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Nucleic Acid Aptamers as Molecular Tags for Omics Analyses Involving Sequencing

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1. Introduction

Since the beginning of the \$1,000 genome project, sequencing technologies have progressed rapidly and equipments capable of massively parallel sequencing have been developed. These machines can perform 3G DNA sequencing corresponding to the entire human genome within only a few hours. Indeed, using such machines, quantitative analysis methods for multiple types of RNA, even in a single cell, are very close to being developed. This would be useful for transcriptome studies including mRNA, rRNA, tRNA, and other non-coding RNA. However, these methods would not be applicable to proteome and other omics analyses because non-nucleic acid molecules, such as proteins and peptides, do not have nucleotide sequences. If non-nucleic acid molecules could be converted to a nucleotide sequence, analytical methods that use massively parallel sequencing could also be applied to omics analyses for non-nucleic acid molecules. Nucleic acid aptamers (specific binders) can be readily amplified by polymerase reaction and decoded by sequencing. Therefore, it is possible to apply them as molecular tags to quantitative biomolecular analyses and single cell analyses, among others. This chapter focuses on the development of nucleic acid aptamers and the outlook for related technologies.

2. Construction of random screening systems

Random screening refers to a methodology for obtaining molecules that perform a desired activity, such as catalysis and specific binding, by displaying masses of diverse molecules called a library and selecting from them. This method involves phage display [Smith & Petrenko, 1997] and split-mix combichem [Geysen *et al.*, 2003] as well as *in vitro* selection [Robertson & Joyce, 1990; Ellington & Szostak, 1990; Tuerk & Gold, 1990], *i.e.*, it is a random screening method for functional nucleic acids.

2.1 Beginning of screening functional nucleic acids

Functions of nucleic acids, such as DNA and RNA, have been considered to be only to preserve, transfer, and express genetic information. The prevailing notion is that proteins are

functional molecules that play an important role in the body and that DNA is the blueprint of proteins and RNA is the mediator. Therefore, the potential of nucleic acids to behave as functional molecules has not attracted much interest. However, since the 1980s, with the discovery by Cech *et al.* and Altman *et al.* of RNA enzymes (ribozymes) that catalyze RNA self-cleavage or RNA transesterification in splicing [Zaug & Cech, 1986; Guerrier-Takada *et al.*, 1983], much interest has been focused on the creation and application of functional nucleic acids with new activities that are different from the typical activities of nucleic acids.

A screening methodology to select functional RNA molecules that can catalyze a specific reaction (ribozyme) or bind to a specific molecule (aptamer) from the RNA library (RNA pool of miscellaneous random sequences) was independently reported by Szostak *et al.*, Joyce *et al.*, and Gold *et al.* around 1990. This random screening method for functional nucleic acids is called *in vitro* selection. This is often referred to as SELEX (systematic evolution of ligands by exponential enrichment), particularly when used for screening aptamers. Using the *in vitro* selection method, successful screenings of functional RNA were demonstrated, followed by the creation of the DNA enzyme as a catalyst and the DNA aptamer as a specific binder [Breaker & Joyce, 1994].

2.2 Random screening of nucleic acid aptamers

Single-stranded RNA and DNA (ssRNA and ssDNA, respectively) with a particular sequence, which can be selected by the SELEX method, exert the aptamer activities by forming a specific steric structure with intramolecular hydrogen bonding, stacking interactions, electrostatic interactions, and metal coordination. Using the SELEX method, various RNA/DNA aptamers, which specifically bind to a broad spectrum of targets involving small molecules such as nucleotides or amino acids, vitamins, and antibiotics; macromolecules such as thrombin; and particles such as Rous sarcoma virus (RSV), have been created artificially [Uphoff *et al.*, 1996]. Since the discovery of the riboswitch early in the 21st century, it has been known that nucleic acid aptamers also exist in nature [Winkler *et al.*, 2002].

In the SELEX method, the screening of nucleic acid aptamer is performed using an ssDNA pool of miscellaneous random sequences as the initial library. Oligonucleotide containing approximately 10^{13-15} diversities of sequences is chemically synthesized. Typically, its length is approximately forty mer to one hundred and several tens mer, though the length can vary depending on the size of the target and the screening system that the researchers want to construct. In the case of DNA aptamer screening (Fig. 1), the initial library is applied to solid support, such as sepharose gel and nitrocellulose membrane, such that the target is immobilized and active species are separated from nonactive ones. The DNA with target activity is amplified by polymerase chain reaction (PCR) to give the corresponding dsDNA, and then ssDNA is prepared by an appropriate treatment of the complementary strand, such as degradation using λ exonuclease and biotin trapping using avidin gels. The resulting ssDNA library is used for the next round, and repeated cycles of separation and amplification lead to enriched sequences that show affinity to the target. The DNA aptamers are individually isolated from the enriched pool using a cloning method, and their sequences and binding properties to the target are analyzed. In the case of RNA aptamer screening, two processes are involved. The initial synthetic DNA library is converted to a RNA library using RNA polymerase and after affinity separation, the active RNA is converted and amplified to DNA using a reverse transcription PCR (RT-PCR); this library is subjected to the previously described DNA aptamer screening scheme.

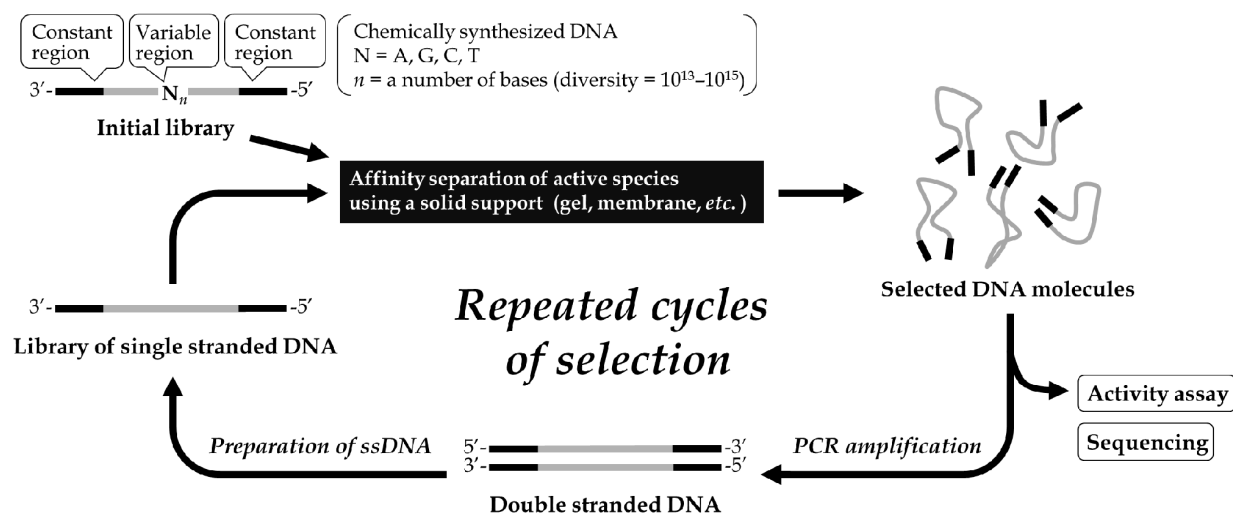


Fig. 1. General scheme of the SELEX method using the DNA library.

3. Chemical modification

Many examples of nucleic acid aptamers have been reported. However, some defects have been revealed. A major defect is physical instability due to degradation by endogenous nucleases. Unmodified nucleic acid aptamers are immediately degraded *in vivo*. Furthermore, a limitation on the binding affinity and specificity are anticipated because nucleic acids consist of a combination of only four nucleotides whereas proteins consist of a combination of twenty amino acids with diverse functional groups in their side chains. Therefore, the development of an advanced screening method to obtain modified nucleic acid aptamers from chemically modified libraries, in which different functionalities that cannot be found in a nucleotide are incorporated, has been performed.

3.1 Polymerase reaction involving modified nucleotides

A key step in the SELEX method is the amplification of selected nucleic acid molecules by affinity separation. This method would make good use of a unique feature of the DNA molecule: it can be amplified and replicated through PCR. Other organic molecules do not possess such a feature. In order to adapt the method to modified nucleic acids as well as to natural RNA and DNA, modified nucleic acids should be enzymatically synthesized from a natural DNA template by polymerase reactions with high efficiency. Conversely, DNA should also be efficiently produced from a modified nucleic acid template. Of course, those conversions are not necessary if the modified nucleic acid used could be directly amplified by PCR in the same manner as DNA.

as a good substrate for the polymerase reaction, the corresponding modified nucleic acids could be efficiently produced. The modification of the sugar and phosphate moieties would tend to decrease the efficiency of the polymerase reaction to a much greater extent than the base moiety does, although this depends on the type of chemical modification [Kuwahara *et al.*, 2006; 2008; 2009]. In particular, 2'-deoxynucleoside-5'-triphosphate analogs with pyrimidine substituted at the 5th position and purine substituted at the 7th or 8th positions of the base moiety tend to be acceptable for DNA polymerase and act as good substrates [Sakthivel & Barbas, 1998; Lee *et al.*, 2001; Tasara *et al.*, 2003]. Furthermore, α -phosphate analogs, where oxygen is replaced with other chemical species, can also be acceptable for DNA polymerase [Andreola *et al.*, 2000]. Analogues with amino (-NH₂), fluoro (-F), and methoxy (-OMe) groups substituted at the 2' position of the sugar moiety are known to work in the RNA polymerase reaction [Aurup *et al.*, 1992; Kujau & Wölfl, 1998].

The chemical structure and replaced position of the modified group as well as the type of polymerase used would affect the production of modified nucleic acids. Among the commercially available thermostable DNA polymerases used for PCR, those belonging to the evolutionary family B, such as *KOD Dash*, *Vent(exo-)*, and *Phusion*, are thought to be suitable for modified nucleic acid production. In particular, *KOD Dash* and mutants of *KOD* DNA polymerases derived from *Thermococcus kodakaraensis* would be the most suitable for modified DNA synthesis as long as misincorporation rates and successive incorporation of modified substrates are investigated. In contrast, *Taq* or *Tth* DNA polymerases, which belong to the evolutionary family A, have been found to be sensitive to chemical modification and not suitable for modified DNA synthesis. In PCR, efficient incorporation of modified substrates into the extending strand is required and the modified nucleotide strands produced should act as templates for the next thermal cycle. Though this double barrier makes modified DNA synthesis difficult, some 2'-deoxynucleoside-5'-triphosphate analogs of C5-substituted pyrimidine, C7-substituted 7-deaza-purine, and α -phosphorothioate have been found to be good substrates for PCR when combined with an appropriate DNA polymerase.

Modification of nucleotides often decreases the reaction efficiency and results in small or reduced amounts of the modified DNA with PCR; the modification would cause sequence dependency on the reaction rate, resulting in a bias in the sequences that emerged from the screening. Indeed, kinetic studies on a polymerase reaction using modified nucleotides showed that the reaction with successive incorporations of modified nucleotides was found to be far more inefficient than the reaction with natural nucleotides. In the SELEX using modified DNA, PCR amplification is performed using natural triphosphates in many cases to circumvent this problem. First, the modified DNA is prepared by a primer extension reaction (PEX) using the modified substrate and natural DNA template. After affinity separation, the natural DNA is synthesized and amplified from the selected modified DNA as a template during PCR using natural triphosphates. Then, the modified DNA for the next selection round can be prepared by transcribing the amplified DNA using PEX. This scheme does not contain the simultaneous use of modified triphosphate substrate and modified DNA template. Therefore, the modified DNA, in which many kinds of functionalities are incorporated, can be synthesized more efficiently compared with the previously described method of direct PCR amplification of modified DNA. Indeed, this has enabled SELEX to be used with libraries of doubly or triply modified DNA prepared using PEX [Perrin *et al.*, 2001; Sidorov *et al.*, 2004; Hollenstein *et al.*, 2009].

For enzymatic synthesis of modified RNA, the template DNA is transcribed using modified ribonucleoside triphosphates instead of natural triphosphates. Fig. 2b shows modified analogs accepted as substrates by RNA polymerase, *e.g.*, *T7* RNA polymerase and *SP6* RNA polymerase. Modified RNA, particularly that prepared using a uridine analog with various functionalities at the 5th position or uridine/cytidine analogs with a fluorine group or an amino group at the 2' position, is often applied to the SELEX method. Although the incorporation efficiency of modified analogs is inferior to that of natural substrates, they can provide a relatively long strand of modified RNA as a full-length product corresponding to template DNA. In the SELEX method using modified RNA, after affinity separation, the selected modified RNA is reverse transcribed to DNA and then amplified by PCR; natural nucleoside triphosphates are used as substrates in these processes. The modified RNA for the next selection round is prepared by transcribing the amplified DNA. In reverse transcription, AMV (*avian myeloblastosis virus*) reverse transcriptase, *Tth* DNA polymerase with reverse transcription activity, or other types of modified RNA polymerases are often used. The aforementioned modified RNAs were found to act as templates for the reverse transcription catalyzed by these polymerases to yield the corresponding DNA strands.

3.2 Modified nucleic acid aptamers

To use nucleic acid aptamers *in vivo*, chemical modification is indispensable. The first aptamer drug, “Macugen,” which is used to treat age-related macular degeneration (AMD), comprises modified RNA including 2'-fluoropyrimidine nucleotides (U, C) and 2'-methoxy purine nucleotides (A, G). This aptamer was created as follows: 1) Sequences that bound to vascular endothelial growth factor (VEGF) using a modified RNA library including 2'-fluoropyrimidine nucleotides were selected by SELEX; 2) Natural purine nucleotides were replaced with 2'-methoxy purine nucleotides, in part, only at sites where its binding affinity was retained after the replacement; 3) Chemical modifications of the 5' and 3' ends with branched polyethylene glycol strands and 3'-thymidylate were introduced [Ruckman *et al.*, 1998].

In step 1 of the aforementioned process, *T7* RNA polymerase was used as an enzyme, and 2'-fluoropyrimidine nucleoside triphosphates and natural purine nucleoside triphosphates were used as substrates to prepare the modified RNA library. Here, modified purine nucleoside triphosphates could not be used due to the limited capability of *T7* RNA polymerase in producing modified RNA. Steps 2 and 3 are called post-SELEX chemical modifications, which can increase nuclease resistance and extend the periods of hemodynamic stasis. In general, chemical modifications have been shown to remarkably enhance biostability and occasionally increase the binding capability of nucleic acid aptamers, as in the following examples.

A sugar-modified RNA aptamer specific to human neutrophil elastase (HNE) is highly stable in serum with a half-life of approximately 20 hours, while natural RNA is degraded within approximately 5 minutes [Lin *et al.*, 1994]. This aptamer includes 2'-aminopyrimidine nucleotides (U, C) instead of the corresponding natural nucleotides. In addition, the improved nuclease resistance of modified RNA aptamers with other types of modifications, *i.e.*, 2'-fluorine and 2'-methoxy groups, were experimentally confirmed. Furthermore, inserting only a single bridged nucleotide analog having 2'-CH(Ph)OCH₂-4' at the 3' end of a thrombin binding DNA aptamer (TBA) could increase the resistance to venom exonuclease up to approximately 30 times (Fig. 3) [Kasahara *et al.*, 2010].

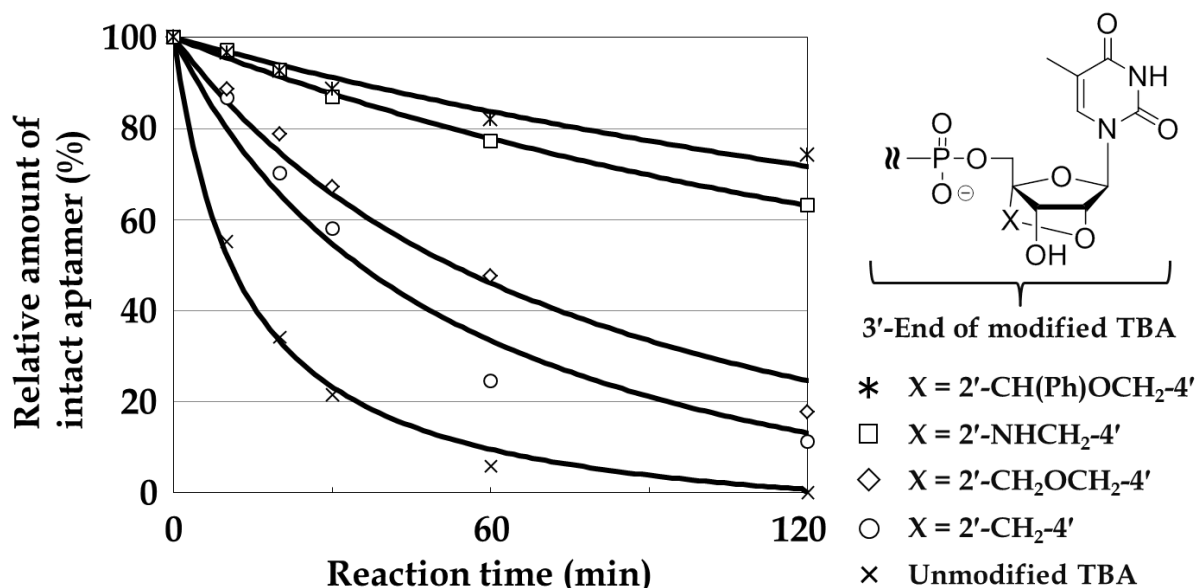


Fig. 3. The time course of the degradation of thrombin binding DNA aptamers (TBAs) by phosphodiesterase I; Total quantities of the products were set at 100% in each reaction mixture.

A base-modified RNA aptamer specific to the human immunodeficiency virus (HIV) Rev protein obtained from a library of modified RNA containing 5-iodouridine instead of uridine could be bound to the target with a somewhat higher binding affinity ($K_d = 0.8$ nM) compared with the corresponding natural RNA aptamer [Jensen *et al.*, 1995]. The unique feature of this base-modified aptamer is to form a cross-link with the target protein by UV irradiation; halogenated uracil is known to form a covalent bond with a nearby electron-rich amino acid residue by photoirradiation.

Such photo-cross-linkable aptamers are called photoaptamers. They are generally created by photoSELEX, which involves a photo-cross-linking process in the conventional selection cycle. Photoaptamers, in particular, would have an advantage for use in protein assays because much more stringent washing of excess or non-specific proteins can be done compared with antibody-based sandwich assays, because photoaptamers can covalently and irreversibly bind to a target.

A few phosphate-modified RNA aptamers have been reported because the commonly used T7 RNA polymerase could accept limited triphosphate analogs modified at the phosphate moiety as substrates. Modified nucleoside triphosphates involving phosphorothioate and boranophosphate [Lato *et al.*, 2002] are normally used as substrates. A phosphate-modified RNA aptamer specific for bFGF was obtained by screening the library of modified RNA exclusively involving phosphorothioate inter-nucleoside linkages, which could greatly enhance nuclease resistance [Jhaveri *et al.*, 1998]. The aptamer bound to bFGF with a binding affinity of $K_d = 1.8$ nM which was approximately five times lower than that observed for a 2'-amino RNA aptamer for the same target ($K_d = 0.35$ nM). However, its strong nuclease resistance was confirmed.

Similarly, various modified DNA aptamers involving 2'-deoxynucleoside analogs with base or phosphate modifications have been selected by SELEX using modified DNA libraries. In both cases with modified RNA/DNA, the inefficiency of enzymatically modified nucleic acid production would occasionally become a bottleneck. Another methodological approach

to create modified nucleic acid aptamers is a mirror-image aptamer coined Spiegelmer. To obtain Spiegelmers, there is no need to enzymatically prepare modified nucleic acid libraries during the selection cycles. Instead, screening is performed using natural RNA/DNA libraries by means of a mirror image of the target molecule. After screening, mirror-image aptamers with the same sequences as the selected aptamers were chemically synthesized using *L*-ribonucleotides or *L*-deoxyribonucleotides. Until date, Spiegelmers that bind specifically to *L*-arginine, *D*-adenosine, *L*-vasopressin, and others have been created; they were found to have improved nuclease resistance [Nolte *et al.*, 1996].

4. Affinity and specificity

Affinities of well-known representative nucleic acid aptamers are shown in Fig. 4, along with those of specific protein binders, such as antibody, streptavidin, and rectin. In general, the binding affinity between molecules can be numerically expressed by the dissociation constant (K_d). At equilibrium for the association/dissociation reaction $R + L \rightleftharpoons RL$, K_d is defined by: $K_d = [R][L]/[RL]$, where $[R]$, $[L]$, and $[RL]$ are concentrations of free receptor, free ligand, and their interaction complex, respectively; smaller K_d values indicate higher binding affinities. For example, K_d values of biotin/streptavidin and digoxigenin/anti-digoxigenin antibody, which are often used as research reagents, are 4×10^{-14} (40 fM) and 1.1×10^{-8} (11 nM), respectively [Holmberg *et al.*, 2005; Tetin *et al.*, 2002]. Those for galactose/jacalin and mannose/concanavalin A are 1.6×10^{-5} (16 μ M) and 2.0×10^{-4} (200 μ M), respectively [Smith *et al.*, 2003]. Immunoglobulin G (IgG) has K_d values of approximately 10^{-7} (100 nM) or less, which is classified as a high affinity antibody.

K_d values of protein binding aptamers specific for keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), and thrombin are 3×10^{-13} (0.3 pM), 5×10^{-11} (50 pM), and 5×10^{-10} (500 pM), respectively; these have excellent binding affinities [Pagratis *et al.*, 1997; Kubik *et al.*, 1994; Ruckman *et al.*, 1998]. The VEGF binding aptamer is Macugen. The K_d value of an anti-VEGF antibody, bevacizumab, which is used for cancer therapy, is 1.1×10^{-9} (1.1 nM). In general, the K_d value required for antibody drugs for molecular targeted therapies should be approximately 10^{-9} (1 nM) or less. Hence, nucleic acid aptamers comparable to antibodies in terms of binding affinity can be created by SELEX with sophisticated techniques as long as the target is a high molecular weight molecule like a protein.

Regarding nucleic acid aptamers specific for small molecules, the K_d value of a biotin binding aptamer, for example, is about 6×10^{-6} (6 μ M), which is 10^8 or more times greater than that of streptavidin [Wilson *et al.*, 1998]. Except for a few examples, the reported small molecule binding aptamers that have been artificially created have K_d values ranging from approximately 10^{-7} to 10^{-4} (several hundred nM to several hundred μ M). To use these as research reagents and diagnostic agents, their binding affinities need to be increased up to at least 10^{-7} or less, hopefully 10^{-8} or less.

Ligand binding sites of riboswitches found in the mRNA of bacteria and the like are considered to be naturally occurring aptamers. Some of these aptamers exhibit very high binding affinities for their small molecule targets, and their K_d values are several nM. For example, K_d values of aptamers specific for S-adenosyl-methionine, flavin mono nucleoside, and guanine are 4×10^{-9} (4 nM), 5×10^{-9} (5 nM), and 5×10^{-9} (5 nM), respectively [Winkler *et al.*, 2003; Winkler *et al.*, 2002; Mandal *et al.*, 2003]. Thus, it is possible that aptamers that bind strongly to small molecules could be created by further improvements in screening methods.

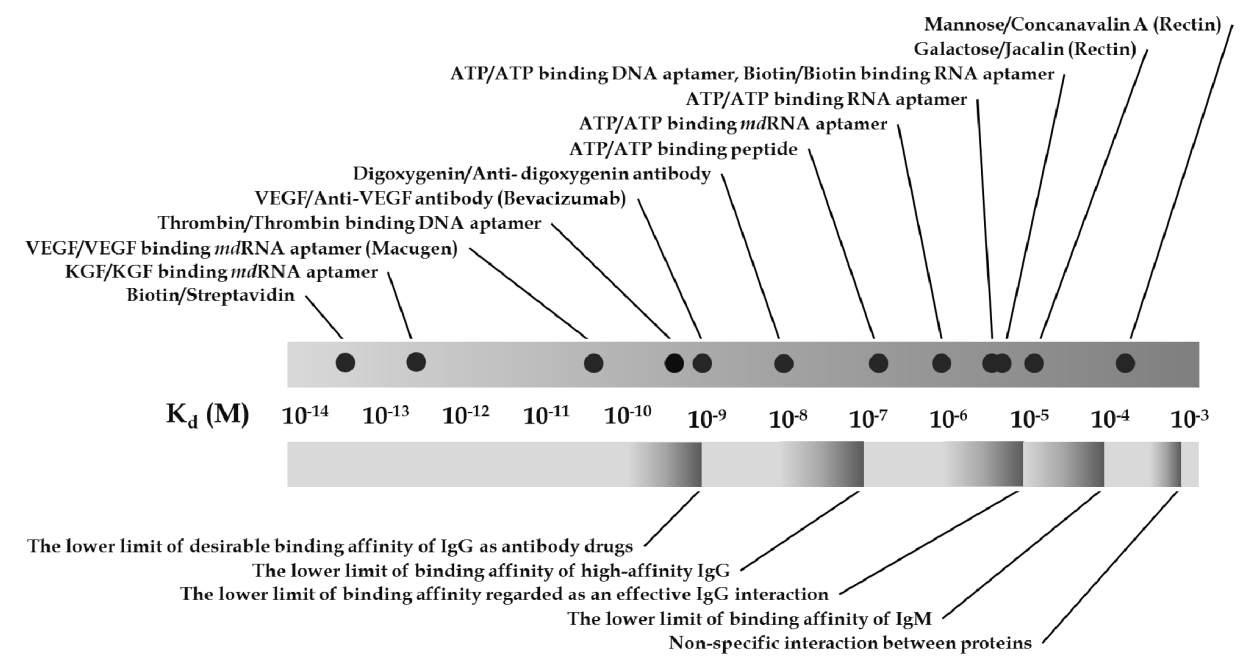


Fig. 4. Comparisons of binding affinities of well-known representative nucleic acid aptamers with those of proteins having specific binding abilities.

Effects of chemical modifications on binding affinity and specificity remain unclear till date. However, it has been suggested that chemical modifications that could constrain structural fluctuations would favorably influence binding affinity. This does not contradict that, in many cases, a target/nucleic acid aptamer complex can be stabilized with divalent metal cations like Mg^{2+} and Ca^{2+} . To characterize effects of chemical species in a library in terms of affinity and specificity, binding properties of aptamers specific for a target, which are respectively selected from libraries different in chemical species using a standardized common screening protocol, should be systematically analyzed.

In previous studies, ATP binding aptamers were obtained respectively from RNA, DNA, and modified RNA and modified DNA, although there were some differences in the screening protocol [Sazani *et al.*, 2004; Huizenga & Szostak, 1995; Vaish *et al.*, 2003; Battersby *et al.*, 1999]. Modified RNA and modified DNA contain 5-(3-amino)propyl-uridine and 5-(3-amino)propyl-2'-deoxyuridine, respectively. K_d values of the obtained aptamers ranged from approximately 10^{-7} to 10^{-6} (several hundred nM to several μ M), and remarkable differences depending on chemical species in libraries were not observed. Incidentally, the K_d value of an ATP binding peptide comprised 62 amino acids obtained using mRNA display selection was 1.9×10^{-7} (190 nM); the binding affinity of this peptide is likely to be higher to some degree than those of the aforementioned nucleic acid aptamers [Chaput & Szostak, 2004].

Fig. 5 shows a comparison of the binding specificities of an RNA aptamer, a modified RNA aptamer, and a peptide that bind to ATP. The affinity of the RNA aptamer is 64-fold lower because of a lack of γ -phosphate in ATP, and is 1100-fold lower because of lack of both β - and γ -phosphates. Although its affinity is 600-fold lower because of a replacement of an adenine base with a pyrimidine base, its specificities for the base and sugar moieties in ATP tend to be less sensitive than that for the phosphate moiety. In contrast, the modified RNA

Recognition site	Phosphate moiety			Sugar moiety		Base moiety			
Target molecule	ATP	ADP	AMP	3'-dATP	2'-dATP	ITP	GTP	CTP	UTP
ATP binding RNA aptamer	1	64	1100	8.5	10	66	19	680	610
ATP binding <i>md</i> RNA aptamer	1	1	2.1	2.5	2.4	150	NB	NB	NB
ATP binding peptide	1	6.8	17	40	190	>5000	>5000	>5000	>5000

5. Development of advanced screening system

Although the SELEX method has been successfully used to create nucleic acid aptamers, further improvements are required to make the screening process much more rapid and convenient. Nucleic acid aptamers that show high activities *in vitro* are not always as effective *in vivo*. Alternative evolutionary approaches to create nucleic acid aptamers that can function in living cells and organisms are also being developed.

5.1 Capillary electrophoresis-SELEX

Capillary electrophoresis (CE)-SELEX and associated improved methods have been developed to obtain protein binding nucleic acid aptamers quickly and easily (Fig. 6a) [Mendonsa & Bowser, 2005]. The common feature of these methods is the use of CE in the affinity separation process, whereas solid supports, such as sepharose gel and nitrocellulose membranes, are used for conventional SELEX methods. Association and dissociation reactions between the target and nucleic acids occur in solution; therefore, enrichment of undesired sequences that bind to the solid support would be avoidable. In addition, use of an automated CE device with high resolving power can greatly reduce the number of repeated cycles of the screening operation. Conventional SELEX typically requires 8–15 selection cycles. CE-SELEX can complete the process in only 2–4 rounds. To date, various DNA aptamers that bind to the human immunodeficiency virus transferase (HIV-RT), immunoglobulin E (IgE), mismatch repair protein (MutS), and ricin, among others, have been obtained by CE-SELEX methods. These resulting DNA aptamers possess a sufficient binding ability as a specific binder. Their K_d values are 0.18 nM, 23 nM, 3.6 nM, and 58 nM, respectively [Mosing *et al.*, 2005; Mendonsa & Bowser, 2004; Drabovich *et al.*, 2005; Tang *et al.*, 2006;].

For CE-SELEX, an uncoated fused-silica capillary can be used because both nucleic acid and the inner wall of the capillary are negatively charged during electrophoresis. Capillaries in which the inner wall is coated are often used for CE separation analyses of biomolecules to suppress peak tailing due to interaction between analytes and the inner wall. The length of the oligonucleotide for the initial library is about 30 to 40 mer including 70 to 90 mer of the random sequence region. In many cases, the 5'-end of the oligonucleotide is labeled with a fluorophore, such as fluorescein (FAM), to detect the active species in the library using a laser induced fluorescence (LIF) detector, which is far more sensitive than a UV-absorbance detector.

In a typical CE-SELEX method of protein binding DNA aptamers, the library is incubated with the target. The mixture is injected into a capillary and subsequently separated using nonequilibrium CE of equilibrium mixtures (NECEEM). The sample and detector are set on the cathode and anode side, respectively, because electroosmotic flow (EOF) toward anode from cathode is generated when voltage is applied (Fig. 6b). Small and positively charged substances would migrate faster in the capillary. In many cases, DNA that forms a complex with the target protein would reach the detector prior to unbound free DNA, resulting in a clear separation between active and inactive species. However, if the molecular weight of the target protein is considerably large, at times, peaks of bound DNA and unbound DNA get closer and cannot be separated enough to conduct the screening. In such cases, the capillary with which the inner wall is coated, *e.g.*, polyvinyl alcohol is used for the

separation. Analytes migrate toward the cathode from the anode in the running buffer at a pH higher than the isoelectric point (pI) of the target protein because EOF does not generate in the coated capillary. Therefore, contrary to the case using a fused-silica capillary, the sample and detector are set on the anode side and the cathode side, respectively.

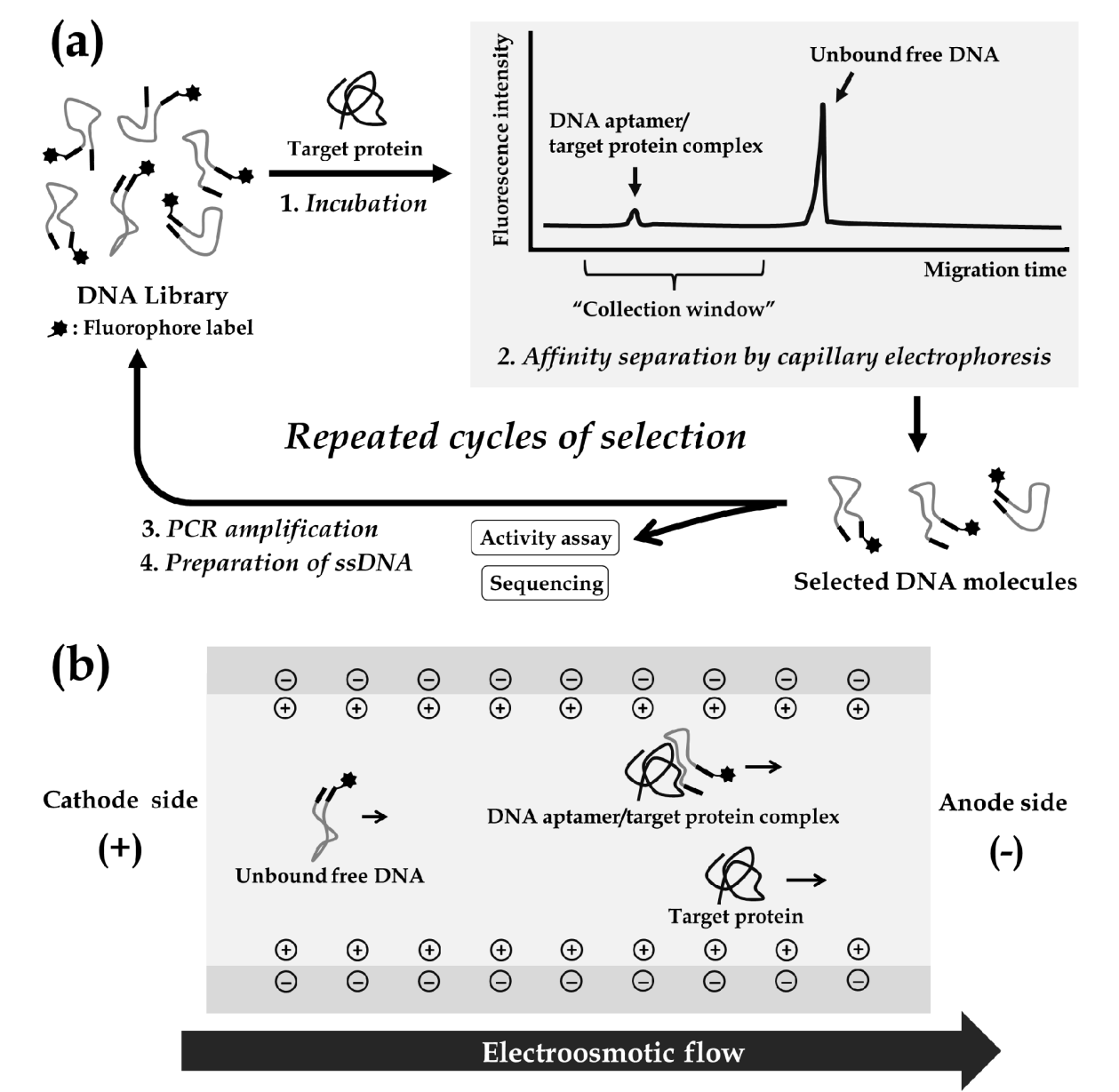


Fig. 6. General scheme of the CE-SELEX method (a); Affinity separation of the bound DNA from the unbound DNA using free zone CE (b).

During electrophoresis, association and dissociation between DNA and target protein is in a non-equilibrium state, and DNA gradually dissociates from the target protein. Therefore, DNA aptamers with a slow dissociation rate are expected to be obtained if a longer capillary is used. However, the migration time is proportional to the length of the capillary. The injection volume of the sample could be increased if a capillary with a larger inner diameter (i.d.) is used. However, the use of a thick capillary makes the release of Joule heating

inefficient. Joule heating should be avoided because the active structure of the DNA aptamer would be destabilized due to heat denaturation. Thus, optimizations of capillary length and i.d. are important to obtain the target DNA aptamer efficiently. In many cases, capillaries with a length of approximately 31–80 cm and an i.d. of 50 or 75 μm are used.

Limitation of the library size is a defect of CE-SELEX; only several nanoliters of sample mixture can be injected per affinity separation. In the conventional SELEX method, the initial library normally contains miscellaneous sequences with 10^{13-15} diversities. If CE-SELEX were performed using a library of the same size, a sample mixture with a concentration of several tens to several hundreds mM has to be injected. With such a high DNA concentration, it is very difficult to separate the DNA aptamer/target protein complex from the free DNA by CE. Therefore, for CE-SELEX, the size of initial library is approximately 10^{10-13} .

Regardless of this limitation, CE-SELEX provides an effective methodology to obtain target DNA aptamers with a high incidence rate in a few selection rounds and there is no requirement of negative selection. For example, in the screening of IgE binding DNA aptamer by CE-SELEX, most sequences obtained in the enriched pool showed binding activity; almost 100% enrichment was achieved. Such fast enrichments of active species have not been reported for the conventional SELEX method in the literature; the enrichment has rarely been found to be above 50% within the first few rounds. Furthermore, non-SELEX selection, with no need to repeat tiresome selection cycles, has been developed as an advanced type of CE-SELEX. Thus, the generation rate for nucleic acid aptamers has dramatically increased, and the process has become more convenient by applying CE, with high resolving power, to the SELEX method.

5.2 Cell-SELEX

Cell-SELEX is a methodology used to obtain nucleic acid aptamers that specifically bind to a particular cell line [Daniels *et al.*, 2003]. This method does not require prior knowledge of targets and would, in principle, generate nucleic acid aptamers for multiple targets simultaneously on the cell surface. Until now, nucleic acid aptamers specific to a number of cell lines, including a T-cell line (human acute lymphoblastic leukemia), B-cell line (human Burkitt lymphoma), and mouse liver hepatoma cell line, have been reported [Shangguan *et al.*, 2006; Raddatz *et al.*, 2008; Shangguan *et al.*, 2008]. One problem with the conventional SELEX technique is that undesired sequences bind to the solid support such that the target becomes immobilized, causing negative selection to be performed. In cell-SELEX, sequences bound to the common cell matrix also need to be excluded. Therefore, in many cases, counter selection using a cell line akin to the target cell line is performed before or after selection using the target.

In the screening of cell-specific aptamers, an ssDNA library containing miscellaneous sequences with 10^{14-15} diversities is incubated with the target cell. Unbound sequences are subsequently removed by washing or centrifugation and bound sequences are amplified by polymerase chain reaction followed by preparation of ssDNA for the next selection round. The cell-specific aptamers can be obtained by repeating these processes approximately 20 times. For the present selection, living cells were used, although the sample was partially contaminated with dead cells. Dead cells are known to strongly and independently absorb

nucleic acid sequences, interfering with enrichment of the target aptamers. To solve this problem, a method using fluorescence-activated cell sorting (FACS) has been developed [Raddatz *et al.*, 2008]. Use of FACS enables dead cells to be removed and active and inactive species to be separated at the same time.

There are many proteins that cause gene expression and a cellular response through signal transductions on the cell surface. Using nucleic acid aptamers specific to these proteins as targets, development of inhibitors that suppress their actions to achieve cell-specific drug delivery has been attempted. To date, nucleic acid aptamers that specifically bind to the cytokine interleukin-17 receptor and human RET receptor tyrosine kinase, for example, have been created by cell-SELEX, and their specific inhibition of the target proteins has been confirmed experimentally [Chen *et al.*, 2011]. As for examples of drug-delivery systems, conjugations of cell membrane protein binding nucleic acid aptamers to siRNAs, toxic proteins, nanoparticles, and antitumor drugs have been developed [McNamara *et al.*, 2006; Chu *et al.*, 2006]. Among these, modified nucleic acids, *e.g.*, RNAs with 2'-fluoro and 2'-amino nucleotides and phosphorothioate DNAs, which possess enhanced biostability, are included.

In the conventional SELEX technique using purified proteins as targets, it is anticipated that the nucleic acid aptamer obtained may not be able to bind to the target on the living cell. In contrast, the cell-SELEX can circumvent this problem because screening is performed using actual living cells. By choosing an appropriate cell for the counter selection, researchers can select nucleic acid aptamers targeting either known or unknown molecules, as well as the matrix structure particular to a certain cell line. Thus, cell-SELEX has provided a viable means to create nucleic acid aptamers with significant potential for *in vivo* application.

6. Outlook

The application range of nucleic acid aptamers can be very broad because of their notable advantages over antibodies. For example, nucleic acid aptamers can be obtained by random screening without using laboratory animals, and they can be produced by organic synthesis at low cost. While further technical innovations are still required to bring the performances of nucleic acid aptamers close to those of antibodies, many applications in bioanalyses ranging from test tube assays such as colorimetric sensing to imaging of living cells and tissues as well as therapeutic drugs have been investigated because of their aforementioned advantages. For *in vitro* assays, natural nucleic acid aptamers are used in many cases; however, chemical modifications assume increased importance in *in cell* and *in vivo* analyses to avoid degradation by nucleases. Therefore, development of rapid/facile screening techniques and *in vivo* screening techniques for creating modified nucleic acid aptamers will contribute to further advances in current bioanalytical measurements. In omics studies for non-nucleic acid molecules such as proteins, peptides, sugars, metabolites, until date, methodologies based on mass spectrometry are mainstreams. However, DNA molecules can readily be amplified by polymerase reactions such as PCR and rolling circle amplification, which generate 10^6 – 10^9 copies of an original sequence. As observed in a riboswitch, RNA molecules cause dynamic structural conversion by association of such small molecules like metabolites to provide multiple transcripts as a molecular offspring. Technologies involving 10^8 or more parallel sequencing and single molecule sequencing reactions are currently established. Considering these unique features of nucleic acid molecules and recent progress

of sequencing technologies, aptamer/sequencing based analyses, which enable integrated omics analyses at single-cell level, may be developed, and these may replace the present methods for omics analyses in future.

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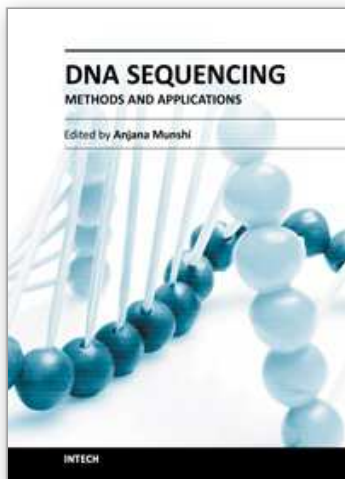
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