

# Advances in Aptamer Screening and Small Molecule Aptasensors

Yeon Seok Kim and Man Bock Gu

**Abstract** It has been 20 years since *aptamer* and *SELEX* (systematic evolution of ligands by exponential enrichment) were described independently by Andrew Ellington and Larry Gold. Based on the great advantages of aptamers, there have been numerous isolated aptamers for various targets that have actively been applied as therapeutic and analytical tools. Over 2,000 papers related to aptamers or SELEX have been published, attesting to their wide usefulness and the applicability of aptamers. SELEX methods have been modified or re-created over the years to enable aptamer isolation with higher affinity and selectivity in more labor- and time-efficient manners, including automation. Initially, most of the studies about aptamers have focused on the protein targets, which have physiological functions in the body, and their applications as therapeutic agents or receptors for diagnostics. However, aptamers for small molecules such as organic or inorganic compounds, drugs, antibiotics, or metabolites have not been studied sufficiently, despite the ever-increasing need for rapid and simple analytical methods for various chemical targets in the fields of medical diagnostics, environmental monitoring, food safety, and national defense against targets including chemical warfare. This review focuses on not only recent advances in aptamer screening methods but also its analytical application for small molecules.

**Keywords** Aptamers • SELEX • Small molecule aptasensors

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## 1 Introduction

As one of the most popular biomaterials for molecular recognition, antibodies have been widely used for more than three decades in various fields, especially in medical diagnostics and therapeutics. The new class of oligonucleotide-based molecular recognition elements has more recently emerged as a rival of antibody-based methods. These oligonucleotide sequences are called “aptamers,” derived from a linguistic chimera composed of the Latin word *aptus* (meaning “to fit”) and the Greek suffix “-mer” [1, 2]. Aptamers can bind to a wide range of target molecules that include proteins, peptides, nucleotides, amino acids, antibiotics, low-molecular organic or inorganic compounds, and even whole cells with high affinity and specificity [3]. Aptamers can be developed in vitro by systematic evolution of ligands by exponential enrichment (SELEX), which makes it possible to isolate functional oligonucleotides against a specific target from a random single strand (ss)DNA or RNA library (usually  $10^{15}$  different sequences). The higher-order structure of oligonucleotides is accomplished by changing intramolecular base pairing. This means that a random library is actually a library of three-dimensional (3D) structural DNA or RNA. Some sequences among the nucleic acid library are folded into unique 3D structures having a combination of stems, loops, quadruplexes, pseudoknots, bulges, or hairpins [4]. The molecular recognition of aptamers results from intermolecular interactions such as the stacking of aromatic rings, electrostatic and van der Waals interactions, or hydrogen bonding with a target compound. In addition, the specific interaction between an aptamer and its target is complemented through an induced fit mechanism, which requires the aptamer to adopt a unique folded structure to its target [4–6].

Over the last 20 years, a large and diverse collection of aptamers for hundreds of targets has been developed from combinatorial ssDNA or RNA pools by basic or advanced SELEX processes. The dissociation constant ( $K_d$ ) of aptamers to their targets is typically from the micromolar to picomolar range, which is comparable

to or sometimes even better than the affinity of antibodies to their antigens. Aptamers have high specificity, which makes it possible to discriminate specific target molecules from their derivatives, as demonstrated in previous reports for a theophyllin binding aptamer, which showed a 10,000-fold higher binding capacity against caffeine (difference of only a methyl group) [7], by an  $\text{L}$ -arginine binding aptamer with 12,000-fold affinity against  $\text{D}$ -arginine [8], and by an oxytetracycline binding aptamer against tetracycline (difference of only an OH-group) [9].

In addition to high affinity and specificity, aptamers have a number of advantages as molecular recognition elements in comparison to antibodies. First, aptamers are easy to handle due to their thermostability. Aptamers can be also regenerated easily within a few minutes after denaturation because aptamers undergo reversible denaturation. This excellent flexibility of the aptamer structure is useful in developing new types of sensing methods. Second, aptamers are easily modified, linked with labeling molecules such as dyes, or immobilized on the surface of beads or substrates for different applications. This is a tremendous advantage for diagnostic and biosensor applications, because the uniform alignment and immobilization of receptors are very important in analytical systems. Labeling with signal-generating molecules is a common method for signal production or amplification in biosensors. Third, there is no or rare immunogenicity when aptamers are applied to an *in vivo* system. Aptamers also enable easy control of bioavailability and delivery durability due to their small size (generally less than 20 kDa). This facilitates their penetration into cells and their delivery or immobilization in any medium, similar to liposomes. Fourth, nucleic acid aptamers can be easily amplified by polymerase chain reaction (PCR), unlike other synthetic receptors such as antibodies, oligopeptides, or molecular imprinted polymers (MIP), and also can be expressed inside cells containing a plasmid that includes the aptamer sequence [10]. Finally, the most important advantage of using aptamers is that these receptors do not require animals for selection and production. There is no need for an *in vivo* immunization to obtain an aptamer, which can be isolated and chemically synthesized *in vitro*. This is the main advantage of aptamers because it is not easy to produce antibodies against some targets, such as proteins, that are structurally similar to endogenous proteins and toxic compounds. In contrast, aptamers are not limited to their targets. *In vitro* synthesis makes it possible for the production of purified aptamers with low cost and without batch-to-batch variation. *In vitro* selection is also proper to develop a high-throughput or automated system for aptamer isolation. Based on these properties of aptamers, the screening method, the SELEX process, has been modified or evolutionarily changed over the years to develop aptamers with higher affinity and selectivity in more efficient, less time-consuming, or automatic ways.

In addition, one of the most successful applications of aptamers is as a recognition probe for medical diagnostics and biosensor development. Various benefits of aptamers as sensing elements (i.e., high affinity and specificity, small size, easy modification and labeling, high stability, no limitation against any kinds of targets, and reversible denaturation) have been verified [11]. Concerning the aptamer-based biosensors, several novel strategies have been devised with

different signal transducers [12]. Most studies on aptamer-based biosensing have focused on protein targets for medical diagnostics. However, the aptamers for small molecules such as organic or inorganic compounds, drugs, antibiotics, or metabolites have not been studied sufficiently, despite the ever-increasing demand for rapid and simple analytical methods for chemical targets in the fields of medical diagnostics, environmental monitoring, food safety, and national defense against targets including chemical warfare agents.

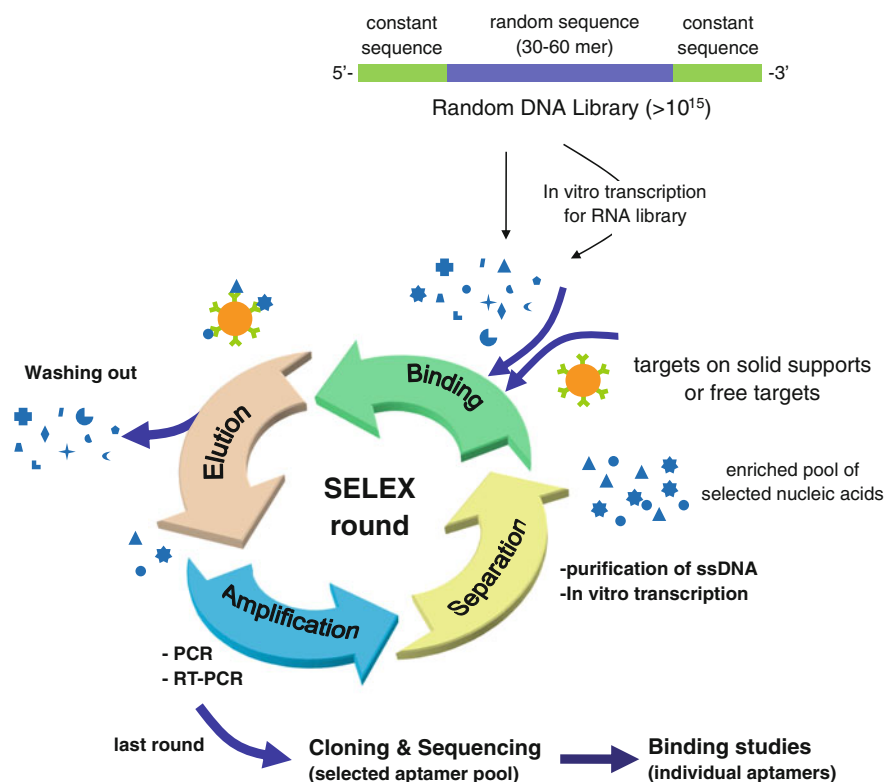
Therefore, this review focuses on recent advances in aptamer screening techniques and aptamer-based biosensors for small molecule detection.

## 2 Recent Advances in Aptamer Screening Methods

### 2.1 General Process of Aptamer Screening

In 1990, three laboratories independently described a technique for isolation of functional oligonucleotides, showing the affinity to their target molecule or enzymatic activity, from a randomly synthesized nucleic acid library composed of more than  $10^{15}$  different sequences [1, 2, 13]. This was accomplished by repetition of selection and amplification. This method of *in vitro* selection is commonly known as SELEX. Since this early introduction of aptamers and the SELEX process, numerous papers about aptamer isolation, their applications in various fields, and the modifications of the SELEX process have been reported. SELEX has become a general and powerful method for isolating nucleic acid aptamers. Figure 1 is a schematic diagram depicting the basic SELEX process including repeated cycles of selection and amplification. This aptamer screening process is affected by many parameters such as target features, design of the random DNA library, selection conditions, and the efficiency of the partitioning methods.

As with any other combinatorial method, the SELEX process starts with the chemical synthesis of a single-stranded (ss)DNA library comprised of random sequences at the center flanked by defined primer binding sites at each 5' and 3' terminus. Individual ssDNA has a different sequence. Compared to other libraries, the randomized ssDNA pool can be easily prepared by a standard DNA synthesizer, because the coupling efficiency of the A, T, G, and C phosphoramidites is very similar. The random ssDNA library can be produced with the mixture of phosphoramidites in a ratio of 1.5: 1.25: 1.15: 1.0 (A: C: G: T) [14]. The diversity of the library depends on the length of the random region. Although one generates  $4^n$  different sequences for  $n$  nucleotides long, about  $10^{15}$  diversity is a practical limitation, which corresponds to a length of about 25 nucleotides. Because 30–60 nucleotides at random regions are a commonly used DNA pool, unfortunately, the full theoretical diversity is not covered for a randomly synthesized DNA library in real experiments [14]. Normally, an initial ssDNA pool comprised of around  $10^{15}$  different sequences allowing a generation of a high possibility of sequences



**Fig. 1** SELEX technology aptamer screening process

specific for a target is used. Only the short part of full aptamer sequences is sufficient for binding to a target [15]. This suggests that a short library, which is cost-effective and easier to manage, can be used for successful aptamer screening as well. However, long random sequences in a library are more appropriate for providing higher structural complexity, which is important in isolating aptamers with high affinity. Therefore, a longer library may increase the possibility for successful aptamer selection [16]. The constant region as a primer binding site should be well designed to avoid primer-dimer pairs and self-priming during PCR amplification, and to reduce the probability of base pairing between the two constant regions. The design of a constant region is important, because the DNA pool should undergo many rounds of amplification. Therefore, any unwanted nucleotides could be amplified in the final aptamer population by hundreds of PCR cycles [17]. An initial random ssDNA library then is incubated with a target molecule. In this step, however, the ssDNA library must be transformed to a RNA library before the incubation with the target is conducted for the selection of RNA aptamers. In an early stage of aptamer research, RNA libraries were frequently used for aptamer selection because RNA is better at folding into complex 3D

structures that provide stronger molecular interactions with the target. RNA also has an additional hydroxyl group that might facilitate the formation of additional hydrogen bonds between the aptamer and the target. For these reasons, the ability of RNA aptamers is generally reported to be superior to DNA aptamers in terms of their affinity and specificity. For *in vivo* applications such as therapeutics, molecular imaging, or drug delivery, both RNA and DNA aptamers have to be modified to achieve resistance in degradation against nucleases, which is very expensive work. For *in vitro* applications such as biosensor development, the modification of DNA aptamers for enhancing stability is not necessary, whereas RNA aptamers should still be modified due to their low stability. Nowadays, therefore, ssDNA pools and DNA aptamers are widely used for the isolation of aptamers and for the development of aptamer-based biosensors or separation systems. In fact, DNA aptamers are much cheaper than RNA aptamers, and DNA is easier to manipulate during the SELEX process [16]. Furthermore, ssDNA folds into a 3D configuration containing stems and loops, even though the folded ssDNAs are less stable than the folded structure of the corresponding RNA sequences [18, 19]. It should also be noted that the conformations of DNA aptamers differ from the corresponding RNA aptamer sequences.

The essential steps of the normal SELEX process are binding, selection, amplification, and partitioning. The most critical step of this process is the selection step. In the first step of SELEX, the random DNA or RNA library is incubated with the target. The nucleic acid–target complex is subsequently partitioned from unbound and weakly bound nucleotides. This is one of the most critical steps to isolate high affinity and specificity aptamers among the extremely diverse oligonucleotide library. During this incubation step, target molecules are either interacted with the nucleic acid pool as a free form or a form that is immobilized on a certain substrate. Fixation of the target on a solid support facilitates easy separation of bound nucleic acids to target from unbound and weakly bound nucleotides. Consequently, this method is very efficient for low-molecular targets. But, the immobilization of the target may result in its conformational change and cause interference on the binding of the library with the conjugation side of the target molecules [20]. The abundant nonspecific interaction of nucleic acid with the solid supports or linker molecules is also notable. Furthermore, it must be remarked that the elution of the strongly bound nucleic acids from the target is difficult, especially in an affinity chromatography type operation, which can restrict the isolation of the extremely high affinity of aptamers. This technique also requires a large amount of targets for elution. In previous studies, therefore, most of the highest affinity aptamers with subnanomolar or picomolar  $K_d$  values were obtained by SELEX with free-form target molecules [21, 22]. In addition, a free-form target can avoid all of these issues. However, the separation of a free target–nucleotide complex from unbound nucleotides is difficult or impossible in some cases, especially for low-molecular targets. Therefore, this method is proper only for macromolecular targets. This method normally produces low separation efficiency, which significantly affects the efficiency of the SELEX

process. An efficient partitioning method can reduce the required number of SELEX rounds.

Filtrations using a nitrocellulose membrane or affinity chromatography column containing the target immobilized beads are traditional and conventional methods for partitioning in the SELEX process [1, 2, 22, 23]. In the filtration method, unbound oligonucleotides to targets are removed from aptamer–target complexes based on the molecular weight difference between nucleic acids and the oligonucleotide–target complex. Affinity chromatography separates oligonucleotides interacted with the target molecules from the pool of nucleic acids by using a column packed with the target-immobilized beads. These separation methods, however, often show low resolution and efficiency in separation. Therefore, many cycles of SELEX rounds are required. In addition, it is not easy to elute nucleic acids that are strongly bound to targets using an affinity chromatography type operation. Recently, many advanced separation methods have been developed to improve separation efficiency, which are detailed later in this review. The affinity of nucleotides to their targets might be affected by the selection conditions. In some cases, the binding and washing conditions (i.e., concentration of target, buffer composition, time, and volume) are changed stringently in later SELEX rounds to obtain the aptamers with high affinity and specificity [16]. The nucleotides that bind to the target can be eluted through the heating, the change of ionic strength or pH, the competitive elution by the excessive addition of target, or the addition of denaturing substances including urea, sodium dodecyl sulfate (SDS), or ethylenediamine tetraacetic acid (EDTA) after the harsh washing step [8, 24–28].

In principle, it is expected that only a few oligonucleotide sequences among the initial oligonucleotide library having an extreme diversity might bind to the target molecules. However, it is very difficult or impossible to separate these from a library readily at a time, due to the low partitioning coefficient of normal separation techniques. Therefore, the repetition of the selection step is required in practical protocols of aptamer screening. To perform this repeated selection, the bound oligonucleotides to the targets should be amplified by reverse transcription (RT)-PCR for a RNA library or PCR for a DNA library, which generates a new population of oligonucleotides for the next round of SELEX. PCR is one of the major steps in the aptamer screening process and, therefore, is important to achieve successive PCR amplification with high efficiency. PCR efficiency in the SELEX process is normally not high, mainly due to the central random sequence regions of the nucleotide pool. Therefore, the PCR conditions including the number of cycles should be optimized depending on the design of the primers and library.

Too many PCR cycles might cause unexpected DNA banding (normally a longer DNA band), whereas the excessive amplification of the selected DNA is not required. Normally, 10–20 amplifications are sufficient within 10–20 cycles [29] for the next round of selection. Improvements of the SELEX process have been accomplished in this amplification step, the preparation of the random nucleotides' pool, or the modification of the SELEX procedure. Normally, for practical reasons, the library of random nucleic acids cannot contain all possible sequences. Mutagenic PCR and nonhomologous random recombination (NRR) have been applied

to increase the diversity of the DNA pool during PCR amplification. In error-prone PCR techniques, point mutations occur with a frequency of 1–10 % per base per PCR reaction. This evolution by mutagenic PCR increases the diversity of the oligonucleotide pool. Consequently, the probability to select the more efficient aptamers can be increased [30]. However, the degree of evolution by point mutation is still not sufficient. Liu and co-workers applied NRR to already defined DNA aptamers for a streptavidin target. NRR enabled random recombination of the nucleic acid fragments in a length-controlled manner. As a result, the affinity of aptamers was improved by 15- to 20-fold compared to aptamers enhanced by mutagenic PCR and 27- to 46-fold higher than the original aptamers [31].

After PCR amplification, the enriched oligonucleotide pool exists as double-stranded DNAs. This dsDNA pool is separated to individual ssDNAs and then a forward strand DNA pool is incubated with targets as the next round of SELEX. For the selection of RNA aptamers, the ssDNA pool has to be converted to a RNA pool by reverse transcription. The streptavidin/biotin interaction is widely used for this. In this method, a biotin molecule is incorporated into the unwanted strands during PCR amplification with biotinylated reverse primer. Then, biotin-labeled dsDNAs (only reverse-strand biotinylated) are incubated or passed through a column of streptavidin-coated beads. Forward strands originating from nucleic acids bound to the target are separated by alkaline denaturation or affinity purification, whereas the biotinylated reverse strands are captured on the streptavidin-coated supports [29, 32–34]. Asymmetric PCR is one possible approach to generate enriched ssDNAs from the eluted ssDNA pool. In this technique, only one or a significant portion of one primer is used for PCR [35]. The forward strand can dominate in a mixture of the ss- or ds-DNA pool by asymmetric PCR amplification. Another method was also developed, based on the size difference between strands. Williams and Bartel used a primer linked with a hexaethylene-glycol (HEGL) spacer and an additional 20 adenine nucleotides to provide a size difference between strands [36]. The different-sized strands were easily discriminated and visible in denaturing polyacrylamide gel electrophoresis (PAGE) by ultraviolet (UV) shadowing or by fluorescence using dye-modified primer in PCR [24, 37–39].

During repeated cycles of selection and amplification, the diversity in an oligonucleotide pool is decreased and a high affinity of oligonucleotides to target can result, because low or no affinity of oligonucleotides have no chance to interact with the target. In general, only a few sequences that can bind to targets with high affinity dominate in an oligonucleotide pool after around 8–15 cycles of selection and amplification. The progress of the SELEX process (enrichment of target-bound oligonucleotides) can be monitored by the quantification of target-bound oligonucleotides among the pools of incubated oligonucleotides at each round of SELEX. Radioactive markers are widely used for the quantification of target-bound oligonucleotides during SELEX due to their high sensitivity, even though they have many drawbacks such as the need for an isotope laboratory, high cost, and health risk for experimenters [1, 40, 41]. Fluorescence dyes are also attractive labeling materials for the quantification of target-bound oligonucleotides, because



they are sufficiently sensitive, relatively economical, and easy to handle and measure [24, 38, 39, 42].

Typically during the SELEX process, a counter-selection (negative selection) is normally involved and is necessary to exclude oligonucleotides that are nonspecifically bound to the membrane or bead surface in the absence of the target [7, 43, 44]. The oligonucleotides that bind to structurally similar compounds or abundant molecules in a real sample such as serum albumin proteins can also be removed by counter-selection. During the counter-selection, these undesirable molecules are incubated with the pool of oligonucleotides instead of the target molecule itself, and the oligonucleotides bound to undesirable molecules are eliminated from the oligonucleotide pool. Counter-selection can enhance the specificity of aptamers, but too much counter-selection might decrease the efficiency of the entire SELEX procedure. Subtractive selection is also similar to counter-selection, but it purposefully improves aptamer selectivity against complex targets such as whole cells. Subtractive selection excludes the oligonucleotides bound to the uninteresting regions of the complex target. One study isolated aptamers that can discriminate target cells (leukemia cells) and other cells using the normal human lymphoma cell line as subtractive cells [45]. This technique has become widely used and is powerful for the isolation of highly selective aptamers against cancer cells or bacterial cells [29, 46].

The selection is stopped when oligonucleotides bound to the target are fully dominant in the pool of oligonucleotides or when no significant enhancement of target-bound oligonucleotides is observed during two or three successive SELEX rounds. At this time, most selected oligonucleotides can be regarded as potential aptamer sequences. These selected oligonucleotides are amplified with unmodified primers. Subsequently, the sequences of individually selected oligonucleotides are identified by cloning and sequencing of the selected clones. The number of different aptamer sequences screened by the SELEX process might depend on the stringency of the selection conditions and target characteristics [47]. In general, about 50 or more colonies among many colonies are sequenced. Sequence analysis can give some useful information about selected oligonucleotides. Regions of homologous sequences differing only by a few points can be identified by sequence analysis. These highly conserved regions or some unique sequence patterns are often an essential part of an aptamer for target binding (see Sect. 2.2.1 for details). DIALIGN and CLUSTAL are frequently used sequence alignment programs (<http://bibiserv.techfak.uni-bielefeld.de/dialign/> and <http://www.ebi.ac.uk/clustalw/>) [48–51]. Secondary structure analysis of aptamers can also provide binding site information that can be predicted easily by a computer program. A representative program “m-fold” for the secondary structure prediction of ssDNA or RNA at various conditions is available at <http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi> [52]. This program is based on a free energy minimization algorithm. Consensus motifs in predicted secondary structures of different aptamers are mainly located in stem-loop structures and are rarely in G-rich structures such as G-quadruplexes or pseudoknot structures [53–55]. These structurally conserved motifs normally correspond

closely with consensus regions of aptamer sequences and are often regarded as binding sites of aptamer to target. After the selection and identification of aptamers, the affinity and specificity of individual aptamers is evaluated by various methods. In some cases, however, the oligonucleotide pool obtained from the last SELEX round can be characterized according to binding ability and used as a mixture, such as polyclonal antibodies [56]. Exact assessment of the aptamer's affinity (dissociation constant,  $K_d$ ) and specificity is very important for further applications of aptamers. These characteristics of aptamers can be influenced by the conditions of the binding assay.

The normal SELEX process for aptamer screening is universally performed by the repetition of three main steps: selection, amplification, and partitioning. However, there is no standard aptamer screening protocol for any target groups. The SELEX process has been continuously modified to improve screening efficiency and make possible aptamer screening against inaccessible targets by using new efficient separation techniques, automation of the process, or new design of oligonucleotide libraries. The following section describes these advances in the SELEX process in detail.

## ***2.2 Advanced Methods for Aptamer Screening***

### **2.2.1 Oligonucleotide Libraries**

Several modifications of the random nucleic acid library in the SELEX process have been developed. Basically, modification is done with the aim of improving aptamer potency by increasing the affinity of aptamers to targets, by offering nuclease resistance, or by providing higher stability. Among the approximately  $10^{15}$  different oligonucleotides, a very small portion of sequences are folded to the 3D structure that allows them to bind to the specific target. Based on this knowledge, Liu and co-workers examined the relationship between the degree of secondary structure in a nucleic acid library and its ability to yield aptamers [57]. They designed a patterned nucleic acid library with a remarkably high degree of secondary structure and ability to accommodate loops and bulges normally observed in the aptamer structures, and demonstrated that the use of a patterned oligonucleotide library had a higher potential to isolate the higher affinity of aptamers than the same size of an unpatterned random library to the same targets.

For in vivo applications, aptamers should be modified to endow nuclease-resistant ability because DNA, and especially RNA, is very sensitive to nucleases [58]. Thus, some methods to transform aptamers into nuclease-resistant moieties by modifying the ribose ring at the 2'-position or by modifying the pyrimidine nucleotide specifically have been reported [59, 60]. Because most nucleases in biological fluids are pyrimidine-specific nucleases, the specific modification with 2'-amino and 2'-fluoro functional groups at the 2'-position of the pyrimidine nucleotide is the commonly used method to increase resistance from nucleases,

increasing half-life up to 15 h [61]. Macugen® marketed by Pfizer and several aptamers currently in clinical development were generated with a library containing 2'-fluoro-pyrimidines. A large number of modifications including substitution at the 2' position (2'-*O*-methyl, 2'-fluoro) and phosphate modifications (phosphorothioate, phosphoraminate, morpholino) have been developed [62–64]. In some cases, these kinds of modifications are limited in their application to the SELEX process because the polymerase cannot effectively amplify DNA with these modified nucleotides. Therefore, some researchers have tried to develop modification schemes that protect DNA or RNA from nuclease-mediated degradation while maintaining the availability of modified nucleotides for PCR amplification by using a special DNA polymerase such as KOD Dash DNA polymerase [65, 66]. These SELEX processes with a modified nucleic acid library can endow not only the nuclease-resistant ability to aptamers, but also accessibility to difficult or impossible targets [67].

A photo-SELEX method performed by incorporating modified nucleic acids activated by absorption of light instead of normal nucleotides has been introduced [68]. In this technique, fluorophore-modified nucleotides, such as 5' bromodeoxyuridineITP or 5' iodouracil, were used. The modified ssDNA aptamers can bind to photocross-linking the target molecule and were identified by photo-SELEX [68]. These modified aptamers can make a photo-induced covalent bond with the target molecules, which is very useful in developing sensitive assays. This method screens the high affinity and specificity of aptamers via strong covalent cross-linking between nucleic acid and the target, but the false-positive rate is also high. So the cross-linking conditions should be optimized well.

Genomic SELEX used ssDNA or RNA libraries derived from the whole genome of a certain organism. *Escherichia coli*, *Saccharomyces cerevisiae*, and the human genomic DNA library were used for genomic SELEX in previous studies [23, 69, 70]. All other steps in the genomic SELEX are similar to the normal SELEX. This method provides great potential for the study of regulation networks between proteins and nucleic acids, and interaction between bioactive molecules and nucleic acids.

The full aptamer sequence is generally not essential for binding to the target [71, 72]. Therefore, several different truncation series of aptamer sequences were arrayed on a chip with high density and DNA microarray experiments with these chips were conducted with dye-labeled target molecules to ascertain which sequence among the full aptamer sequence was essential for the target binding. Fluorescence intensity at each spot well represented the binding ability of truncated aptamer sequences to the target. These massively parallel sequence-function analyses with an aptamer microarray demonstrated that the consensus sequence and common stem-loop structures of aptamers were important for target binding.

In addition, the exclusion of primer sequences was generally not necessary for target binding. However, truncation of primer sequences did not affect the binding ability in all cases. Wen and Gray developed a primer-free SELEX with a bacteriophage-derived genomic library to avoid the influence of primers [73]. Recently, Pan and co-workers reported a minimal primer and primer-free SELEX

protocol with a random DNA library [74]. In these techniques, primer sequences were excluded from the DNA pool before incubation with the target and were incorporated again at the amplification step. Tailored SELEX as a similar approach was successively demonstrated with a random library flanked by short oligonucleotide sequences (only 10 nucleotides) that formed complementary base pairing [75]. In this manner, primers were added to both ends by bridge sequences during amplification; then these primers were eliminated by an alkali treatment. This newly prepared short oligonucleotide pool can then be used in the next round of SELEX. As a result, short aptamer sequences originated from all randomized DNA libraries not incorporated with primer sequences could be screened directly, with no need for a truncation study as a post-SELEX process to use screened aptamers in practical applications.

Burke and Willis varied the SELEX protocol in the generation of a starting nucleic acid pool to develop bifunctional aptamers [76]. At first, they prepared a chimera RNA by simple junction of aptamers previously identified for different targets. These chimera RNAs showed some binding ability to both targets, but their binding activity was not satisfactory probably due to misfolding. To solve this problem, a recombined RNA population was generated by an overlap extension method in PCR with different two aptamers and was used in the SELEX process to both target molecules, instead of a random nucleic acid library (chimeric SELEX). By this technique, dual-functional aptamers having high-binding ability to both targets were screened. Similar to the chimeric SELEX, multistage SELEX was introduced [35]. In this method, each SELEX process was first performed individually with different targets. Then, after five to six rounds of SELEX, two nucleic acid pools were fused to form longer oligonucleotides that were used in the next round of SELEX for both targets.

### 2.2.2 Selection Methods

In the normal SELEX process, counter-selection is strongly suggested to improve the specificity of aptamers to their target, which is essential for aptamer use, especially in medical diagnostic or therapeutic applications. However, in some applications such as bioseparation and environmental monitoring, the universal binding (low specificity to single target molecules) of aptamer to a group of structurally similar molecules can be more useful [38, 77]. In order to screen the aptamers having high universality, the sequential screening of the nucleic acid pool should be performed with a group of target analogues [22, 25, 38]. Based on this strategy, White and co-workers suggested a toggle-SELEX process, in which two different target molecules are switched during alternating rounds of selection [22]. This method allowed the identification of aptamers that can recognize both human and porcine thrombin with  $K_d$  values of 1–4 nM and less than 1 nM, respectively. This technique suggests a useful approach for new therapies when aptamers isolated against human targets may not progress to clinical trials due to their therapeutic efficacy in animal models. Gu's group also developed DNA

aptamers that can bind to two structurally similar antibiotics, oxytetracycline and tetracycline [38]. These aptamers might be more useful in aptasensors to estimate the total concentration of three different tetracycline antibiotics, meaningful information in environmental monitoring, than a combination of aptamers to individual tetracyclines. Toggle-SELEX is available for structurally similar targets whereas chimeric SELEX and multistage-SELEX are less limited to structural similarity between targets.

SELEX is a time-consuming and laborious process because it requires many repetitions of selection and amplification. Therefore, time, cost, or labor can be effectively reduced if the round of SELEX is decreased by enhancement of separation efficiency. If the separation efficiency of the selection method is low, more cycles are needed to screen the aptamers having high affinity and specificity. To this end, many separation tools were applied in the SELEX process. Stoltenburg and co-workers introduced a FluMag-SELEX process based on magnetic beads and fluorescence-labeled forward primers [24], which is a more advanced method than the technique using magnetic beads [78]. In this method, target molecules are immobilized on the surface of the magnetic beads. Unbound oligonucleotides can be efficiently removed from oligonucleotides bound to targets immobilized on magnetic beads by magnetic separation, which is very simple and also does not require a large amount of target, expensive instruments, or skilled people. The evolution of an oligonucleotide pool amplified by using fluorescence-labeled forward primer is also monitored by measuring fluorescence during SELEX rounds [9, 24, 38, 39]. Many kinds of magnetic beads, functionalized to immobilize any target molecules having a functional group, are commercially provided. Tok and Fischer suggested a similar method based on a single microbead to which target proteins were immobilized [79]. They used only a single bead to reduce the amount of targets incubated with the nucleic acid library. It was asserted that small amounts of targets were better to isolate most strongest and specific aptamers. After two cycles, eight aptamers were identified.

Improvement of the partitioning coefficient of the selection methods can also decrease the rounds of SELEX. In this regard, capillary electrophoresis is an attractive method for aptamer researchers. The first application of capillary electrophoresis in the SELEX process (CE-SELEX) was demonstrated by Mendonsa and Bowser [80]. In this separation system, unbound oligonucleotides can be easily partitioned from oligonucleotide–target complexes with high resolution based on the difference of migration velocity between unbound oligonucleotides and oligonucleotide–target complexes [81–83]. The high efficiency of CE-SELEX aptamer selection was verified by the isolation of IgE specific aptamers identified after only four cycles, whereas normal SELEX required 18 cycles to obtain a similar affinity of aptamers. In addition, CE-SELEX does not need to consider the nonspecific adsorption of oligonucleotides on the matrix because the target interacts with oligonucleotides as a free form. However, CE-SELEX is limited in terms of its properties and target size. Especially, low-molecular weight targets such as small organic compounds are not suitable in CE-SELEX because the migration velocity of oligonucleotide–target complexes is not effectively

discriminated with one of the unbound oligonucleotides. In addition, because a very small volume of sample (nL level) can be injected without overloading the capillary, only a small amount of the library having low diversity (typically  $10^{13}$ ) has a chance to interact with the target.

Non-SELEX is also a highly efficient and fast method for aptamer screening. This process is performed by repetitive selection without an intervening PCR amplification step. The selected target-bound oligonucleotide pool selected from the previous round is directly used in the next round of selection. In this method, nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is used for separation. This method enables the selection of aptamers with predefined kinetic/thermodynamic parameters (such as  $K_d$ ), because the migration time of the aptamer–target complexes in the capillary depends on the  $K_d$ . Berezovski and co-workers first showed that the high affinity of aptamer ( $K_d$ : 0.3 nM) for hRas was screened in only three selection cycles [21]. However, the mechanism of this method is still unclear and reports of aptamers screened by this method are very few. Therefore, additional investigations of non-SELEX for various targets are required to establish an efficient method for aptamer screening.

Faster and greater high-throughput methods for aptamer screening are strongly demanded. To this goal, automatic, high-throughput, and simultaneous aptamer selection methods have and continue to be devised. The first automated SELEX process was developed based on the Biomek 2000 pipetting robot (Beckman Coulter) by Cox and Ellington in 1998 [84]. The authors reported that this robotic workstation could carry out aptamer screening for eight targets simultaneously with the process completed in about 12 rounds of selection in two days. The authors immobilized biotinylated lysozyme on streptavidin-coated beads and then conducted an automated repetition of cycles of separation and amplification [85]. Even if lysozyme aptamers were isolated by automatic SELEX, just pipetting, not the whole SELEX process, was automatically operated in this system. This automated SELEX process using a robotic workstation was also demonstrated for some other protein targets such as CYT-18, MEK-1, and Rho [86]. The robotic workstation for automatic SELEX was improved by the addition of a generation part of protein targets that are directly produced from the respective gene in vitro on station [87]. This can accelerate the SELEX process for protein targets.

Eulberg and co-workers developed a modified automatic SELEX system whose design was based on the optimum aptamer screening conditions including buffer components, pH, and PCR conditions, which could be varied according to the target's features [88]. In this system, the Amp 4200E robotic workstation (MWG Biotech Ebersberg) was incorporated with an ultrafiltration system, fluorescence detector, and semi-quantitative PCR under some flexibility [89]. Recently, an upgraded version of the automatic SELEX process was developed using a microfluidic system [90]. This automatic SELEX system, as a microfluidic, microline-based assembly, enables a start-to-finish SELEX including transcription, selection, RT-PCR, and partitioning. The prototype of this automated system is smaller, simpler, and relatively less expensive than previous robotic workstations. In spite of the rapid improvement of the SELEX process, it has still not been

standardized for any type of target. The automated SELEX system can be a favorable approach to establish a standard SELEX protocol. In addition, the automated SELEX system enables fast and high-throughput aptamer screening, and improves the accessibility of SELEX to many nonspecialized researchers.

Most recently, a sol-gel microfluidic chip was also adapted for the SELEX process, in the form of a “SELEX-on-a-chip” [91]. In this system, the binding and elution are performed on a microelectromechanical systems (MEMS) chip for the simultaneous examination of multiple targets. The microfluidic chip was designed to incorporate five sol-gel droplets in which different molecules were embedded as targets or control. The droplets are located on top of individually addressable electrical microheaters used to elute target-bound nucleic acids in the sol-gel droplets. They have demonstrated the specific binding of aptamers to their respective protein targets, and the selective elution by microheating. This SELEX-on-a-chip system demonstrated high selection efficiency and consequent decrease of selection cycles to isolate high-affinity aptamers. This method needs only a very small reagent volume for selection and induces the competitive binding of oligonucleotides to multiple targets embedded in each droplet, which is useful to increase the specificity of aptamers. In addition, the process can be easily extended for larger arrays of sol-gel-embedded proteins. Another microfluidic chip-based aptamer selection method, termed microfluidic SELEX (M-SELEX), was developed [92, 93]. In this system, the magnetic bead-based SELEX process (using target immobilized micromagnetic beads for selection) is integrated with microfluidics technology, which enables the precise manipulation of a small number of beads and selection of target-bound oligonucleotides from the library with a high partitioning coefficient. M-SELEX successively isolated high-affinity DNA aptamers (nM level  $K_d$  values) that strongly bound to the light chain of recombinant *Botulinum* neurotoxin type A after only a single round of selection.

### 2.2.3 Target Features

The SELEX process has no limitation on various classes of targets. Large molecules such as proteins are the best-suited targets in the SELEX process because they provide a large surface for interaction with aptamers. However, low-molecular-weight targets such as inorganic components ( $Zn^{2+}$ ,  $Ni^{2+}$ , As) [94–96] and small organic molecules including cholic acid, cocaine, theophylline, tyrosinimide, ethanolamine, malachite green, and oxytetracycline [1, 7, 9, 97–101] are also proper in the SELEX process. The affinity of these aptamers for small molecules is normally low in a range of submicro- to micromolar  $K_d$  values. The major difficulty in aptamer screening for low-molecular-weight targets is that the target should be immobilized on a solid substrate to separate the target unbound oligonucleotides from the oligonucleotide–target complex. If a target interacts with oligonucleotides as a free form, it is very difficult to separate them due to the lack of a significant difference between their molecular weights. Unfortunately, the fixation of targets (especially small molecular targets) has the potential to decrease



the specificity of aptamers, especially towards the immobilization part of the target [20]. In addition, many small molecular targets such as pesticides are not easy to immobilize on any solid substrates.

To overcome this problem in SELEX for small molecular targets, Gu and co-workers recently developed an immobilization-free aptamer screening method using graphene oxide (GO-SELEX) [102]. It had already been proven that ssDNA can avidly adsorb on graphene or a graphene oxide sheet via  $\pi$ - $\pi$  stacking interactions [103]. Based on this knowledge, the ssDNA library was adsorbed on graphene oxide and the DNA species were interacted with target molecules. ssDNA binding to target was target-induced detached from the graphene oxide sheet and separated from the unbound ssDNA pool still adsorbed on the graphene oxide sheet by centrifugation. By this GO-SELEX method, ssDNA aptamers having submicromolar  $K_d$  values were successively isolated for Nampt, an adipokine protein. Even in the absence of reports about aptamers for small molecules screened by GO-SELEX, the approach can be easily extended to low-molecular-weight targets because GO-SELEX is based on the competitive interaction among oligonucleotides, graphene sheet, and target molecules, and not on the size difference between unbound oligonucleotide and an oligonucleotide target complex. Another useful approach for immobilization-free SELEX was introduced by Nutiu and Li in 2005 [104]. This method was based on the structural-switching property of aptamers. In this reported SELEX method, an oligonucleotide library was designed as a 15 nt specific sequence flanked by two random sequences of 10 and 20 nt. The central specific sequence was complementary to a biotinylated capture oligonucleotide. Thus, the library could be immobilized on avidin-coated beads by hybridization. Oligonucleotides binding to targets were released from the beads via structure-switching for the formation of a complex that was subsequently separated from the library.

Sometimes the preparation of pure protein is not easy, or the aptamers for a pure protein are not useful (e.g., membrane proteins and ion channel proteins). As well, some researchers are more interested in complex targets such as whole cells. Fortunately, the SELEX process is also suitable for these complex targets or mixtures when detailed information about their individual target is not available, which is very attractive in studies of diseases such as cancer. The structural or molecular change on the surface of cells is normally associated with their state, but information is lacking in many cases. In recent years, an interest in aptamers for complex targets such as mammalian cells, tissues, bacteria, and viruses has spurred the development of a new SELEX process dubbed cell-SELEX or whole cell-SELEX [46, 105–107]. This method is very similar to the normal SELEX process, except for the use of whole cells or tissues, instead of purified targets. But, serious hurdles remain. Because receptors or proteins expressed on the cell surface are so complex and multifarious, it is not easy to isolate the aptamers specific to target proteins. Cerchia and co-workers solved this problem using a new strategy in which target proteins were overexpressed on the cell surface [106]. Another study reported improvements by excessive negative selection using counter-cells having almost same composition of membrane proteins on the cell surface, except for a



specific target receptor [107]. These kinds of cell-SELEX processes are very useful approaches for diagnostics, imaging, drug delivery, and therapy for cancer or any other disease.

### 3 Recent Advances in Aptasensors for Small Molecule Detection

The need for the development of chemical biosensors is continuously increasing because the rapid and on-site detection of low-weight compounds, such as residual antibiotics or medicines, illegal drugs, environmental toxicants, chemical warfare agents, and heavy metals, is increasingly important in aspects that include public health, environmental monitoring, food safety, and antiterrorism. In addition, the need for the accurate detection of small molecules, such as disease-related metabolites or medically relevant bioactive compounds, is also increasing in disease diagnosis through the multiparameter analysis of disease-related proteins and newly discovered metabolite biomarkers. However, biosensors for these low-weight compounds have not been extensively studied as much as protein targets have. In addition to the fact that proteins are very significant compared to other molecules, another critical reason is that the recognition elements for these targets are often very difficult to develop. Antibody- or enzyme-based assays are still regarded as standard and well-established assays for the detection of proteins and small chemicals, but they are restricted to some targets, such as toxicants or nonimmunogenic compounds and also often show low specificity due to structurally diverse similar analogues that can exist in the sample. One more important concern for chemical sensing is related to the transducer platform for signal production. For the detection of small molecules, mass-dependent detection methods, including surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and cantilevers, are not efficient because it is very difficult for small molecular targets to generate a signal effectively upon the molecular interaction, due to their low molecular weight in mass-dependent affinity-based assays [108]. A sandwich assay format, one of most powerful methods in immunoassay, is also unsuitable for the detection of low-weight compounds because these targets are normally pocketed inside the cleft of the capturing probes, leaving little space for interaction with secondary probes [109]. The single-site binding, which is the normal assay configuration for small molecules, is not suitable for signal amplification. Subsequently, the highly sensitive detection of small molecules is very restricted. However, environmentally hazardous components can cause a significant problem as they are present in very low concentration in many cases.

Nucleic acid aptamers are very attractive bioreceptors for low-weight compounds due to their many advantages in biosensor development. Aptamer screening for toxic target molecules or for molecules with no or low immunogenicity is possible. Denatured aptamers can be regenerated easily within minutes, which is a useful property in a bioassay. Aptamers are also promising because of their high specificity to low molecular targets from structurally similar analogues.

Concerning signal production, the structural flexibility of aptamers enables the development of novel and unique aptamer-based sensing platforms [110]. Nucleic acid aptamers fold into a flexible but well-defined three-dimensional structure upon binding to their target molecules. Another approach is the use of the inherent property of aptamers as nucleic acids. Nucleic acid aptamers can form a double-helix structure by hybridization with their complementary sequences. This double-helix structure can be easily destroyed by the binding of aptamers to targets by competitive interaction. There are few reports about aptasensors for low-weight compounds, but some compelling aptasensor developments for small molecules have been introduced based on the conformational change of aptamers, hybridized aptamers, or other strategies with optical or electrochemical platforms.

### ***3.1 Fluorescence-Based Analysis***

Fluorescence analysis is the most popular technique in bioanalytical chemistry. Several conventional fluorescent or quencher molecules are available and their detection is very sensitive. These various dye molecules can be easily conjugated with nucleic acid aptamers and are also inherently suitable for real-time detection. In addition, the high flexibility of the aptamer structure is very useful to establish various types of fluorescent aptasensors, such as a fluorescence resonance energy transfer (FRET) assay, which is based on the energy transfer between two fluorescent molecules (donor and acceptor). A frequently adopted method is an aptamer-based molecular beacon (aptabeacon). If aptamers have a hairpin structure, the aptamer can be used as a molecular beacon by labeling with a fluorescent compound and quenching dye at the 5' and 3' end of the aptamer, respectively. In the presence of targets, the aptamer undergoes a conformational change from the hairpin structure to the unfolded form, and fluorescence of the dye molecules is recovered because the distance between the fluorescence dyes and quencher molecules is beyond that for efficient quenching [111]. Similarly, for aptabeacons, a theophylline-specific aptamer double-labeled with a fluorophore and a quencher dye was examined for the detection of theophylline with a target-dependent allosteric ribozyme [112]. In the presence of theophylline, the ribozyme domain was altered to an active conformation by the action of a theophylline-specific aptamer domain. Thus, the quencher was positioned away from the fluorophore by substrate cleavage, resulting in an increase of the fluorescence signal.

Nutiu and Li developed a more generalized aptamer-based FRET assay by DNA displacement [113]. They hybridized aptamers with two partial complementary sequences labeled with fluorescence dye and quencher, respectively. In the absence of the target, the fluorescence was quenched by the nearby quencher molecule hybridization, whereas the aptamer preferred to form an aptamer–target complex when the target was added. As a result, a strong fluorescence signal could be generated by dissociation of short quenching DNA. This approach can be adopted in sandwich fluorescence assay in which fluorescent nanoparticles and

quenching (or capturing particles) are networked by sandwich hybridization between aptamers and their complementary DNAs. This network is broken by the formation of the target–aptamer complex, resulting in the enhancement or quenching of the fluorescence signal. This target-induced displacement strategy might be useful to improve the universality of the methods and to develop aptasensors when an aptamer has no beacon structure or prior information about its secondary or tertiary structure [114, 115]. Most recently, another general excimer signaling approach for aptasensors was reported by Wu and co-workers [116]. In this method, the aptamer was split into two fragments conjugated with pyrene molecules, abrogating the aptamer's binding ability. Target molecules (cocaine) induced the self-assembly of the split pieces, allowing the pyrene molecules to approach close enough to establish a pyrene excimer. This resulted in a wavelength shift of fluorescence.

Gold nanoparticles (AuNPs) have been widely used in an aptamer-based FRET assay as a fluorescence acceptor, based on the knowledge that AuNPs can superquench fluorescence through an energy/charge transfer process [117]. In general, the quenching effect of AuNPs is several orders of magnitude higher than that of an organic quencher [118]. AuNPs have a broad quenching ability for almost all organic dyes, which enables a multiplex detection of several analytes in homogeneous solution by the anchoring of multiple aptamers on AuNPs without rational design and optimization of fluorophore–quencher pairs [119–121]. Besides AuNPs, carbon nanotube (CNT), graphene, and graphene oxide have been recently applied in aptamer-based FRET assays as effective fluorescence acceptors. Yang and co-workers demonstrated the potency of a CNT–aptamer complex-based fluorescent biosensor. In this method, dye-labeled aptamer was adsorbed on CNT via  $\pi$ – $\pi$  stacking interaction between bases of aptamer and CNT sidewalls. Fluorescence was quenched by the electron or energy transfer from the fluorophore to the CNT. The fluorescence signal was recovered by the addition of the target due to the competitive interaction between aptamer and target [122]. The FRET assay using graphene and graphene oxide has been applied in aptasensors using almost the same mechanism as the CNT-based assay [123, 124]. In some cases, target-induced conformational change of aptamers brings the donor and acceptor in close proximity, which causes fluorescence quenching (signal-off mode). One example is a cocaine-binding aptamer having a three-way junction [125]. This aptamer, which is double labeled with the donor and acceptor at each end, exists as a free form in the absence of cocaine. But the addition of cocaine induces a conformational change of the aptamer, leading to the close apposition of the acceptor and donor, and consequent quenching of the fluorescence.

A double-labeled thrombin-binding aptamer was also used as a signal-off mode to detect  $\text{Pb}^{2+}$  and  $\text{Hg}^{2+}$  [126]. The conformation of this random coil structure aptamer was changed to a G-quadruplex by the interaction with  $\text{Pb}^{2+}$  or to a hairpin structure by interaction with  $\text{Hg}^{2+}$ , respectively. As a result, the fluorescence was decreased by FRET between the fluorophore and quencher. Similarly,  $\text{L}$ -argininamide-binding aptamer was used for detection of  $\text{L}$ -argininamide [127]. Although some of the aptamer-based FRET assays have been conducted only for a

protein target (mostly thrombin), these principles have no restriction on being expanded for small molecular detection.

All of the aforementioned methods used fluorescence and quencher-labeled nucleic acids. But, label-free aptamers involving fluorescence aptasensors for low-weight components using intercalating agents have been studied [128–130]. In these techniques, fluorescence dyes such as N,Ndimethyl-2,7-diazapyrenium dication (DMDAP) and 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND) are intercalated in single or duplex form of aptamers and quenched by acceptors. These intercalating agents are displaced upon target binding, resulting in increased fluorescence. Another approach of the label-free aptamer-based fluorescence assay was developed based on target-binding induced quencher deactivation or removal. In these methods, quenchers were located close to donors, such as fluorescence dye and quantum dots, by conjugation on beads or hybridization with aptamer [131, 132]. In some cases, label-free fluorescent aptasensors were developed using fluorescent dyes including 4',6-diamidino-2-phenylindol (DAPI), Hoechst, malachite green (MG), acridine orange (AO), and OliGreen (OG) without quencher molecules [133–137]. These fluorescent dyes emit very weak fluorescence when they are free in solution, but their fluorescence is significantly enhanced by interaction with specific regions of aptamers such as stem structure or capturing with dye-binding aptamers. As one example, the aptamer has no stable stem structure for dye binding in the absence of targets ( $\gamma$ -argininamide), whereas target binding to an aptamer leads to stable stem formation, resulting in enhancement of fluorescence by the binding of dye (DAPI or Hoechst). The interaction between aptamer and fluorescent dye does not affect the binding ability of the aptamer because the stem structure of aptamers is not normally a binding domain. Conversely, AO and OG dyes interact well with the free form of aptamers in the absence of target, but are displaced by the formation of an aptamer–target complex (signal-off mode) [138, 139].

Recently, an interesting fluorescence assay was reported, which is based on selective fluorescent CuNP formation by accumulation in the major groove of the DNA duplex [140]. Based on this phenomenon, small molecular targets including ATP and cocaine were sensitively detected using dsDNA formed by hybridization between aptamers and their complementary ssDNA. In the absence of targets, the formation of CuNPs associated with the dsDNA template induces the fluorescence emission from CuNPs. But the fluorescence signal cannot be observed in the presence of the target because the dsDNA is broken by the formation of the aptamer–target complex, in which CuNPs cannot be grown due to the absence of dsDNA.

### 3.2 Colorimetric Assay

The colorimetric assay is a very attractive method because detection can be accomplished simply using the naked eye, which eliminates the need for expensive analytical instruments. Based on these features, colorimetric biosensors are a

compelling point-of-care (POC) analysis, which is frequently requested for the real-life application of biosensors. Based on these advantages, colorimetric aptasensors have been recently studied for the detection of low-molecular-weight components, such as small chemicals and heavy metal ions. An initial colorimetric aptasensor for a small molecule (cocaine) was developed based on the intermolecular displacement of the dye from an aptamer–dye complex in the presence of the cocaine target [99]. In this method, cocaine displaced the dye by forming a cocaine–aptamer complex, causing an immediate attenuation of absorbance and eventual precipitation of the dye. In a similar approach, heavy metal ions ( $\text{Pb}^{2+}$  and  $\text{Cu}^{2+}$ ) were also detected using DNAzymes [141, 142].

Nowadays, AuNPs are more widely used materials in colorimetric assays due to their unique optical properties, the size-dependent SPR. AuNPs have high extinction coefficients and show size-dependent colors [143]. The dispersed AuNPs that are approximately 13 nm in diameter appear red in color due to their intense SPR absorbance at 520 nm, but the red color changes to purple when they aggregate. This red-to-purple color change can be easily observed by the naked eye. Based on this optical property of AuNPs, as a more recent technology, aptamer-based colorimetric analyses using AuNPs have been extensively described. In this technique, AuNPs are connected to each other by hybridization between aptamers and two different sequenced DNA probes, which are individually immobilized on AuNPs. Due to the aggregation, the solution of AuNPs appears purple. In the presence of target, the networking between AuNPs is broken and they disperse, changing the color to red [144]. This principle was adopted in a “dipstick” type of aptasensor for the detection of small molecules (adenosine and cocaine) [145]. In this system, target-specific, aptamer-linked NP aggregates were loaded onto a lateral flow device, resulting in a simpler and, more excitingly, more sensitive dipstick test than the colorimetric assay in solution. In the absence of target, aggregated nanostructures did not move along the membrane and show a purple line in the conjugation pad. This dipstick type of aptasensor is very attractive in a practical application format, similar to the pregnancy test strip.

Similarly, aptamer was partially hybridized with a probe immobilized on AuNPs. In this state, the AuNPs are not aggregated by the addition of salt. Upon binding of the target, aptamers that are partially hybridized with probes are released from the AuNPs. Then, the AuNPs will aggregate in the salt addition, which screens the electrostatic repulsion between AuNPs. Consequently, the color of the AuNP solution is changed from red to purple [146]. In a converse format for networking between AuNPs, some macromolecular targets having multibinding moieties for aptamers including thrombin and platelet-derived growth factor can induce interparticle cross-linking aggregation of AuNPs by sandwich binding, resulting in the colorimetric change from red to purple in the presence of the target [147]. Unfortunately, this method cannot be adopted for small molecular detection because small molecules do not possess sufficient binding moieties to enable sandwich binding. However, some metal ions, such as  $\text{Hg}^{2+}$  and  $\text{Ag}^+$ , can be detected by this target-induced cross-linked aggregation of AuNPs. Thymine–thymine (T–T) and cytosine–cytosine (C–C) base pairs selectively capture

mercury and silver ions, respectively, and the metallo-base pairs, T-Hg<sup>2+</sup>-T and C-Ag<sup>+</sup>-C, are formed in DNA duplexes with high stability [148]. This phenomenon was successfully adopted in colorimetric metal ion detection [149–151]. In this method, Hg<sup>2+</sup> and Ag<sup>+</sup> led to the aggregation of AuNPs by the formation of T-Hg<sup>2+</sup>-T and C-Ag<sup>+</sup>-C, respectively, resulting in the red-to-purple color change.

In most cases, AuNP-based colorimetric aptasensors used the modified AuNPs with aptamers or their specific probes for the cross-linking of AuNPs. However, the use of unmodified AuNPs should be more convenient, cost-effective, and time-saving because the synthesis of thiolated aptamers is very expensive and the immobilization of aptamers on AuNPs is time-consuming. Li and Rothberg have described a new strategy of colorimetric assay using unmodified AuNPs for DNA detection, which is based on the different electrostatic interactions between ssDNA and dsDNA on AuNPs. The selective adsorption of ssDNA on AuNPs prevents the aggregation of AuNPs at a salt concentration that screens the repulsive interactions of citrate ions [152]. Inspired by this phenomenon, many unmodified AuNP-based colorimetric aptasensors have been recently developed. Dong's group has developed a simple unmodified AuNP-based colorimetric assay for Pb<sup>2+</sup> detection using 17E DNAzyme, which cleaves its substrate, 17DS, in the presence of Pb<sup>2+</sup> [153]. The ssDNA released from the 17E – 17DS duplex is adsorbed on the AuNPs, preventing the aggregation of the AuNPs. In another approach, the target-induced conformational change of aptamers was applied in an unmodified AuNP-based colorimetric aptasensor for the detection of small molecules or ions.

The detection for several low-weight components (K<sup>+</sup>, Hg<sup>2+</sup>, oxytetracycline) was accomplished using unmodified AuNPs and aptamers with a simple experimental design [154–157]. In the assay, unfolded aptamers were strongly adsorbed on AuNPs and stabilized the AuNPs in a high salt buffer. In the presence of targets the conformation of aptamers transits to a folded state that is not easily adsorbed on AuNPs. Subsequently, the red color of the dispersed AuNPs is changed to a purple color due to the aggregation of AuNPs.

However, not all of the aptamers undergo a sufficient conformational change. To overcome this limitation, Fan and co-workers have developed a displacement-based assay [158]. In this strategy, aptamers were hybridized with their complementary DNA. By the addition of targets, the aptamers detached from the duplex form to interact with target molecules. A released complementary ssDNA from complex stabilizes AuNPs against aggregation and the color of the AuNPs appears as red. This strategy can be generally applied to any kind of aptamer structure. Most recently, Fan's group have reported a more advanced design of an unmodified AuNP-based colorimetric assay. In this assay, aptamers are partitioned to two pieces of ssDNA, which reassemble into the original tertiary structure of aptamers in the presence of target molecules [143]. By this strategy, small molecules (cocaine, adenosine, and K<sup>+</sup>) were detected in the micromolar range within minutes, which is based on the knowledge that the short-sized DNA fragments are more rapidly adsorbed on AuNPs. Surprisingly, almost all of colorimetric aptasensors used only a few aptamers as cocaine, adenosine, and K<sup>+</sup> binding aptamers, because these aptamers have been extensively studied concerning their structure

and binding. Unfortunately, however, it is true that the information on the structure of many aptamers is not available or elucidated. As an answer to this issue, Gu's group demonstrated that long-sized aptamers (76 mer) can also be used in an unmodified AuNP-based colorimetric assay directly after SELEX without studies on the truncation and structure, although the sensitivity for some targets was not satisfactory for use in real samples. They also showed signal enhancement by modulating the interaction between aptamers and AuNPs through the exchange of AuNP capping agents. [156–158]

In addition to AuNP-based assays, other colorimetric aptasensors have been reported using polydiacetylene (PDA) liposomes and colored polymers. Kim and co-workers introduced colorimetric aptasensors incorporated with PDA liposome, which is very sensitive and changes its color by external stimuli including ligand interaction, temperature, solvent, and pH. In this technique, aptamers are conjugated on the surface of PDA liposomes. Binding of target to aptamers induces the color change of the liposomes. The detection of  $K^+$  was successfully performed by this method [159, 160]. In another way, a colorimetric detection of mercury ion was accomplished using conjugated polymer, poly(3-(3'-*N,N,N*-triethylamino-1'-propyloxy)-4-methyl-2,5-thiophene hydrochloride) (PMNT) and  $Hg^{2+}$  specific T-rich ssDNA. The optical properties of polythiophene are highly sensitive to conformational change of its conjugated backbone. In the absence of  $Hg^{2+}$ , T-rich ssDNA has a stretched structure that readily forms an electrostatic complex with cationic PMNT in aqueous solution. This leads to a planar conformation of PMNT with a characteristic red color. In the presence of  $Hg^{2+}$ , T-rich ssDNA forms a stem-loop structure by binding with  $Hg^{2+}$  and is surrounded by PMNT. This conformational change of PMNT induces a color change from red to yellow [161].

Colorimetric aptasensors are very useful for the on-site detection of low-weight components with high simplicity, but the relatively low sensitivity compared with other methods remains a significant hurdle for real applications.

### 3.3 *Electrochemical Analysis*

Electrochemical analysis is one of the most attractive sensing platforms due to its great simplicity, rapidity in detection, cost-effectiveness, and the ease of miniaturization, which are necessary for POC applications of biosensors. An electrochemical biosensor is also suitable for multitarget detection through the design of arrayed electrode chips. Recently, many papers have described the incorporation of aptamer-based biosensors in electrochemical detection systems based on distinct properties of aptamers. In electrochemistry, the state change of an electrode surface that occurs by the interaction between targets and immobilized receptors modifies the resistance and capacitance of the electrode–solution interface. Therefore, voltammetry and impedance analyses are very effective and widely used techniques in electrochemical biosensors. The first electrochemical aptasensors developed were mainly based on the analysis of current or faradic



impedance change occurring by simple binding events. In these systems, the electron transfer resistance between redox mediators such as  $[\text{Fe}(\text{CN})_6]^{-3/-4}$  in solution and electrode is changed by the capturing of target by aptamers immobilized on the electrode. The direction of current or resistance change is greatly affected by electrical properties of the targets [162, 163]. These single-binding methods result in very sensitive and simple detection at the fM level for some protein targets such as thrombin [164]. However, this electrochemical detection based on simple binding is still limited for the development of sensitive aptasensors for small molecules, because the electron transfer resistance or current that can be changed by the simple binding of small molecular targets is generally insignificant, compared to macromolecules [165, 166]. To overcome this problem of low sensitivity, various strategies for the design and signal amplification methods of electrochemical signal production have been incorporated in novel and sensitive electrochemical aptasensors, based on the signals produced by the direct conformational change of aptamers and target-induced displacement.

Hegger and Plaxco have developed new types of electrochemical aptasensors based on the conformational change that occurs when aptamers are bound to target molecules [167–169]. In this technique, the current is changed depending on the distance between electroactive compounds (methylene blue or ferrocene) labeled with aptamers and the electrode surface, which is well distinguished in the absence or presence of any type of target. One example is a thrombin detection using redox-active methylene blue- (MB) labeled thrombin-binding aptamer immobilized on an electrode. The flexible conformation of the aptamer labeled with MB enables the electrical contact of the MB with the electrode, and a voltammetric response of the MB. This sensing principle was successfully expanded to low-weight components such as cocaine and  $\text{K}^+$ . But, this signal-off sensing format has the disadvantage of a negative readout signal.

To solve this defect, several signal-on aptasensors have been developed. One approach is to use a bifunctionalized aptamer labeled with a terminal electroactive ferrocene as a redox group and a thiol group at the second terminus of the aptamer [168, 170]. The long, flexible aptamer strand prevents electrical contact of the ferrocene with the electrode. The formation of an aptamer–target complex makes a rigid configuration and results in the orientation of the ferrocene towards the electrode. This leads to the generation of a positive signal in the presence of targets such as cocaine. In another approach, a DNA duplex structure consisting of a ferrocene-labeled aptamer and its complementary DNA was used [171–173]. In the absence of target molecules (cocaine, ATP,  $\text{Pb}^{2+}$ ), the hybridized aptamer adopts a partially unfolded state, whereas in the presence of targets, the aptamer folds to bind to the targets. Consequently, an electroactive ferrocene is closed to the electrode, and the signal is increased. As another approach, a target-induced displacement format is a very effective method. This strategy is based on the separation of the two strands of duplex nucleic acids, composed of an aptamer strand and partially complementary sequence, induced by the presence of target molecules. In the presence of a target, duplex DNA separates and the aptamer folds and binds to the target. The displacement of a complementary sequence decreases



the electron-flow resistance. The separation of duplex DNA is dependent on target concentration. Thus, this method enables the sensitive detection of small molecules [173–176].

The other method for an electrochemical aptasensor uses a redox-active reporter that intercalates into double-stranded DNA rather than being covalently tethered to the aptamer. In this method, intercalating redox-active molecules such as MB are used for signal enhancement. The hairpin structure of aptamers is immobilized on a gold electrode, and MB intercalated in the duplex stem structure of the probe hairpin. The binding of target with the aptamer opens the hairpin structure, thus releasing the intercalated redox-active MB. As a result, the electrochemical signal decreases proportionally to the target (theophylline) concentration [177]. Shao and co-workers suggested a sensitive chronocoulometric aptasensor for adenosine monophosphate (AMP) detection using  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  (RuHex) [178]. In this report, the aptamers hybridized with short complementary ssDNA were immobilized on a gold surface and many RuHex molecules were associated with the duplex form of the aptamer complex to produce a coulometric signal. Short complementary ssDNA was displaced from the aptamer with RuHex by addition of the target (AMP). Thus, the charge was reduced in proportion to the AMP concentration. Similarly, the sensitivity of adenosine detection was improved as low as 1 nM by MB intercalation into the DNA duplex formed by hybridization between an adenosine-binding aptamer and probe DNA immobilized on the electrode [179]. The addition of adenine induced the displacement of adenine-binding aptamers from the duplex form, followed by the releasing of MB. As a result, the current was decreased.

Other effective signal amplification methods in electrochemical detection have been accomplished by the incorporation of NPs. AuNPs are the most popular NPs used for signal amplification in electrochemical aptasensors due to their large surface area, favorable electronic properties, and electrocatalytic activity. The strategies of AuNP-based signal amplification in electrochemical aptasensors are classified into two different approaches. The first method is an attachment of AuNPs on an electrode for enlargement of the electrode surface. The second method is the use of AuNPs as the carrier of signaling materials for amplification of the electrochemical signal. Zhang and co-workers reported a sensitive electrochemical aptasensor for cocaine detection based on the signal enhancement supported by the self-assembly of AuNPs on a gold electrode [180]. In this report, cocaine-binding aptamers conjugated with a redox-active ferrocene and thiol group, respectively, were immobilized on the AuNP-modified electrode. The target-induced conformational change of aptamers may increase the current due to the close proximity between ferrocene and the electrode in the presence of cocaine. As a result, the sensitivity of aptasensors using an AuNP-modified electrode was enhanced tenfold compared to aptasensors using a naked gold electrode. As a converse method, a signal-off electrochemiluminescence (ECL) aptasensor was developed for the detection of small molecules using a gold electrode modified by the complex of AuNPs and ECL substrate  $(\text{Ru}(\text{bpy})_3^{2+})$  [181]. An adenine-binding aptamer conjugated with ferrocene was immobilized on a modified gold electrode

and stretched by the hybridization with complementary DNA. In the presence of adenine, complementary DNA was dehybridized by the formation of an adenine–aptamer complex. The ferrocene probe was closed to the electrode by the folding of the aptamers. Consequently, the ECL intensity was decreased by the quenching effect of ferrocene to the  $\text{Ru}(\text{bpy})_3^{2+}$ .

AuNP-based electrochemical signal amplification in aptasensors has been developed mainly in the sandwich configuration. Use of AuNPs conjugated with secondary aptamers is a very simple and powerful method for signal amplification in electrochemical aptasensors, but they are not available for small molecular detection due to the sparse binding space of small molecule-binding aptamers. Therefore, almost all sandwich structures in aptasensors consist of duplex DNA formed by hybridization between aptamers and their complementary sequences (as capture and reporter probes conjugated with AuNPs). Zhu and co-workers proposed a method for the detection of  $\text{Hg}^{2+}$  using AuNPs [182]. In this method, mercury-ion-specific ssDNAs were heavily loaded on AuNPs which were linked on the electrode. This led to the extensive capture of a large amount of  $\text{Hg}^{2+}$ , resulting in a decrease of the electrochemical signal. The sensitivity of this method for  $\text{Hg}^{2+}$  was improved by three orders of magnitude compared with one of simple binding of  $\text{Hg}^{2+}$  by  $\text{Hg}^{2+}$ -specific ssDNA on an electrode. Similarly, the sensitive electrochemical aptasensor for ATP detection was also reported [183]. In this method, many ATP-binding aptamers were grafted on AuNPs by the hybridization with partially complemented ssDNA. Then the aptamer-grafted AuNPs were held on the gold electrode by the hybridization between the single-sequenced region of aptamer and another partially complemented capture DNA immobilized on the electrode. The MB bound to the dsDNA and also bound to guanine bases for the generation of strong electrochemical signal. The duplex structure was destroyed by the addition of ATP, which decreased the amount of MB on the electrode. As a result, a peak current was significantly decreased. This aptasensor could detect ATP as low as 0.1 nM, which is a markedly amplified sensitivity compared with use of a single ATP-binding aptamer.

## 4 Future Perspectives

Many researchers have demonstrated that aptamers are very useful bioreceptors in various fields such as therapeutics, medical diagnostics, biosensors for environmental monitoring and food safety, bioseparation, and bioimaging. In addition, the success of Macugen® (Pfizer), the first FDA-approved aptamer drug, in the pharmaceutical industry has accelerated researchers' interest in the isolation and applications of aptamers. However, aptamers for other targets remain few compared with antibodies, and only a few aptamers have been isolated and used in reports describing aptamer applications. Therefore, aptamers for more varied targets are required because it is expected that the spectrum of analytes will be greatly expanded in various applications. To meet the extensive requirement of

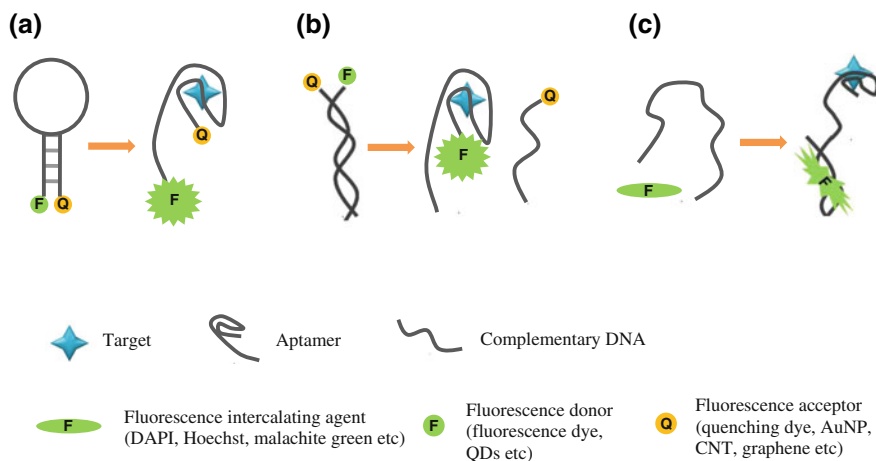
**Table 1** Examples of modified SELEX process

Designation	Description	References
Negative-SELEX	<ul style="list-style-type: none"><li>- Include selection without targets to exclude nonspecifically bound nucleic acids to solid matrix</li></ul>	[76]
Counter-SELEX or	<ul style="list-style-type: none"><li>- Exclude the nucleic acids bound to structurally similar or abundant molecules in real samples</li></ul>	[29, 43–47]
Subtractive SELEX	<ul style="list-style-type: none"><li>- To improve the specificity of aptamers</li></ul>	[69]
Photo-SELEX	<ul style="list-style-type: none"><li>- Using photoreactive molecule-modified nucleic acids library to induce cross-linking between targets and nucleic acids</li></ul>	[23, 70, 71]
Genomic SELEX	<ul style="list-style-type: none"><li>- Using nucleic acids library derived from organism's genome</li><li>- Useful for studies on regulation or interaction between biological target molecules and nucleic acids</li></ul>	
Primer-free SELEX	<ul style="list-style-type: none"><li>- Primer sequences are excluded from library before incubation with target and are incorporated at PCR step</li></ul>	[74, 75]
Tailored SELEX	<ul style="list-style-type: none"><li>- Useful for screening of short aptamers</li><li>- Using random library flanked by short bridge sequences for primers that are linked and removed during SELEX process</li></ul>	[76]
Chimeric SELEX	<ul style="list-style-type: none"><li>- Useful for screening of short aptamers</li><li>- Using recombined nucleic acids library that originate from two or more different aptamer sequences</li></ul>	[35]
Multistage SELEX	<ul style="list-style-type: none"><li>- Generate bifunctional aptamers</li><li>- After five to six rounds of SELEX, two nucleic acid pools are fused to form a new longer nucleic acid library used in the next round for both targets</li></ul>	[77]
Toggle-SELEX	<ul style="list-style-type: none"><li>- Generate bifunctional aptamers</li><li>- Switching between different targets during alternating rounds of selection</li></ul>	[22, 38]
FluMag-SELEX	<ul style="list-style-type: none"><li>- Generate aptamers having high universality</li><li>- Targets are immobilized on magnetic beads</li><li>- Unbound nucleic acids are removed by magnetic separation</li><li>- Using fluorescein-modified nucleic acids pool</li></ul>	[9, 24, 38, 39]

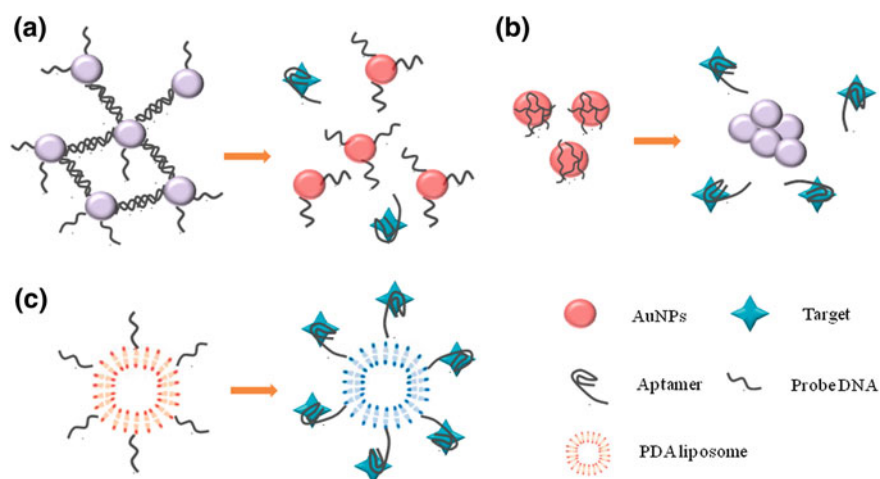
(continued)

**Table 1** (continued)

Designation	Description	References
CE-SELEX	<ul style="list-style-type: none"> <li>- Using capillary electrophoresis to separate target-bound nucleic acids from unbound or weakly bound nucleic acids pool</li> </ul>	[81–84]
Non-SELEX	<ul style="list-style-type: none"> <li>- Not suitable for low-weight molecular targets</li> <li>- Performing repetitive selections without PCR amplification</li> <li>- Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is used for separation</li> </ul>	[21]
Automatic SELEX	<ul style="list-style-type: none"> <li>- Using robotic workstation for repetitive separation and amplification</li> <li>- To improve SELEX efficiency</li> </ul>	[85–91]
SELEX-on-a chip	<ul style="list-style-type: none"> <li>- Performing competitive binding for multiple targets and individual elution on MEMS chip</li> </ul>	[92]
Microfluidic SELEX	<ul style="list-style-type: none"> <li>- Targets are embedded in sol-gel droplets</li> <li>- Integration of microfluidics chip with magnetic separation</li> <li>- Manipulating small amount of beads with high partitioning coefficient</li> </ul>	[93, 94]
GO-SELEX	<ul style="list-style-type: none"> <li>- Graphene oxide (GO) used as an absorbent of nucleic acid pool</li> </ul>	[103]
Cell-SELEX or Tissue SELEX	<ul style="list-style-type: none"> <li>- Target induces the displacement of nucleic acids from GO</li> <li>- For aptamer screening to complex targets such as whole cells or tissues</li> <li>- Various molecules on cells or tissues are targets of selected aptamers</li> </ul>	[47, 106–108]

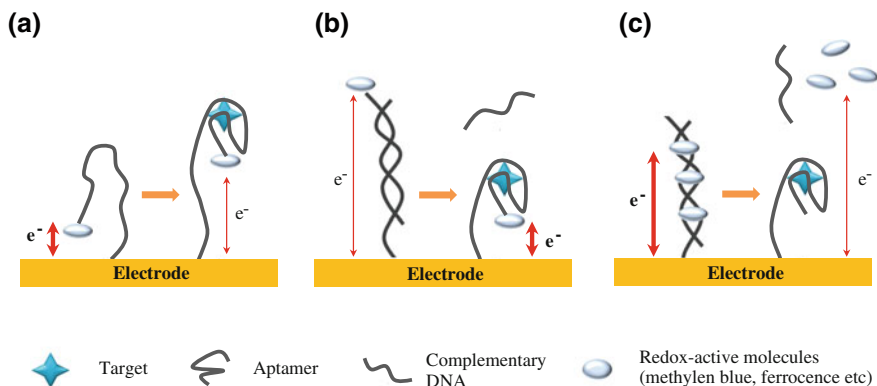


**Fig. 2** Various types of fluorescent aptasensors. (a) FRET assay using aptabeacon conjugated with fluorescence donor and acceptor; (b) fluorescent assay based on target-induced displacement of complementary DNA (CD) from aptamer-CD duplex; (c) label-free fluorescence aptasensor using intercalating fluorescence dye



**Fig. 3** Various types of colorimetric aptasensors. (a) Colorimetric assay using AuNP aggregates cross-linked via aptamers and probe DNA; (b) unmodified AuNP-based colorimetric aptasensing; (c) PDA liposome-based colorimetric aptasensing

aptamers for various targets, researchers will be continuously trying to advance the SELEX process by improving the automation degree and efficiency, development of more high-throughput processes and new screening techniques, and reducing the limitation to targets. Towards these goals, a clearer understanding of the binding mechanism of aptamers to targets and their binding structure in various conditions is needed.



**Fig. 4** Various types of electrochemical aptasensors. (a) Target-induced conformation change-based electrochemical aptasensing using redox active molecules modified aptamer (signal-off mode); (b) electrochemical aptasensing based on target-induced displacement format (signal-on mode); (c) electrochemical signal amplification in aptasensors using intercalating electroactive component

There is no doubt that studies on aptasensors will be continuously and actively increasing based on various merits of aptamers as bioreceptors. In spite of these bright perspectives, aptamer-based biosensors also have some hurdles to overcome. Aptasensor technology is immature compared to immunoassays, and the biosensing and diagnostic market is still largely dominated by antibodies. Only a few aptamer-based biosensors are commercially available.

However, aptasensors have one promising area for detection of small molecules in biological systems, the environment, and food safety because it is very difficult to produce highly specific antibodies for small molecules. For successful commercial applications of aptasensors for low-molecular-weight components, some technical issues should be overcome. First, a stronger affinity of aptamers for small molecules is needed because the affinity of aptamers significantly affects sensitivity. In many cases, especially in the environment, small molecules exist in very low concentrations. Related to this, the post-SELEX including truncation and study of the binding site will become a more conventional step to downsize the aptamers and improve the aptamers' affinity. Truncations of isolated aptamers can not only reduce the synthetic cost meaningfully, but also raise the affinity of original aptamers to targets in some cases. More powerful signal amplification methods are expected to be developed by implementation of various NPs as powerful probe materials, and new sensing mechanisms. Secondly, most aptasensors were tested in defined buffer solution. They should be examined on complex real samples for practical applications. The sensitivity of aptasensors often declines remarkably in real biological or environmental samples. To overcome this problem, aptamer screening has been attempted in real samples, not defined buffer solution. Finally, simpler, more cost-effective, and high-throughput aptasensors are needed. To meet these requirements, the development of

aptasensors should be label-free, real-time, multiplex, and miniaturized systems. All of these efforts on aptasensors will facilitate their commercialization (Table 1, Figs. 2, 3, 4).

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