 40, K_d = 203 \text{ nM}$).\textsuperscript{20} Regarding cell targets, Kim et al. recently suggested that repetitive centrifugation, to partition DNA bound to bacterial cell targets and unbound DNA, combined with non-SELEX is effective for removal of unbound DNA ($N_{lib} = 45, N_{round} = 20$ [partition cycles but without PCR], $K_d = 3.9–10 \text{ nM}$ for \textit{Escherichia coli}).\textsuperscript{21}

To improve the separation of protein–target complexes from free ssDNA, low pH CE-SELEX (Lp-CE-SELEX) was developed. In this process, the positively charged protein, protein–ssDNA complex, and negatively charged ssDNA library migrate oppositely without EOF at pH 2.6.\textsuperscript{22} While separation and partitioning were conducted for three model proteins (transferrin, bovine serum albumin, and cytochrome c) in the proof-of-concept study ($N_{lib} = 40$), there is room for discussion about the feasibility of this selection mode since no aptamer sequence was determined and the affinity was not evaluated. Recently, Le et al. reported a separation mode using CE in which each of complexed and nonbinding ssDNA (and ssDNA dissociated from the complex) migrates in opposite directions because of appropriate pressure applied to the capillary, termed ideal-filter capillary electrophoresis (IFCE).\textsuperscript{23} With this method, a high-affinity DNA pool was successfully obtained by one step (single round) selection for the MutS protein. The efficiency of partitioning was shown to be $10^9$ partition efficiency (PE; the ratio of the number of DNA served for selection to the number of partitioned DNA) and is the highest value reported so far; a PE of $10^2$ is typical for solid-phase partitioning methods. However, more solid evidence of the usefulness of IFCE selection has yet to be reported, since aptamer sequences, $K_d$, and $k_{off}$ have not been determined. In addition, it is unknown what kinds of targets are suitable for IFCE selection.

An online, reaction-based, single-step CE-SELEX (ssCE-SELEX) mode was
proposed using human thrombin as a model target. In ssCE-SELEX, the six procedures in CE-SELEX (sample mixing, incubation, reaction, separation, detection, and collection) are performed in a single CE run by on-capillary zone mixing to identify thrombin binding aptamers ($N_{lib} = 40$, $N_{round} = 2$, $K_d = 56–177$ nM). Additionally, ssCE-SELEX has been successfully employed in the acquisition of aptamers for bovine lactoferrin ($N_{lib} = 40$, $N_{round} = 2$, $K_d = 20$ nM) for CE-aptasensor application.

The fraction collection (partitioning) approach in CE has also improved. For streptavidine, Luo et al. undertook a multiple-partitioning approach, called FCE-SELEX, with 12 fractions and an oil seal in a recovery vial to avoid contamination. The results from a single round showed two fractions with an enriched DNA pool, monitored by real-time PCR ($N_{lib} = 40$, $N_{round} = 1$, $K_d = 30.6$ nM for the obtained pool without determination of the sequencing). Saito et al. reported a new on-capillary concentration-fractionation method combining capillary transient isotachophoresis (ctITP) concentration of ssDNA with precise partitioning using two UV/Vis detection CE systems, which was successfully applied to aptamer selection and purification of a ssDNA library before selection.

The effect of the ctITP concentration mode occurring prior to the transition to separation mode (including fraction collection) in CE-SELEX was evaluated. It was shown that ctITP improved the separation efficiency to obtain a narrower ssDNA peak, and that aptamer sequences for thrombin as a model protein were effectively accumulated in the pool by a factor of $10^6$ with high resolution and reproducibility of fraction collection.

With respect to cell targets, Saito et al. succeeded in obtaining aptamers with a single-round selection for gram negative and gram positive bacteria and fungi as well as human lung cancer cell lines using a unique separation mode called polymer-enhanced capillary transient isotachophoresis (PectI). While the effectiveness of CE
separation on cell-SELEX has been implied by other research groups (without determination of sequences and $K_d$). The electrophoretic control of cells was very difficult because of low reproducibility, resulting in the appearance of a lot of peaks in the electropherogram. In PectI mode, a single peak for the cell was observed with high repeatability; the target cells and unbound ssDNA were each concentrated into a narrow zone, and the zone of the cells was retained by the addition of a water-soluble polymer (polyethyleneoxide 600,000) to the migration buffer, providing a simple electropherogram with only two peaks for the cells and ssDNA. Since the cell peak contains cell–DNA complexes, the peak was partitioned using the two-point detection CE system to obtain the selection pool. The advantages of this approach compared with cell-SELEX are: 1) high resolution by concentration of both cells (particles) and ssDNA (molecules) without immobilization; 2) detectability of the target–DNA complex peak, which is undetectable in most CE-based selection methods; and 3) sufficient sequence patterns from the large injection volume (500–1000 nL) accompanied with on-capillary concentration. Consequently, acquisition of DNA aptamers for bacterial (E. coli and Saccharomyces cerevisiae) and mammalian cells (non-small lung cancer cell line PC-9) was achieved with a single round ($N_{lib} = 45, N_{round} = 1, K_d = 70–350$ nM for PC-9 and 9–170 nM for E. coli and S. cerevisiae).

CE-SELEX suffers from small injection volumes, and thus only a limited number of aptamers can be served in each round. While the magnetic bead-based approach is often associated with tedious procedures and nonspecific binding, Li et al. proposed a hybrid approach that combines the two selection methods to overcome the drawbacks: one single-round selection by boronate affinity magnetic nanoparticles (BA-MNPs) was first performed, followed by CE-based selection rounds. Aptamers for
alkaline phosphatase (ALP) and ribonuclease B (RNase B) were acquired \( (N_{\text{lib}} = 39, N_{\text{round}} = 4, K_d = 65–72 \text{ nM and } 80–102 \text{ nM for ALP and RNase B, respectively}) \); however, it is uncertain whether aptamers that recognize sugar chains can be selected. While the linear coupling of two different approaches in SELEX has often been reported, Wakui et al. merged bead-based and CE-based SELEX in a process called microbead-assisted capillary electrophoresis (MACE)-SELEX. Magnetic beads (1 \( \mu \text{m} \) in diameter) immobilized by thrombin via an amine-coupling reaction were incubated with the DNA library, followed by separation by CE. While the complex peak could not be detected in the first round in conventional CE-SELEX, the bead peak containing the complex (slightly split but within one minute) was detectable even in the first round in MACE-SELEX. The faster enrichment of aptamer sequences in MACE-SELEX than in CE-SELEX was observed by means of high-throughput screening (HTS). The obtained aptamer sequences showed high affinity for the target \( (N_{\text{lib}} = 30, N_{\text{round}} = 3, K_d = 4.5–8.2 \text{ nM}) \), and one of the aptamers showed an anticoagulant ability higher than that of well-known thrombin-binding aptamers. Interestingly, it was also suggested that a number of efficient aptamers would be missed in conventional CE-SELEX because of the adsorption of the complex on the capillary surface.

In the past decade, various chemically modified (unnatural) nucleic acids were applied to SELEX to obtain aptamers with higher affinities than those of native (natural) DNA aptamers; such as SOMAmer, XNA, Ex-SELEX, AEGIS. Kuwahara et al. achieved aptamers of XNAs using CE-SELEX. Libraries containing 2'-O,4'-C-methylene-briged/linked bicyclic ribonucleotides (BNA/LNA) in the primer region and/or C5-modified thymidine with \( N^6 \)-ethyladenine in the randomized region were employed for thrombin, and high-affinity aptamer sequences were successfully obtained.
(\(N_{\text{lib}} = 30\), \(N_{\text{round}} = 4\)–7 with three collections for each round, \(K_d = 0.093\)–34 nM). Interestingly, high-affinity aptamers with non-G4 structures were acquired, although the G4 structure is often observed for thrombin-binding native DNA aptamers. Subsequently, BNA/2'-deoxy-2'-fluoro-ribonucleic acid (FNA) chimeric aptamers for thrombin, exhibiting RNA-like conformations, were obtained using a library containing FNA in the randomized region (\(N_{\text{lib}} = 30\), \(N_{\text{round}} = 5\) with three collections for each round, \(K_d = 10\)–45 nM).46

2-2. Techniques supporting and improving capillary electrophoresis-based selection

Various methods supporting and improving CE-based SELEX have also been developed. One remarkable improvement in CE-based selection is the development of the nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) method for the evaluation of interactions between DNA and protein (for both selection DNA pools and aptamer candidates) in free aqueous solution, as reported by Krylov et al.47,48 In NECEEM, the reactants (target and aptamer) and product (complex) at equilibrium in the sample are rapidly separated by CE, and the trace of aptamer that dissociates from the complex during the course of the separation appears as a bridge-shaped feature in the electropherogram, located between the complex and the free aptamer peaks. Thus, the concentrations of each species in the equilibrium state, along with that of the dissociated aptamer arising during migration, can be calculated by analysis of the electropherogram to determine \(K_d\) and \(k_{\text{off}}\). While determination of \(K_d\) and \(k_{\text{off}}\) based on the electropherogram in CE had been proposed for various reactions49–52 before NECEEM was reported, it was systematically proven that NECEEM is very useful in determining these parameters, especially for protein–DNA complexes within a single CE run. It should be noted that the
$K_d$ values determined by NECEEM are frequently different from those measured by other methods such as SPR. Z. Szeitner et al. suggested that post-selection labeling or immobilization of aptamers may significantly affect their binding ability.\textsuperscript{53} Ric et al. reported that the $K_d$ value for complexes of the well-known 29-mer aptamer and thrombin significantly depended on the electrolyte concentration, and the $K_d$ obtained by NECEEM using a relatively low electrolyte concentration in the separation buffer was larger by more than one to two orders of magnitude compared to that measured by a nitrocellulose filter binding assay.\textsuperscript{54} The advantages of NECEEM are that it does not require the immobilization, and isolation of products and reactants can be achieved, leading to accurate parameter measurements. The disadvantages include the use of a low electrolyte concentration that does not resemble physiological conditions, and the need for a mobility shift. The disadvantages of the SPR assay are the need for immobilization of the target or aptamer, a mixture of signals for some chemical species, and adsorption on the substrate surface, leading to inaccurate thermodynamic and kinetic parameters due to denaturation and incorrect signals. Interestingly, Liu et al. recently suggested that some targets (arsenic\textsuperscript{55} and small molecules\textsuperscript{56}) and aptamers adsorbed on the gold surface led to misinterpretation of binding in the aptamer binding assay. Thus, careful treatment and comparison of various binding assays are also important for aptamer evaluation in the future.

Belobrodov et al. recently reported a mathematical (but empirical) model to predict the $\mu$ of protein–aptamer complexes.\textsuperscript{57} Although $\mu$ is one of the most important parameters for CE-based selection to determine the partitioning position in an electropherogram, aptamers are needed to measure $\mu$. To resolve this dilemma, Belobrodov et al. established a model for predicting the $\mu$ of the complex by assuming a
spherically shaped complex composed of some known parameters: \( N_{\text{lib}} \), \( \mu \) of free DNA, and a total molecular weight for the complex. The validity of their equation was proven using seven aptamer–protein complexes with only 2% standard deviations.

Post-CE separation processes have also been optimized and improved. Yufa et al. found that emulsion PCR (ePCR) is more suitable for NECEEM-based aptamer selection (CE-SELEX) compared to conventional PCR, which generates many byproducts and preferentially amplifies non-binding sequences because of primer-dimer and nonspecific primer binding.\(^{58}\) The efficiency of ePCR for selection, which reduces byproduct formation, was demonstrated by successful aptamer selection for the DNA repair enzyme ABH2 (\( N_{\text{lib}} = 40, N_{\text{round}} = 4, K_d = 35–630 \text{ nM} \)), for which the aptamer cannot be obtained by NECEEM-based selection using conventional PCR. Eaton et al. avoided this problem even with conventional PCR by optimizing the number of PCR cycles for each round.\(^{59}\)

The applicability of HTS (also known as NGS, deep sequencing, or massively parallel sequencing) technology after selection in CE-SELEX has been evaluated by several research groups, because HTS is a promising and developing technology for aptamer selection (ex. see ref. 60 as a review article). Jing et al. combined CE-SELEX with HTS to select aptamers for the rhVEGF165 protein.\(^{61}\) The sequencing results provided important insight into why selection pools partitioned by CE-SELEX frequently have reduced affinities after many rounds (typically >4). Preferential amplification of a particular short PCR product allows this nonbinding sequence to overtake the pool in later rounds of selection, suggesting that further refinement of primer design or amplification optimization is necessary.

Riley et al. demonstrated that CE-SELEX/HTS coupled with cTTP concentration
is useful in enriching aptamer sequences. In a subsequent study, the selection for a glycoprotein, vitronectine, using the ctITP-SELEX/HTS approach was successful ($N_{\text{lib}} = 40$, $N_{\text{round}} = 3$, $K_d = 405$ nM). They suggest that less populous sequences have higher affinities than more populous sequences do, as other researchers have reported. The obtained aptamer was successfully applied to a cytotoxic agent for targeting MDA-MB-231 BC cells. Eaton et al. also performed the CE-SELEX/HTS approach for the ovarian cancer biomarker HE4. After five rounds, the sequences determined by HTS were analyzed using a bioinformatics pipeline. The top 1000 most enriched sequences were clustered using the CD-HIT-EST program, which successfully discovered aptamer sequences ($N_{\text{lib}} = 25$, $N_{\text{round}} = 5$, $K_d = 300$–870 nM). Recently, Ric et al. evaluated CE-SELEX/HTS in detail by investigating the results of HTS after partitioning a randomized library with the addition of a well-known thrombin-binding 29-mer aptamer to monitor the enrichment of the G4 sequence and the sequence accuracy. Some significant mismatches in HTS were observed, and the authors suggest that they were due to PCR amplification.

Since CE-based selection is attractive and reasonable for the acquisition of DNA aptamers, many improvements have been reported as described above. In addition to affording high resolution in free solution without immobilization of molecules, CE fractionation is semi-automated. Furthermore, there are many options for the choice of separation mode, on-capillary concentration, and fractionation, as well as NECEEM-based binding assays, to evaluate selection pools and aptamer candidates under the same conditions of selection. The operation of the CE instrument is relatively simple, and the separation capillary itself is easy to replace in order to minimize the negative effect of contamination. In addition, the PE and the separation time, which strongly affect the $k_{\text{eff}}$
of the obtained aptamers, are also tunable by changing the length of the capillary. However, there are disadvantages, which include: a relatively low diversity of initial DNA sequence patterns to be used for selection (typically $10^{11}–10^{13}$ sequences); selection conditions with low electrolyte concentration (typically some tens of millimoles, which is very different from physiological saline); the need for a significant electrophoretic mobility shift between the complex and unbound DNA (as a result, it is generally difficult to apply CE selection to small molecules); and a very large driving force to dissociate the complex in order to obtain low-affinity aptamers.

2-3. Chip-based microfluidic selection

Microfluidic separation technologies (M-SELEX) have also been developed using chip-based devices. Since the first prototype of SELEX using an automated, microfluidic, microline-based device, many studies using chip-based selection have been reported (see, for example, ref. 65 as a review of researches reported from 2002 to 2010).

Micro free flow electrophoresis (µFFE), which is a plate electrophoresis system with micrometer thickness able to provide continuous injection of a large volume sample (3 µL, $1.8 \times 10^{14}$ sequences), was developed and succeeded in acquiring IgE binding aptamers ($N_{\text{lib}} = 40$, $N_{\text{round}} = 1–4$, $K_d = 20–136$ nM). In µFFE, aptamers with high affinity comparable to those obtained by conventional and CE-SELEX, have been obtained even after only a single round.

Studies have been undertaken on the magnetic bead-based SELEX process conducted on microfluidics devices. Soh et al. reported a continuous-flow magnetic activated chip-based separation (CMACS) device. By this method, target-immobilized magnetic beads are effectively separated from ssDNA and eluted into a microchannel by
a high-intensity local magnetic field gradient to accurately control the transport of a large number of micromagnetic beads. This method successfully obtained DNA aptamers binding with recombinant botulinum neurotoxin type A ($N_{\text{lib}} = 60, N_{\text{round}} = 1, K_d = 34–86 \text{nM}$) with PE comparable to that of CE-based selection.

Another M-SELEX using a micromagnetic separation chip to trap target-modified magnetic bead–DNA complexes with microfabricated ferromagnetic structures identified aptamers binding with streptavidin ($N_{\text{lib}} = 60, N_{\text{round}} = 3$ with a counter selection round, $K_d = 25–65 \text{nM}$). A multifunctional microfluidic selection platform was also developed, on which the negative and positive selections were conducted in one round using target protein (MUC1)-immobilized magnetic beads captured in the chip in the presence of a continuous flow. In addition, the selection process was evaluated via in situ monitoring in real time. The aptamers obtained ($N_{\text{lib}} = 40, N_{\text{round}} = 2, K_d = 22–65 \text{nM}$) could capture exosomes from the MCF-7 cancer cell line. Recently, Hong et al. developed magnetism-controlled selection microfluidic chips, possessing dual selection zones to simultaneously acquire DNA aptamers for two proteins (EBOV GP [type I transmembrane protein] and NP [nucleoprotein]). The target-coated magnetic beads were trapped by the Ni pattern in the chip and separated from unbound ssDNA ($N_{\text{lib}} = 60, N_{\text{round}} = 3, K_d = 10–76 \text{nM}$). These aptamers were applied to the detection of Ebola virus with a 4.2 ng/mL detection limit by a magnetism-controlled detection chip.

M-SELEX has been combined with other post-separation techniques. Soh et al. proposed the combined use of M-SELEX with HTS followed by an array-based discovery platform for multivalent aptamers (AD-MAP) to identify aptamer pairs that recognize distinct epitopes on human angiopoietin-2 ($N_{\text{lib}} = 40, N_{\text{round}} = 4, K_d = 37.6–57 \text{nM}$). The 235 most enriched sequences according to copy number in the HTS data were synthesized.
on an Agilent custom DNA aptamer array. Aptamer pairs on the array were screened by competitive binding assay. Aptamers bound to secondary epitopes were employed in the development of bidentate aptamers by connecting the aptamer pairs via the T25 linker, resulting in a 200-fold improvement of $K_d$ (97 pM) compared to that of individual aptamers.

The M-SELEX system, in which the positive and negative selection units are integrated into one channel to be achieved simultaneously, was developed by using pinches set for fixing target-coated beads for myoglobin ($N_{lib} = 40, N_{round} = 7, K_d = 4.9–6.4$ nM).

M-SELEX has also been integrated with the microarray system. The targets are dotted and immobilized on a microfluidic chip by physical adsorption to prepare the protein microarray, and the resulting microarray is further integrated into a microfluidic chip for the SELEX (called PMM-SELEX) process. The interaction between aptamer candidates and targets is monitored using a fluorescence microarray scanner. The PMM-SELEX process was performed to obtain DNA aptamers for lactoferrin from bovine milk ($N_{lib} = 40, N_{round} = 7, K_d = 0.63–5.48$ nM).

M-SELEX can also be applied to cell-SELEX. On-chip cell-SELEX for ovarian cancer cells (TOV21G, TOV112D, IGROV1, and BG01 cell lines) has been demonstrated ($N_{lib} = 40, N_{round} = 5, K_d = 1.3–150$ nM). In this system, complexes of target cell-coated immunomagnetic beads with DNA are trapped by a magnet. It should be noted that $N_{round}$ is significantly decreased for aptamers specific to cancer cell lines.

Further integration of SELEX processes is of interest. To this end, some automated M-SELEX systems have been reported. Sinha et al. designed an automated, miniaturized SELEX platform for the screening of aptamers for three protein biomarkers associated with cardiovascular diseases (N-terminal pro-peptide of B-type natriuretic
peptide, human cardiac troponin I, and fibrinogen). In the platform, not only separation (screening) but also reagent transport, mixing, incubation, and even PCR amplification are processed; that is, all processes for the “round” are automated using target protein-immobilized magnetic beads ($N_{lib} = 40, N_{round} = 5$ [one negative and four positive selections], $K_d = 2.9–4.4$ nM). Olsen et al. developed integrated M-SELEX in which the binding and separation stages as well as the PCR amplification stage of SELEX are automated. DNA bound to the target (IgA) and immobilized on magnetic beads was eluted by heat and then isolated using electrokinetic transfer. While an enriched DNA pool was obtained using this SELEX of closed-loop of rounds, the aptamer sequences and $K_d$ values were not determined ($N_{lib} = 36, N_{round} = 2$).

While the PE of M-SELEX is often comparable to that of CE separations, it is superior in terms of the diversity of the initial randomized ssDNA library. In addition, unique contrivances are possible in M-SELEX. For example, effective devices such as multiple flow lines, trapping, and successive reactors suitable for different targets (small molecules, viruses, and cells) are expected. In this respect, the potential of M-SELEX is different from that of CE-based SELEX which are higher resolution with simple homogeneous separation. In addition, there are some issues with conventional M-SELEX: for example, there can be nonspecific adsorption of DNAs on beads, flow lines, and tubes, which require negative selection rounds in most cases; unexpected bead aggregations; and a distorted flow stream by microbubbles. Although the automated M-SELEX system is promising, the evaluation of the affinity of each selection pool per round is difficult, and determination of the causes of problems can be complicated because of the complexity of the device.
2-4. Solid-phase separation approach using a unique library or new material

While the acquisition of DNA aptamers for small molecules, even using CE-SELEX and M-SELEX, is more difficult than that of proteins, several effective separation approaches to removing free ssDNA have been reported. A unique, randomized ssDNA library was designed for a small molecule target. The target is immobilized on magnetic beads through hybridization by a complementary oligo DNA for the docking (fixed) sequence, which is incorporated into the divided two randomized regions in the library in the absence of the target molecule. When specific aptamer sequences exhibit sufficient affinity for the target to induce a secondary structure via interaction between the two randomized regions, the small molecule–ssDNA complexes are released from the beads into the aqueous phase to be partitioned. This nonbinding ssDNA capturing (and removing) approach is called Capture-SELEX. In Capture-SELEX, no immobilization of target molecules and no washing of nonbinding sequences are needed. Using this method, aptamers for aminoglycoside antibiotic kanamycin A (MW 484.5) were successfully obtained ($N_{\text{lib}} = 40, N_{\text{round}} = 13, K_d = 68–343 \mu M$). Capture-SELEX coupled with SPR and HTS was reported for tobramycin (MW 467.5) ($N_{\text{lib}} = 60, N_{\text{round}} = 12, K_d = 200 \text{ nM}$). Lee and Zhen improved Capture-SELEX by means of a short, randomized DNA library for docking sequences complementary to a randomized aptamer library without dividing the randomized region; that is, the fixed docking sequence was changed to randomized. This strategy, termed double library (DL)-SELEX, was shown to work successfully in the acquisition of aptamers for a protein (VEGF$_{165}$) and small molecule (doxycycline [DOX]; MW 444.4) ($N_{\text{lib}} = 40, N_{\text{round}} = 8$ and $7, K_d = 54–150 \text{ nM}$ and $71 \text{ nM}$ for DOX and VEGF$_{165}$, respectively).

Similar ssDNA removal approaches based on adsorption of DNA on graphene
oxide (GO) have been reported (GO-SELEX), since GO can adsorb ssDNA via $\pi-\pi$ stacking.\textsuperscript{81} Park et al. reported that unbound ssDNA (adsorbed on GO) is easily removed by centrifugation (typically 12,000–14,000 rpm) without use of expensive instruments at the separation stage in SELEX.\textsuperscript{82} Using GO-SELEX, DNA aptamers for an adipokine protein, Nampt, were successfully obtained ($N_{\text{lib}} = 30$, $N_{\text{round}} = 5$, $K_d = 71.7$–117.4 nM), and those for okadaic acid (MW 805) were obtained and successfully applied to an enzyme-linked aptamer assay (ELAA) ($N_{\text{lib}} = 40$, $N_{\text{round}} = 13$, $K_d = 42$–88 nM).\textsuperscript{83} GO-SELEX was also applied to obtain aptamers that bind to ephedrine (MW 165.2) ($N_{\text{lib}} = 40$, $N_{\text{round}} = 9$, $K_d = 2.86 \mu$M), assisted by the resonance Rayleigh scattering (RRS) method to monitor the screening process.\textsuperscript{84} It should be noted that the affinity of the aptamer to ephedrine was four times higher than that of the stereoisomer, pseudoephedrine ($K_d = 11.86 \mu$M), although affinities in the range of micromoles are not very high.

An improved GO-SELEX, magnetic reduced graphene oxide (MRGO)-SELEX, for low-molecular-weight marine biotoxins (domoic acid [MW 311.3], saxitoxin [MW 299.3], and tetrodotoxin [MW 319.3]) was developed ($N_{\text{lib}} = 40$, $N_{\text{round}} = 15$, $K_d = 40$–60 nM).\textsuperscript{85} MRGO synthesis occurs via a one-pot process in which the Fe$_3$O$_4$ nanoparticles and reduced GO are connected by gluconic acid. MRGO could then be used for magnetic separation to simplify the separation step and improve separation efficiency. Recently, Song et al. succeeded in applying GO-SELEX to cell-SELEX for \textit{Vibrio parahaemolyticus} bacterial cells by means of polyethylene glycol (PEG)- and chitosan (CTS)-modified GO combined with rolling circle amplification ($N_{\text{lib}} = 60$; $N_{\text{round}} = 8$, including two counter selection rounds; $K_d = 10$–25 nM).\textsuperscript{86} Since PEG-GO and CTS-GO are water-soluble materials, the modified GO (adsorbing ssDNA unbound to the target) is not precipitated from the aqueous phase by centrifugation; that is, the cell and cell—
DNA complex can be separated.

Inspired by GO-SELEX, researchers have also employed single-walled carbon nanotubes (SWCNTs) in the removal of unbound ssDNA. In SWCNT-assisted cell-SELEX, unbound or nonspecific ssDNA is adsorbed onto SWCNTs by $\pi-\pi$ stacking interactions between the nucleotide bases and SWCNT sidewalls, while the bound ssDNA remains on the cell surface. In a proof-of-concept study, DNA aptamers binding to a nasopharyngeal carcinoma cell line, CNE2, were obtained ($N_{\text{lib}} = 40$, $N_{\text{round}} = 6$, $K_d = 31$–120 nM).

The separation modes of free target-unbound ssDNA using bead or solid phase, such as Capture-SELEX and GO-SELEX, are effective for selecting aptamers, especially for small molecule targets. While immobilization of small molecule targets is needed in conventional SELEX and biases the selected sequences, no target molecules or complexes are immobilized, and thus they retain their original conformation in aqueous solution in the free DNA-removing approach. In addition, since the separation is conducted in an equilibrium state without a strong driving force for dissociation, as in the case of CE-based SELEX with rigorous separation (washing), aptamers possessing relatively weak interactions with the target (even with relatively large $K_d$ and $k_{\text{off}}$) can be obtained. (In contrast, aptamers with slower dissociation rates are preferentially accessible in CE-based SELEX). However, it should be noted that the aptamers, which are accompanied by higher-order structural changes (structure induction or structure switching) during binding, are exclusively obtained since aptamer sequences with natively formed rigid structures are not discriminated from target binding sequences. Furthermore, aptamer sequences not involved in the docking region during binding cannot be selected in Capture-SELEX, and, in principle, no aptamers for targets (or complexes) adsorbed on
the GO surface are selected. Higher rounds seem to be necessary for selection in GO- and Capture-SELEX compared with CE-based and M-SELEX.

2-5. **Fluorescence-activated cell sorting-based selection**

SELEX using FACS has been proposed for cell-SELEX. For example, aptamers binding to CD19+ Burkitt’s lymphoma cells grown in suspension or for primary cell isolates have been acquired with fewer rounds than the 20-30 rounds that are typical of conventional cell-SELEX ($N_{\text{lib}} = 49, N_{\text{round}} = 10$).88,89 Inspired by yeast90 and bacterial display,91 Soh et al. reported an innovative approach called particle display (PD)-SELEX,92 in which each particle displays many copies of a single sequence on its surface by emulsion PCR technology, to create a library of PD. A mixture of PD and fluorescence-labeled proteins was separated by FACS, and acquisition of high-affinity DNA aptamers for four different proteins (thrombin, ApoE, PAI1, and 4-1BB) was successful ($N_{\text{lib}} = 60, N_{\text{round}} = 3, K_d = 7.04 \text{ pM to } 2.32 \text{ nM}$). Thereafter, they applied PD-SELEX to select structure-switching (higher order structure-induced) aptamers (SSAs) binding to metal ions by means of a DNA library in which the randomized region was designed to be a sequence of a 10-mer randomized region, a 10-mer fixed region, and a 20-mer randomized region.93 A fluorescence-labeled oligo DNA complementary to the fixed region was hybridized as a reporter sequence, which (similar to Capture-SELEX) was released when the two randomized regions recognized the target metal ion to form a higher-order structure. The particles were separated based on the fluorescence of FACS, and SSAs for Cu$^{2+}$ and Hg$^{2+}$ were obtained ($N_{\text{lib}} = 30, N_{\text{round}} = 4, K_d = 47 \mu\text{M} \text{ and } 1.5 \text{ nM} \text{ for Cu$^{2+}$ and Hg$^{2+}$, respectively}$). Recently, PD using alkyne-modified dUTP substituted with dT in the library and chemical modification through alkyne-azide cycloaddition (click chemistry),
called Click-PD, was performed, and a boronic acid-modified aptamer for epinephrine (MW 183.2) and a mannose-modified aptamer for lectin concanavalin A (Con A) were obtained. The strong interaction was caused by chemical modifications ($N_{\text{lib}} = 40$, $N_{\text{round}} = 4$, $K_d = 1.1$ μM for epinephrine and 3.2–20 nM for ConA). These results suggest that novel base-modified DNA aptamers can be effectively acquired by Click-PD-SELEX. In addition, PD-SELEX was applied to RNA aptamer selection with gen-linked RNA aptamer particles to generate a fluorescence-enhancing RNA aptamer binding with fluorescent small molecules.94

In FACS-based SELEX, the advantage is direct monitoring of the binding between DNA and the target to be separated by highly efficient sorting. Thus, the selection seems to be very reliable compared with other selection methods, although it is often observed that selection results in failure. In addition, it has been demonstrated that PD-SELEX can be applied to various targets and libraries, including proteins, metal ions, small molecules, and chemically modified libraries. The disadvantages are the high instrument cost, the negative effect of immobilization (as with other solid phase selection methods), and the need for a traditional bead-based selection round before FACS selection due to the relatively low diversity of sequences to be served (~$10^7$ particles per hour).

2-6. Other separation approaches

As described above, chemically modified nucleic acid aptamers have been developed. It has been proven that such unnatural aptamers provide higher affinity, novel higher structure, and better anti-biodegradability than natural DNA aptamers. SOMAmers are acquired by means of naphtyl-, phenyl-3-propyl-, 1-morpholino-2-ethyl-, 4-hydroxy-2-ethyl-, and S-2-hydroxypropyl modified-5-carboxamide-modified $dC$TP and $dU$TP with
unique separation conditions. The separation is performed using traditional bead-based selection, with a unique incubation step (called kinetic challenge) in the presence of a high concentration of dextran sulfate as a polyanion (5–10 mM at 37 °C for 15–30 min), which competes with aptamers to bind to the target in order to select SOMAmer sequences \(N_{\text{lib}} = 40 \text{ and } 30, N_{\text{round}} = 7 \text{ and } 6, K_d = 7.3 \text{ nM and } <1 \text{ nM for interleukin-1}\alpha \text{ and proprotein convertase subtilisin/kexin type 9, respectively).}

For virus targets, Nitsche et al. reported a one-step (single-round) chromatographic method to isolate aptamers (called MonoLEX) against Vaccinia virus (VACV). The affinity column that traps the aptamer–virus complex, which is coated with heat-inactivated VACV particles, is physically sliced for 40 fractions to be recovered \(N_{\text{lib}} = 20, N_{\text{round}} = 1\).

For a small molecule target, a sol-gel droplet medium, which is deposited onto a porous silicon chip to entrap a metabolite target (xanthine, MW 152.1), is employed to circumvent the chemical modification of targets in SELEX (sol-gel SELEX). The sol-gel silicate material contains both nanoscale pores and microscale channels. DNA can freely move through micron-sized channels, and aptamers interacting with targets that are entrapped within nanoscale pores can be retained. An aptamer highly selective for xanthine over purine metabolites was successfully obtained using this method \(N_{\text{lib}} = 40, N_{\text{round}} = 7, K_d = 4.2 \mu\text{M).}

DNA aptamer selection by atomic force microscopy (AFM) was first reported by Peng et al. A DNA library labeled with a fluorophore and a bead reacts with the target-coated substrate. The target-binding DNA is monitored by fluorescence microscopy to directly extract the aptamer–bead conjugates using an AFM tip. Proof-of-concept experiments of this selection system, called NanoSelection, using a mixture of...
thrombin-binding aptamer and nonsense DNA, suggest successful detection and extraction of aptamer sequences. Miyachi et al. employed AFM for DNA aptamer selection using a different approach. The biotinylated ssDNA library, which is immobilized on a streptavidine-coated cantilever in AFM, is approached by a target (thrombin)-immobilized gold chip for binding. If the affinity between the target and the aptamer is stronger than the avidin–biotin interaction, the aptamer sequences remain on the gold chip. It was reported that the acquisition of a high-affinity aptamer was successful using this method ($N_{\text{lib}} = 60$, $N_{\text{round}} = 3$, $K_d = 0.2 \text{ nM}$).

3. Outlook
In this decade, many improvements in the separation step of SELEX-based DNA aptamer selection have been reported. Consequently, the selection procedure has become more feasible, and the suitability of separation techniques for different targets has been revealed. For protein targets, CE-based, microfluidic chip-based, and FACS-based selection, which are superior in terms of their high efficiency of separation, provide a strong driving force for dissociation during separation to access high-affinity (low $K_d$ and $k_{\text{off}}$) aptamers. For small molecule targets, strategies such as GO-SELEX and Capture-SELEX, in which unbound DNA is removed by a weak driving force to dissociate complexes (i.e., separation in a quasi-equilibrium state) by solid phase, seems effective since most DNA aptamers binding with small molecules generally have high $K_d$ and $k_{\text{off}}$ values.

To minimize the number of selection rounds, single-round selection has been proposed: IFCE,$^{23}$ μFFE,$^{67}$ and CMACS$^{68}$ for proteins; Pectl$^{30,31}$ for bacterial and cancer cells; and MonoLEX$^{95}$ for viruses. A common property of these methods is their very high PE. It could be estimated that PE greater than $10^6$–$10^7$ is needed for single-round selection,
which implies that the population of aptamer sequences is sub-ppm or lower in the initial randomized library. The single-round approach is important not only in simplifying the selection procedure but also in retaining the diversity of aptamer sequences. In addition, bias originating from PCR amplification and contamination of non-aptamer sequences, which often cause failure of selection, are minimized.

However, challenges in the separation technique in SELEX-based selection remain. For example, the present selection technologies are not comprehensive; that is, it is unknown whether the aptamer obtained is the best aptamer with the highest affinity, because the number of DNAs used for the selection (typically $10^{11} - 10^{15}$) is significantly smaller than that of the massive library (ex. $4^{30} = 10^{18}$ sequences for $N_{\text{lib}} = 30$). This is supported by the fact that a well-known anticancer aptamer, AS1411, which is a G-rich 26-mer that recognizes the cell surface protein, nucleolin, was not generated by the SELEX approach but was developed based on observations of guanosine-rich oligonucleotides.\textsuperscript{99} From a different point of view, there is still a high possibility that unidentified high-affinity and highly functional aptamers exist in the library. A key for comprehensive selection may be to develop a separation method to effectively extract desirable aptamer sequences from a library solution without losing its large diversity. The compatibility of various separation techniques with post-SELEX sequence analysis using bioinformatics and machine learning (including deep learning)\textsuperscript{101–103} should be evaluated to find high-affinity and highly functional aptamer sequences.

The development of a unique separation technique to access functional aptamers is also a challenging subject.\textsuperscript{104} Reports about functional aptamers are increasing and address higher affinity and stability through a variety of means, including: DNA nanoscaffold\textsuperscript{105} and circular DNA;\textsuperscript{106} structure-switching aptamers (typically in Capture-
SELEX and PD-SELEX) and affinity rulers for applicability in ELONA using aptamers selected by EX-SELEX,\(^{107}\) different epitope-binding\(^ {108}\) and isotope-binding aptamers,\(^ {109,110}\) and RNA mimics of green fluorescent protein.\(^ {111-113}\) However, the compatibility of separation techniques used to acquire these functional aptamers is unclear, and it could be expected that unique separation modes could be identified to extract unique aptamers in the future.

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Figure 1. Processes of SELEX-based DNA aptamer selection and constituting techniques (bullet symbols) cited in this review.

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Graphical abstract

Which separation method is suitable for DNA aptamer selection?