



Review

SELEX methods on the road to protein targeting with nucleic acid aptamers



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ABSTRACT

Systematic evolution of ligand by exponential enrichment (SELEX) is an efficient method used to isolate high-affinity single stranded oligonucleotides from a large random sequence pool. These SELEX-derived oligonucleotides named aptamer, can be selected against a broad spectrum of target molecules including proteins, cells, microorganisms and chemical compounds. Like antibodies, aptamers have a great potential in interacting with and binding to their targets through structural recognition and are therefore called “chemical antibodies”. However, aptamers offer advantages over antibodies including smaller size, better tissue penetration, higher thermal stability, lower immunogenicity, easier production, lower cost of synthesis and facilitated conjugation or modification with different functional moieties. Thus, aptamers represent an attractive substitution for protein antibodies in the fields of biomarker discovery, diagnosis, imaging and targeted therapy. Enormous interest in aptamer technology triggered the development of SELEX that has underwent numerous modifications since its introduction in 1990. This review will discuss the recent advances in SELEX methods and their advantages and limitations. Aptamer applications are also briefly outlined in this review.

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1. Introduction to SELEX techniques

Aptamers are short single-stranded DNA (ssDNA) or RNA sequences generated by an *in vitro* process called Systematic Evolution of Ligands by EXponential enrichment (SELEX). The term aptamer is derived from the Latin words *aptus* and *meros* (meaning to fit and particle, respectively). These molecules can bind to a broad spectrum of target molecules such as proteins [1], peptides [2], nucleotides [3] antibiotics [4], toxins [5] and small molecules [6] with high affinity and specificity. The SELEX process was first developed by three independent groups in 1990 to isolate RNA sequences that could specifically bind to target molecules [7–9]. The conventional SELEX contains the repetitive cycles of incubation, binding, partitioning and amplification steps. A random pool of oligonucleotide sequences (initiating library) containing 10^{14} to 10^{15} different sequences is incubated with target molecules. During incubation, some of the sequences are bound to the target molecules whereas a number of sequences are weakly bound or do not interact. Partitioning is a step wherein bound sequences are isolated from weakly bound or unbound ones. The eluted oligonucleotides are amplified by polymerase chain reaction (PCR) (in case of DNA sequences) or reverse transcription PCR (RT-PCR) in case of RNA sequences) to enrich the library [10,11]. There are several methods to generate ssDNA from the resulting double-stranded DNA (dsDNA) including asymmetric PCR [12], denaturing urea-polyacrylamide gel [13], lambda exonuclease and T7 Gene 6 exonuclease digestion [14,15], and magnetic separation with streptavidin-coated beads [16]. The obtained ssDNAs are then used for the next round of selection. In most cases, the isolated aptamers *via* SELEX possess high affinity and low dissociation constant (K_D), ranging from micromolar to nanomolar. Therefore, aptamers are known as chemical antibodies. In contrast to antibodies, the aptamers are non-immunogenic, a feature that makes them superior to antibodies and attractive alternatives for various applications [17]. A number of successful therapeutic aptamers commonly used in clinic or clinical trials has been listed in Table 1. Among them, Pegaptanib, as a vascular endothelial growth factor (VEGF)-binding RNA aptamer approved by Food and Drug Administration (FDA), is used in the treatment of age-related macular degeneration (AMD) [18]. In addition to therapeutic applications, aptamers are applicable for many other purposes including diagnosis of microbial infections [19], disease biomarkers discovery [20], protein

detection in Western blot [21], surface plasmon resonance (SPR) assays [22], microarrays [23] and biosensors [24]. Since SELEX invention, many changes have been made in this process to improve the efficacy and shorten the duration of SELEX from several days to several hours. In this review, we describe several types of oligonucleotide pool used as SELEX library and modifications of nucleic acid aptamers to increase their resistance to nuclease degradation and renal filtration. Then, some SELEX methods from the early conventional methods to the most sophisticated techniques are briefly explained. At the end, we will provide the audience with aptamer applications in a wide variety of fields, ranging from biomarker discovery, diagnosis, imaging to therapy.

2. Oligonucleotide pool design

The emergence and development of SELEX technology, especially during its first decade, was characterized by a dominance of RNA aptamers [25,26]. The inclination to RNA aptamers was likely because of the common opinion that only RNA molecules could form functional spatial structures [27]. Before long, it was demonstrated that ssDNA molecules are capable of folding into target-binding structures as well [28]. From the beginning of SELEX era to 2007, about 70% of all studies were associated with RNA aptamers. Since then, DNA aptamers have occupied 70% of SELEX studies [26]. Nevertheless, the isolated DNA and RNA aptamers against a number of small-molecule targets indicated similar affinities [29]. Therefore, both ssDNA and RNA library are applied in SELEX experiments.

The design of a suitable oligonucleotide pool (library) is the first step of a successful SELEX process. Oligonucleotide library used for SELEX process mainly contains a central region with a random sequence flanked by two regions with fixed sequences. The fixed sequences act as primer binding sites (PBSs) during amplification steps. By standard methods of single-stranded oligonucleotide synthesis, the ssDNA library is obtained. Synthesizing the random region is achieved by adding the mixture of all four deoxyribonucleotide derivatives to the reaction mixture. In the case of RNA library, the promoter sequence for T7 RNA polymerase is introduced into the 5'-end of ssDNA library. Double-stranded DNA is generated by PCR and *in vitro* transcription is performed to obtain RNA library. Different types of library used for aptamer selection are as follows: 1) Classical library: this type of library usually contains sequences

adapters are used for sequence amplification and removed before the next round [37].

Another key parameter in library design is the incorporation of modified nucleotides into the library sequences. To protect both DNA and RNA aptamers from nuclease degradation [38,39], rapid renal clearance [40] and to improve the aptamer binding affinity [41], a large set of chemical modifications of nucleic acid library has been developed. These modifications can be performed in two ways: pre-SELEX and post-SELEX chemical modifications. In pre-SELEX modification, the modified library is directly used during selection. The main limitation of this approach is that the modification can affect the ability of the nucleotide to serve as a substrate for DNA or RNA polymerase. As an example, a modified nucleotide pool for generating RNA aptamers cannot be recognized by wild-type T7 RNA polymerase. To overcome this limitation, some mutant T7 RNA polymerases such as Y639F T7 RNA polymerase for 2'-fluoro pyrimidines [42] and Y693 F/H784A or R425C T7 RNA polymerases for 2'-O-methyl nucleotides [43,44] were applied. In post-SELEX modification approach, the previously obtained aptamers are modified. In this case, modification of aptamers can reduce the binding affinity of aptamers, and so the modification pattern optimization in every particular case is indispensable which makes the SELEX process more time-consuming and laborious. Some of the most common modifications of nucleic acid aptamers are as follows:

2.1. Nuclease resistance-related modifications

A number of modifications including 3'-end capping by inverted thymidine [45] and 3'-biotin conjugates [46] can improve the resistance of the aptamers to 3'-exonuclease. Moreover, the 3'-biotin-streptavidin conjugates decelerated the clearance rate of aptamers in blood circulation system [46]. The modifications on sugar ring are another trick to enhance the stability of aptamers. Some examples of these modifications are 2'-substitutions on sugar ring such as 2'-fluoro pyrimidines [47], 2'-amino pyrimidines [48], 2'-methoxy nucleotides [49] and 4'-thio nucleotides [50]. The modification of 2'-NH₂ was the first SELEX-compatible modification that became less favorable due to problems related to the chemical synthesis of 2'-NH₂-modified aptamers and negative impact of 2'-NH₂ on the ribose conformation [51]. By contrast, 2'-F modification proposed almost at the same time, became more popular because it provided sufficient nuclease resistance, had not unpleasant effect on RNA aptamer spatial structure and could be incorporated even by a non-modified T7 RNA polymerase under optimized conditions [52]. Structures of locked nucleic acid (LNA), unlocked nucleic acid (UNA) and 2'-deoxy-2'-fluoro-D-arabinonucleic acid (2'-FANA) have shown great resistance to nuclease attack. LNA is an analog of ribonucleotide with a methylene linkage between 2'-O and 4'-C of the sugar ring which confers a desired resistance to nuclease and increased thermostability [53]. Unlike the LNA that makes the modified aptamer rigid, UNA misses a bond between 2' and 3' of the sugar ring, resulting in a more flexible aptamers. This flexibility can alleviate strain in tight loop structures [54]. It was demonstrated that UNA modifications on the loop regions of thrombin-binding aptamer increased its thermodynamic stability. However, UNA modification is unfavorable for quadruplex formation [55]. Peng et al. indicated that in thrombin-binding aptamer, the modification of 2'-deoxy-2'-fluoro-D-arabinonucleic acid (2'-FANA) could increase the binding affinity and nuclease resistance up to four folds and seven folds, respectively. As a result, 2'-FANA modification was very suitable to improve the biological and physicochemical properties of DNA G-quartets [56].

In addition to sugar ring modifications mentioned above, a set of modifications can be applied on the phosphodiester linkage such as

its replacement with methylphosphonate, phosphorothioate [57] and triazole [58] analogs. Sacca et al. discovered that introduction of phosphorothioate analog into G-quadruplex structure might influence its thermal stability in a molecularly-dependent manner [57]. Although complete replacement of phosphate backbone with thiophosphorylated oligonucleotides is restricted because of their high toxicity, partial replacement with maximum thermal stability was selected to evaluate the stability and anti-thrombin activity of thrombin-binding aptamer under conditions of nuclease RQ1 DNase hydrolysis [59]. Replacement of phosphodiester linkage with triazole analogs has been also promising. The triazole internucleotide modifications can be performed via automated phosphoramidite synthesis with modified dinucleoside blocks [60] or the click reaction between azide- and alkyne-bearing nucleosides [61,62]. In 2013, Varizhuk et al. synthesized a series of triazole-bearing DNA aptamers that were structurally similar to the famous thrombin-inhibiting G-quadruplexes TBA15 and TBA31 (thrombin-binding aptamers). The modified aptamers were evaluated in terms of their secondary structure stabilities, binding affinities and anticoagulant effects. Although the modified aptamers possessed anti-coagulant activity and thrombin-binding affinity similar to those of TBA15, they obtained an increased nuclease resistance [63].

Mirror image aptamers (called Spiegelmers), the aptamers consisting of non-natural L-ribose nucleotides, can show high resistance to nuclease degradation and maintain their binding affinities. Based on the sequences of the respective D-aptamer against a mirror image target of interest, the L-enantiomeric oligonucleotide aptamers are then chemically synthesized [64]. A 67-nucleotide-long Spiegelmer with a K_D value of 20 nM was isolated against gonadotropin-releasing hormone (GnRH) through an *in vitro* selection process that was an effective antagonist to GnRH in Chinese hamster and rat models [65]. Olaptosed pegol (NOX-A12), a 45-nucleotide-long Spiegelmer to CXCL-12 chemokine, is in two phase II clinical trials for the treatment of both chronic lymphocytic leukemia and refractory multiple myeloma. NOX-A12 binding to CXCL-12 can block its receptor binding and thus avoiding CXCL-12 concentration gradient thereby decreasing cancer cell homing [64]. NOX-E36 is another L-form aptamer that binds to monocyte chemoattractant protein 1 (MCP-1), a chemokine involved in inflammation by recruiting leukocytes, and inhibits MCP-1-induced inflammation. Phase II clinical trials of NOX-E36 for the treatment of type-2 diabetes are in progress [66].

2.2. Renal clearance resistance-related modifications

Small size of aptamers causes their rapid renal clearance through glomerular filtration. Increasing the aptamer size through 5'-end formulation with bulky moieties including cholesterol, dialkyl lipids and polyethyleneglycol (PEG) is a strategy to overcome the rapid renal filtration and extend circulation time of the aptamers. Adding cholesterol to 5'-end of an aptamer can form a cholesterol-oligonucleotide (CholODN) conjugate. Lee et al. generated a cholesterol-modified 29-mer RNA aptamer which could be efficiently absorbed by the target cells and inhibits replication of Hepatitis C virus. Administration of resulting modified aptamer was well tolerated in mice and showed a nine-fold decrease in clearance rate from the bloodstream [67]. In one study, it was demonstrated that conjugation of VEGF aptamer (NX213) with a dialkylglycerol (DAG) contributes to stable anchoring of respective aptamer into bilayers of liposomes. DAG-modified aptamer was prepared via solid phase phosphoramidite method in which aptamer was attached to two 18-carbon saturated unbranched hydrocarbon chains by a tetraethylene glycol linker. The obtained DAG-modified aptamer was then incorporated into the bilayers of liposomes,

which resulted in a dramatic improvement in plasma circulation time and inhibitory activity of modified aptamers compared with non-modified ones [68]. One strategy to increase the size of aptamers is to add high molecular weight PEG to 5'-end of the aptamer. For instance, unmodified aptamer MP7 with the capability of binding to extracellular domain of murine programmed death protein 1 (PD-1) displayed *in vivo* half-life less than 1 h, whereas its conjugation with PEG limited the renal filtration rate and extended the half-life up to 24–48 h [69].

3. SELEX methods

3.1. Conventional SELEX

Conventional SELEX was independently developed by two groups more than two decades ago. Aptamers generation by conventional method takes from few weeks up to one month. In this method, a chemically synthesized pool of oligonucleotides (called library) is applied for aptamer isolation. The library contains up to 10^{15} different oligonucleotides that each of them comprises a central region of random sequence (20–80 nucleotides in length) flanked by two constant sequences (15–25 nucleotides in length) which enable primer annealing during amplification step of PCR [70]. Depending on the type of desired aptamer, several selection steps are implemented in conventional SELEX (Fig. 1A). In the case of DNA aptamers, a random DNA library is incubated with target molecule. The unbound sequences are removed and the bound ones are detached from the target. Uncoupled sequences are amplified by PCR and used for the next round of selection. Counter selection step is usually performed to remove target-unspecific oligonucleotides. After several rounds of selection, the obtained oligonucleotides are sequenced and their affinity for the target is determined by various methods. For RNA aptamers selection, a random ssDNA library is converted into dsDNA library by PCR. Then, the dsDNA library is transcribed into RNA sequences by means of a RNA polymerase (e.g. T7 RNA polymerase). Resulting RNA library is used to initiate RNA SELEX process. After each round of RNA SELEX, the isolated sequences should be reverse transcribed and then amplified by reverse transcription PCR (RT-PCR). Transcription of multiplied DNA molecules is carried out to generate RNA aptamers on which the next round is started. Up to 20 rounds of selection can be performed to enrich aptamers with high affinity and specificity [71]. Although the conventional SELEX is a well-established, it is considered as a time consuming and labor intensive method. Therefore, the development of alternative methods for aptamer isolation has been indispensable. The following sections deal with a number of more effective methods that can overcome the limitations of the aforementioned conventional SELEX method.

3.2. Nitrocellulose filter binding SELEX

Nitrocellulose filter binding SELEX is a simple and rapid method that was developed in the early stages of molecular biology studies in the 1970s. This method relies upon the fact that proteins and protein-DNA complex are bound to nitrocellulose filter whereas free DNA molecules are not retained. The unbound aptamers pass through the filter and are removed from the library. Then the mixture of protein-DNA is separated by electrophoresis and blotted onto nitrocellulose membrane (Fig. 1B) [72].

In addition to the convenience and time-saving properties of this method, other advantages are listed as follows:

- a) The true equilibrium binding of aptamers to its target in solution

- b) A relatively cheap and medium-throughput binding assay
- c) Isolation of aptamers against multiple targets in parallel
- d) Required no special equipment

In spite of aforementioned advantages, there are drawbacks that limit application of nitrocellulose filter binding assay including:

- a) Experimental conditions affect protein binding to membrane and protein capture yield differs from one protein to another as well.
- b) Molecules smaller than filter pore size (0.22–0.45 μm) are not captured by the filter [73,74].
- c) Non-selective binding of aptamers with specific 3D structure to filter (e.g. aptamers with a multi-G motif that nonspecifically bind to the membrane).
- e) Large number of selection rounds (8–20 rounds) is necessary to find high affinity aptamers [75].

A considerable number of aptamers have been isolated through nitrocellulose filter binding SELEX method and it is introduced as an efficient technique to isolate versatile aptamers (Table 2).

3.3. Bead-based SELEX

One of the common methods for SELEX is immobilization of target molecules on a solid support such as agarose, sepharose, and sephadex. This kind of strategy is appropriate for various target molecules due to the availability of different solid supports (resins) along with various tags comprising histidine (His), glutathione S-transferase (GST), biotin or maltose-binding protein (MBP) for Nickel-nitrilotriacetic acid (Ni-NTA), glutathione, streptavidin and amylose resins, respectively [76,77].

In bead-based SELEX, two scenarios are proposed: 1) pre-immobilization of target molecule on a solid support and then incubation with aptamer library 2) incubation of target molecule with aptamer library and then capturing target-aptamer complex by the affinity resin. In both scenarios, steady-state equilibrium binding occurs whereas non-equilibrium binding under flow conditions only occurs in pre-immobilization of target molecule on solid support allowing the users to select aptamers on the basis of kinetic parameters such as fast on-rate or slow off-rate, if needed. In the case of pre-immobilization, it is noteworthy to mention that the density of target molecule on the solid support plays a pivotal role in the enrichment of high-affinity aptamers since at unsuitably high concentration of target, cooperative binding may take place that in turn, retains undesired low-affinity aptamers and decreases the enrichment of high-affinity aptamers in each round of selection (Fig. 1C) [78]. The isolation of DNA aptamers to Staphylococcal enterotoxin B (SEB) is a good example of aptamer selection based on bead-based SELEX. The isolated anti-SEB aptamers indicated a limit of detection of less than 10 pg when were used in electrochemiluminescence assay [79]. Another example is isolating DNA aptamer against programmed death ligand 1 (PDL-1) as a tumor biomarker after 10 rounds of SELEX, leading to isolation of PDL-1 specific aptamer with high affinity ($K_D = 64.77 \text{ nM}$). Specificity and sensitivity of the isolated aptamer was to the extent that it can be considered as an aptasensor to detect tumor cells as low as 10 tumor cells per mL [80].

Some advantages that have made bead-based SELEX attractive are as follows:

- 1) Applicable to most targets such as small molecules, peptide, protein and cells
- 2) Serial and parallel selection of aptamers

- 3) The convenience of fine-tuning of selection stringency through changing flow rate and incubation parameters

3.4. Microcolumn bead-based SELEX

In an improved version of bead-based SELEX, resin is packed in a microcolumn, allowing all rounds of selection to be automated by means of pumps and/or centrifuges. Target molecule is immobilized on packed resin and then aptamer library is pumped through the microcolumn. Unbound aptamers are washed out and bound aptamers are collected and used for next round of selection. Microcolumn bead-based SELEX minimizes the amount of resin and aptamer and thereby reduces dead volume and non-specific binding while increases the efficiency of aptamer enrichment. In this respect, GST-tagged heat shock factor (HSF) proteins were immobilized onto glutathione-agarose resin and the conjugate was then packed into the microcolumn. The starting RNA library was loaded onto the microcolumn. The unbound RNA molecules were washed and RNA-protein complexes were eluted from microcolumn by elution buffer. The bound RNA aptamers were reverse transcribed, amplified and used for the next round of selection. After the completion of 5 selection rounds, two high-affinity RNA aptamers with K_D less than 20 nM were obtained [81]. Although several microcolumns can serially be connected to each other enabling multiplex selections to be carried out by a single aptamer library, this manipulation is tedious to be employed for large-scale multiplex experiments. To overcome this limitation, a scaled-up version of microcolumn called MEDUSA (microplate-based enrichment device used for the selection of aptamers) was designed. MEDUSA can directly be coupled to a 96-well microplate in addition to all benefits of the individual microcolumns. This coupling not only provided high-throughput selections, but also boosted plate-based methods for sample handling that can be automated by liquid-handling robots [82]. In MEDUSA method, it should be noted that density of target molecule significantly affects aptamer enrichment. For instance when target molecule is protein, 0.5–1 $\mu\text{g}/\mu\text{L}$ of the target protein (independent of molecular weight of target protein) would improve the recovery of the specific aptamers [83]. On the contrary, any arbitrary increase in density of the target protein could lead to molecular accumulation and decrease in aptamer recovery. A possible explanation is that multiple weak interactions between a low-affinity aptamer and several juxtaposed protein targets cooperatively increases aptamer binding strength as much as three orders of magnitude that culminates in retention of low-affinity aptamer in final pool [75]. Therefore, it is strongly recommended that density of target molecule be optimized to achieve successful enrichment of aptamer.

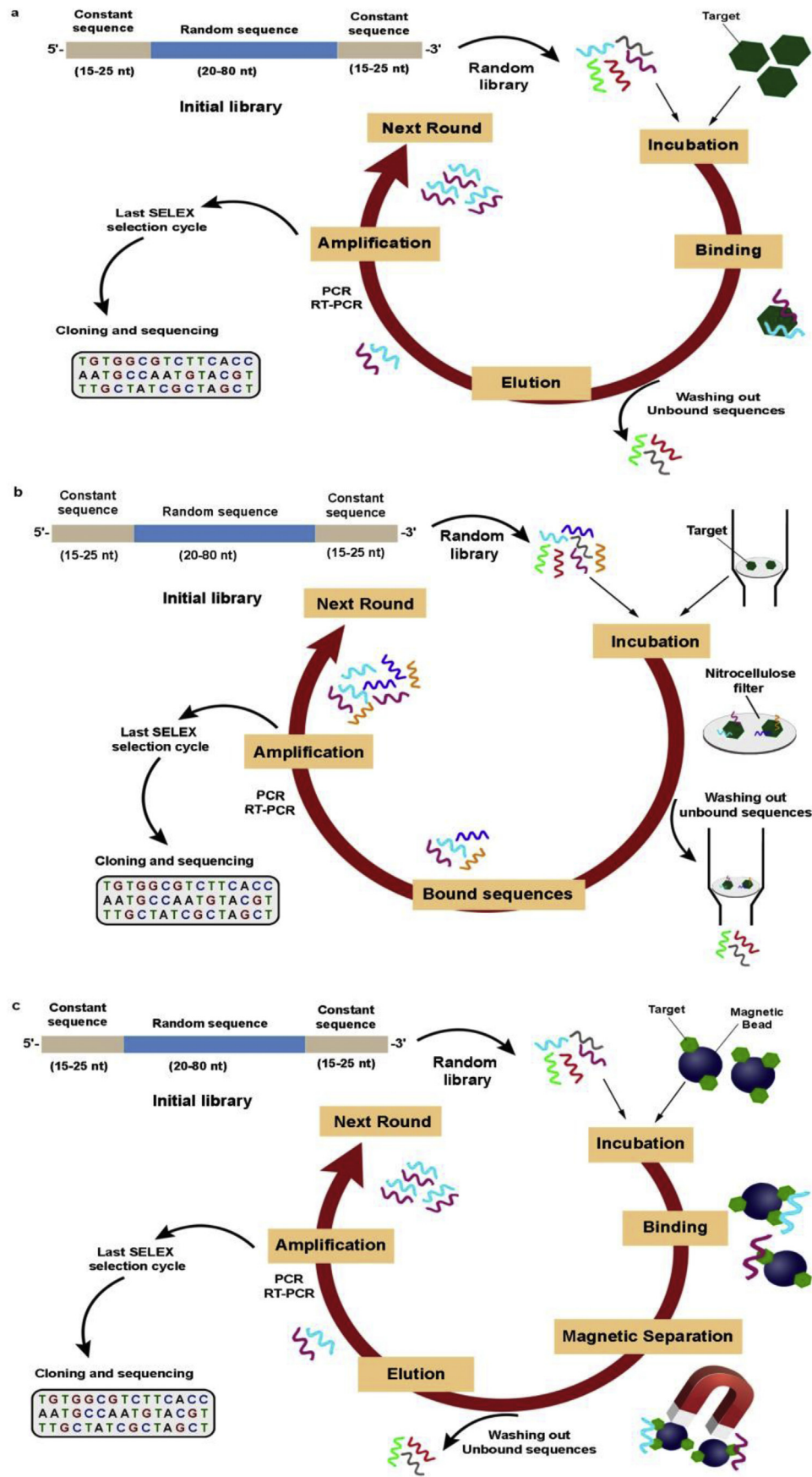
3.5. Particle display

In most SELEX methods, several rounds of selection need to be performed (about 8–15 rounds) to obtain desired aptamers that, in turn, is accompanied with disadvantages including loss of rare sequences [70], PCR bias [84] and retention of non-specific and low-affinity aptamers [85]. In an effort to overcome these limitations, an alternative method has been developed called particle display in which relative affinity of each candidate aptamer is quantitatively measured and individually sorted in a high-throughput manner. Inspired by yeast [86] and bacterial [87] display techniques used in protein engineering, particle display converts individual aptamers into aptamer particles (APs) wherein each particle presents as many as 10^5 copies of a single aptamer. Each aptamer particle is synthesized *via* emulsion polymerase chain reaction (ePCR) where water-in-oil emulsion with PCR reagents is prepared. In ePCR, each

droplet contains (in most cases) only one DNA template and one magnetic bead coated with forward primer. After ePCR within each droplet and generating about 10^5 copies of each aptamer, emulsion is broken and unreacted PCR reagents are removed. By NaOH addition, denaturation occurs and reverse strand is released. Then aptamer particles are collected by magnetic separation. The collected APs are incubated with fluorescently labeled target molecule and sorted on the basis of target binding by FACS (Fluorescence-Activated Cell Sorting) instrument. It should be noted that the fluorescence intensity of bound target to aptamer particle is in direct proportion to binding affinity of the aptamer. This characteristic allows the user to quantitatively identify and sort the aptamers with the highest affinity using FACS. The sorted aptamer particles are amplified by PCR to generate an enriched pool of aptamers for the next rounds of selection (Fig. 2A). Particle display method has advantages over other conventional SELEX methods that make it attractive. Firstly, due to individual measurement of each aptamer, better discrimination can be achieved between aptamers with similar affinity while it is possible to isolate the highest-affinity aptamers in fewer rounds. Secondly, unlike other conventional SELEX methods that a considerable percentage of unwanted and low-affinity aptamers are enriched, in particle display method such low-affinity aptamers generate low fluorescence and will therefore be removed during FACS process. Finally, visualizing the screening process *via* implementing FACS enables the users to determine the desired aptamer affinity threshold in a quantitative and reproducible manner [88]. The obtained aptamers by using particle display method exhibited considerably higher affinities than those of previously discovered aptamers *via* other conventional SELEX methods against the same targets. For instance, the isolated anti-thrombin aptamer by particle display method exhibited a K_D value of 7.04 pM whereas K_D values of the isolated aptamers by Bock [89] and Tasset [90] to the same target were 2.6 nM and 5.4 nM, respectively, when they were measured by the same binding assay. In addition, although many attempts were unsuccessful to generate aptamers against PAI-1 and 4-1 BB, particle display method generated aptamers with high affinity to PAI-1 ($K_D = 339$ pM) and 4-1 BB ($K_D = 2.32$ nM) [88].

3.6. Capillary electrophoresis SELEX

Capillary electrophoresis SELEX (CE-SELEX) relies upon the shift in electrophoretic mobility pattern of nucleic acid following target molecule-nucleic acid complex formation [91]. In CE-SELEX, nucleic acid library is incubated with target molecule in solution and then the mixture is injected onto a CE capillary. Under the establishment of high voltage, components of the mixture are separated. The concept of separation is based on the electrophoretic mobility pattern of bound aptamers which is distinct from that of unbound ones, allowing them to be collected as different fractions in the selection process. Bound aptamers are amplified by PCR and purified for the next round of selection (Fig. 2B) [92]. CE-SELEX has successfully been used to isolate aptamers with low nanomolar affinity against large protein targets such as IgE [93] and protein farnesyltransferase [94] but it can also be used to isolate aptamers that bind to small molecules such as neuropeptide Y [95] and N-methyl mesoporphyrin IX (NMM) [96]. In comparison with bead-based SELEX, the isolation of high-affinity aptamers can be carried out only in 1–4 rounds using CE-SELEX method reducing the selection rounds and time needed [92]. Moreover, in CE-SELEX, the aptamer binding to target takes place in free solution in which aptamer-target interaction occurs without steric hindrance. In-solution aptamer-target interaction reduces the opportunity for non-specific bindings and eliminates the need for target immobilization strategies. Until now, two distinct CE-SELEX methods have



been used for aptamer selection: non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) [94] and equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) [97]. The major difference between ECEEM and NECEEM method is that, in the case of the former, the equilibrated mixture of DNA library-target is injected into a capillary prefilled with a run buffer containing the target at the concentration identical to that of target in the equilibrium mixture whereas the latter is conducted without target in the run buffer [97].

3.7. Non-SELEX

Non-SELEX aptamer selection is a process in which amplification of sequences between repetitive steps of partitioning has been excluded. Omitting iterative steps of amplification speeds up the procedure of aptamer isolation as well as removes quantitative errors related to the nature of PCR amplification [98]. Additionally, non-SELEX can offer a viable substitution for SELEX in the commercial development of aptamers [99]. Considering the outstanding partitioning ability of CE methods and no need for amplification steps of non-SELEX, a new method called NECEEM-based non-SELEX was designed to improve the affinity of aptamer to a target protein (h-Ras protein) by more than four orders of magnitude. Surprisingly, NECEEM-based non-SELEX process took only 1 h to complete compared to several days or weeks required for SELEX procedure by common partitioning methods. Moreover, the method allowed to accurately measure the aptamer abundance in the library. Using NECEEM-based non-SELEX, DNA aptamer was selected against h-Ras protein (Ras proteins are small GTPases that regulate growth, proliferation and differentiation of cells). The selection process was started with a pre-selection step wherein migration times of Ras protein, fluorescently labeled DNA library and Ras-fluorescent DNA complex were measured. The peak areas of Ras-DNA complex and unbound DNA were used to determine bulk K_D value, while the migration times of the two peaks defined the aptamer collection window. In the selection steps, multiple steps of NECEEM without PCR amplification in between were performed to partition the candidate oligonucleotides from the rest of the library. After each step of partitioning, the collected fractions are kept for subsequent analysis in which all the collected DNA fractions are amplified by PCR, double-stranded products are separated to produce single-stranded aptamer pools and, finally, the affinity of the obtained pools is measured. Pools with the best affinity are cloned and sequenced [98].

3.8. Micro free flow electrophoresis SELEX

Small size of the initiating library ($\sim 10^{12}$ sequences) is the major limitation of CE-SELEX. In an effort to circumvent this limitation, a micro free flow electrophoresis (μ FFE) device has been developed. This device has increased the amount of the initiating library up to 300 times (1.8×10^{14} sequences). Analytes are continuously introduced, separated and collected in μ FFE method, resulting in higher yield of collected aptamer in contrast to CE-SELEX. In brief, the analytes are continuously injected into a separation chamber. An electric field is perpendicularly established to flow direction by which analytes are laterally diverted according to their

Table 2

Examples of obtained aptamers using nitrocellulose membrane filtration method.

Target molecules	Year	Ref.
T4 DNA polymerase	1990	[8]
HIV-1 Rev	1991	[212]
Reverse transcriptase (HIV-1)	1992	[213]
Rev-binding element of HIV-1	1993	[214]
Human α -thrombin	1994	[215]
Rous sarcoma virus (RSV)	1995	[216]
Human IgE	1996	[217]
NS3 of hepatitis C virus	1997	[218]
Vascular endothelial growth factor (VEGF)	1998	[188]
Rex fusion protein	1999	[219]
Human factor VIIa	2000	[220]
Drosophila transformer 2 (Tra2)	2001	[221]
Human coagulation factor IXa	2002	[222]
Angiopoietin-2	2003	[223]
Mouse prion protein	2006	[224]
4-1 B B (CD137)	2007	[225]
HIV-1 gp120	2009	[226]
HBS antigen	2010	[227]
Hfq	2011	[228]
Avian influenza virus H5N1	2013	[229]
Shiga toxin	2014	[230]
Extracellular vesicles	2017	[231]
Human α A-crystallin	2017	[232]
Lactate dehydrogenase of <i>Plasmodium falciparum</i>	2018	[233]

electrophoretic mobility. Jing et al. used μ FFE SELEX to isolate aptamers against human IgE. The high-affinity aptamers with K_D of 20 nM were obtained after 2–4 rounds of selection [100]. Larger size of the starting library, lower electric field and shorter separation time are the advantages of μ FFE over CE-SELEX [75]. Unfortunately, μ FFE has shortcomings that highly limit its application in an ordinary laboratory due to high tech requirements including design and fabrication of sophisticated μ FFE device and specific electric equipment. In addition, although μ FFE augmented the size of starting library compared to CE-SELEX, there are SELEX methods that provide the application of even larger size of starting library up to 10^{16} sequences.

3.9. Microfluidic SELEX

Time-consuming and labor-intensive are two common disadvantages of most SELEX methods. In recent years, microfluidic SELEX (M-SELEX) technology has been designed to accelerate the process of aptamer selection by providing highly stringent selection conditions by the use of very small amount of reagents [101]. In addition, this method enables the users to control the flow rate to remove weakly or non-specifically bound aptamers with minimal target loss. These features make the isolation of high-affinity aptamers possible within 1–6 rounds of selection [102]. Microfluidic SELEX can be performed in two ways: 1) immobilization of target molecules on micromagnetic beads and then incubation of coated beads with library [102] and 2) encapsulation of target molecules in sol-gel and then adding library to sol-gel-entrapped target molecules [103]. Unlike the CE-SELEX, wherein electrophoretic mobility shift is a fundamental parameter to obtain high-affinity aptamers, M-SELEX is applicable to small molecules that do not cause significant change in electrophoretic mobility of

Fig. 1. Schematic representation of SELEX. A) In general, SELEX process is composed of incubation, binding, partitioning and amplification steps. In the incubation step, target molecules are exposed to random library to provide the opportunity for oligonucleotide-target binding. The bound sequences are isolated from unbound ones during the partitioning step. The bound aptamers are then amplified to generate an enriched pool of target-binding sequences for the next round of selection. After several rounds of selection, the enriched pool is cloned and the obtained clones are separately sequenced. B) Nitrocellulose filter binding SELEX: target molecules are immobilized on nitrocellulose filter and then incubated with the library. The unbound sequences are washed out and pass through the filter whereas the target-bound sequences are retained. The bound sequences are eluted and amplified for the next round of SELEX. C) Bead-based SELEX: the target molecules are covalently or non-covalently immobilized on the magnetic beads. After incubation of oligonucleotide pool with bead-target complexes, the partitioning is done with the aid of a magnet. The bound sequences are eluted and amplified for the next round.

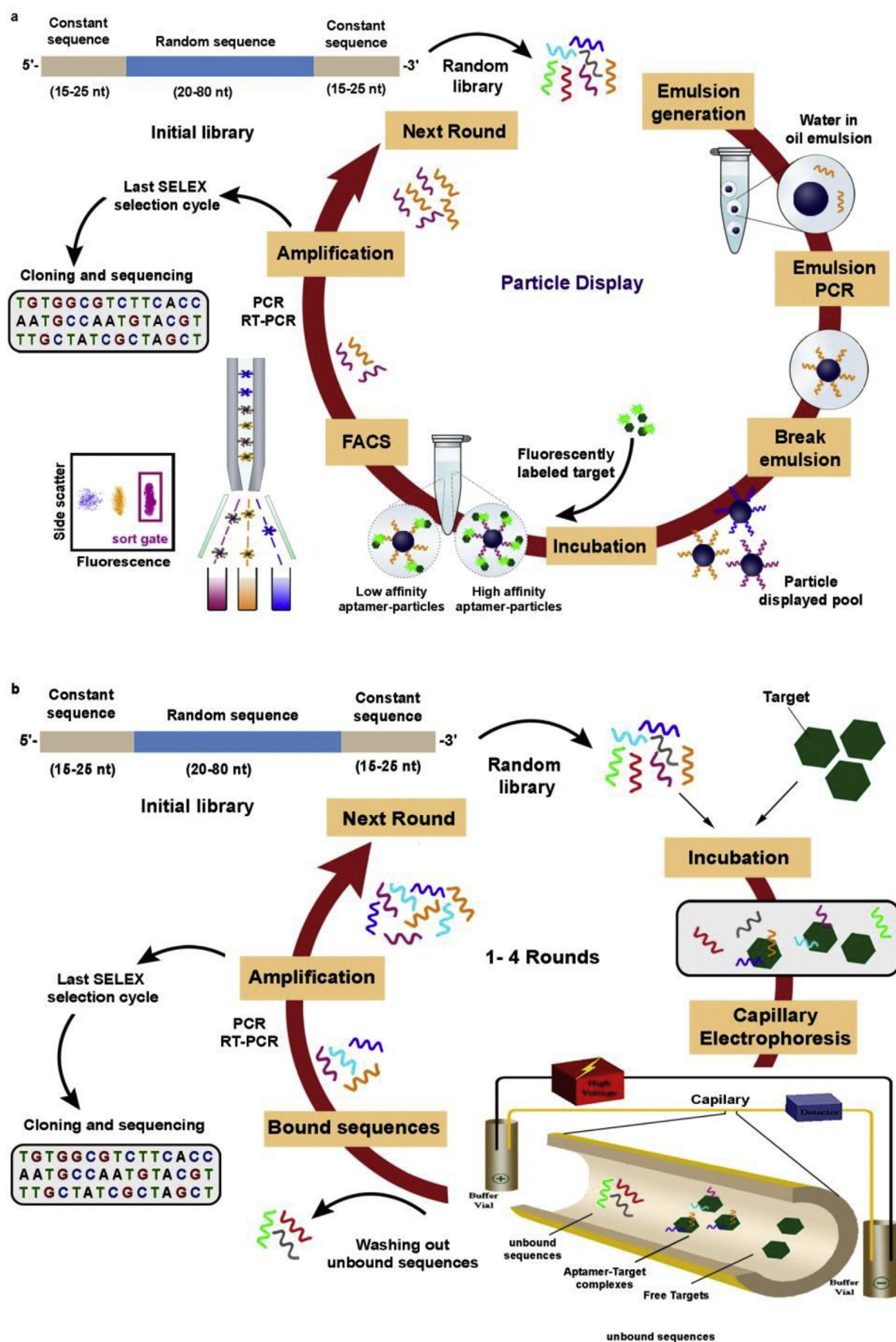


Fig. 2. Schematic representation of A) Particle display: in this method, the aptamers of solution-phase library are converted into aptamer particles (Aps) using emulsion PCR (ePCR) reaction. In most cases, each droplet contains one sequence and one forward primer-coated magnetic bead. Upon ePCR within the droplet, as many as 10^5 copies of a single aptamer sequence are generated on the surface of each particle. After breaking the emulsions, APs are released and reverse strands are removed by NaOH treatment. The APs are incubated with fluorescently labeled target molecules. Fluorescence-Activated Cell Sorting (FACS) is then used to individually measure the aptamer affinity. The fluorescence intensity of

target-aptamer complex. In micro-magnetic bead-based microfluidic SELEX process, a random library of aptamer is incubated with target protein conjugated with magnetic beads. In the next stage, a continuous-laminar-flow, magnetically-activated, cheap-based separation (CMACS) device is exploited to isolate bound aptamers from unbound ones. Under the effect of magnetic field, bound aptamers to magnetic bead-protein complex move at the center of chip and are eluted through middle outlet whereas unbound aptamers are eluted through the side waste outlet. Then, the bound aptamers are amplified by PCR and single stranded aptamers are generated (Fig. 3A). DNA aptamer against the light chain of recombinant botulinum neurotoxin type A (BoNT/A-rLc) with low-nanomolar affinity was isolated after a single round of selection by the use of micro-magnetic bead-based microfluidic SELEX strategy [104]. Although this method is rapid, highly efficient, and applicable to a wide variety of targets and can be automated, its major drawbacks are diversion of flow direction by microbubbles and low purity and recovery of aptamers due to aggregation of bead in the microchannel. To overcome these limitations, an improved microfluidic separation device was developed by Soh et al. called micromagnetic separation (MMS) chip which represented several advantages including reproducible molecular separation with very high bead recovery (about 99.5%) and high partition efficiencies ($PE \sim 10^6$). For instance, aptamer against streptavidin with low nanomolar affinity ($\sim 30 \pm 5$ nM) was obtained in just three rounds using the MMS device [101].

In a new strategy, M-SELEX was integrated with both next generation sequencing (NGS) and *in-situ* synthesized aptamer array to create a quantitative parallel aptamer selection system (QPASS) which enables simultaneous measurement of specificity and affinity of thousands of aptamers in parallel. QPASS was used to isolate and characterize aptamers against angiopoietin-2 (Ang-2) as a human cancer biomarker. Angiopoietin-2 was immobilized on magnetic bead and four rounds of M-SELEX were performed. All resulting aptamers were sequenced by NGS and then analyzed for copy number and sequence homology. To characterize the affinity and specificity of obtained aptamers, a chip containing eight identical *in-situ* synthesized aptamer arrays of 15000 aptamers was fabricated. Each array represented the 235 top candidate aptamers from each round of selection and 65 control sequences in triplicate. By QPASS, six high-affinity aptamers against Ang-2 were identified with affinity less than 30 nM and excellent specificity [105].

3.10. Sol-gel based microfluidic SELEX

Sol-gel is a silica material that is prepared by hydrolysis and polycondensation reactions at room temperature. Sol-gel matrices contain nanoscale pores and microscale channels [106]. Nanoscale pores act as a support to retain target molecules whereas aptamers can freely move through microscale channels until reaching the entrapped target molecules [107]. The process of sol-gel provides an aqueous environment that conserves biological activity of entrapped molecules and may enhance their biomolecular stability [108]. In sol-gel method, affinity tags are added to reaction mixture during synthesis of matrices. Then affinity tags are encapsulated within silica-based materials (Fig. 3B). Hence, a wide variety of targets such as proteins and aptamers can be captured on the surface of sol-gel chips with no need for biomolecular modifications [109]. Xanthine as a purine metabolite was successfully encapsulated within sol-gel and the mixture was spotted onto the

porous silicon chip. After seven rounds of SELEX, high-affinity aptamers ($K_D = 4.2 \mu\text{M}$) against xanthine was isolated [103]. Very recently, in an effort to develop a fluorescent aptamer as an aptasensor to detect botulinum neurotoxin E (BoNTE), porous silicon-sol-gel (PS-SG) SELEX was used. Like other PS-SG SELEX methods, after encapsulation of BoNTE within sol-gel, spotting the mixture onto the porous silicon chip was performed. Then, aptamers with low nanomolar binding affinity (~ 53 nM) were obtained after five rounds of selection [110]. One of the main advantages of sol-gel based microfluidic SELEX is the possibility of isolating aptamers for multiple targets in one experiment with multiplex selection.

Regardless of all advantages of both micromagnetic bead-based and sol-gel based microfluidic SELEX, there are limitations that decrease applicability of the aforementioned methods including: 1) Requirement of nanofabrication of microfluidic devices and respective electronic instruments, 2) The possibility of cross-contamination in microfluidic SELEX methods because selection process is performed in micro scale, 3) The partitioning step is carried out under non-equilibrium flow conditions, and 4) In sol-gel based method, the chemical reactions during sol-gel formation may be followed by potential undesired effects on target molecules [75].

3.11. SPR-SELEX

One of the reasons making the conventional SELEX time-consuming is that selection and binding evaluation of isolated oligonucleotides are performed in two discrete steps. At first, the sequences binding target are isolated after several rounds of selection and then binding enrichment of selected sequences is assayed at the end. SPR-SELEX has bypassed this limitation through real-time (round by round) evaluation of pool enrichment during the selection process. SPR-SELEX allows evaluation of binding via monitoring of SPR signal when the pool is injected while partitioning is carried out when the injection is stopped during the dissociation phase of the sensograms. If no further evolution takes place in SPR signal, the selection process can be stopped. In conclusion, by means of SPR-SELEX method, aptamer isolation is accelerated because the selection and binding evaluation steps are done simultaneously. This method can be applied for all types of targets provided that oligonucleotide-target dissociation does not occur faster than the dead time of the instrument to recover them. If complex dissociation is too fast, it could be decelerated by lowering the temperature of the microfluidic cartridge. A high-affinity RNA aptamer (K_D value = 8 nM) that forms highly stable loop-loop complexes with the target was identified by SPR-SELEX within six rounds of selection [111].

3.12. Microarray-based SELEX

Microarray technology paved the way for simultaneous analysis of a large number of parameters from an insignificant amount of sample within a single experiment. In recent years, DNA and RNA microarrays have been developed as a new approach for aptamer selection [112,113]. In this method, DNA or RNA aptamers are immobilized on a slide using chemical linkage and then fluorescently labeled target proteins are added to spotted microarrays. The signal intensity of each spot is determined and strongly bound aptamers are selected accordingly. For example, DNA aptamers with high affinity and specificity were isolated against the

captured target molecule is directly proportional to the binding affinity of the aptamer. Accordingly, the high-affinity aptamers are determined and amplified for the following rounds. B) Capillary electrophoresis SELEX: random library is incubated with target molecules in solution and the mixture is then loaded into a capillary electrophoresis system in which mobility pattern of the bound oligonucleotides is different from that of unbound ones, allowing their collection as distinct fractions. The collected oligonucleotides are amplified for the next round.

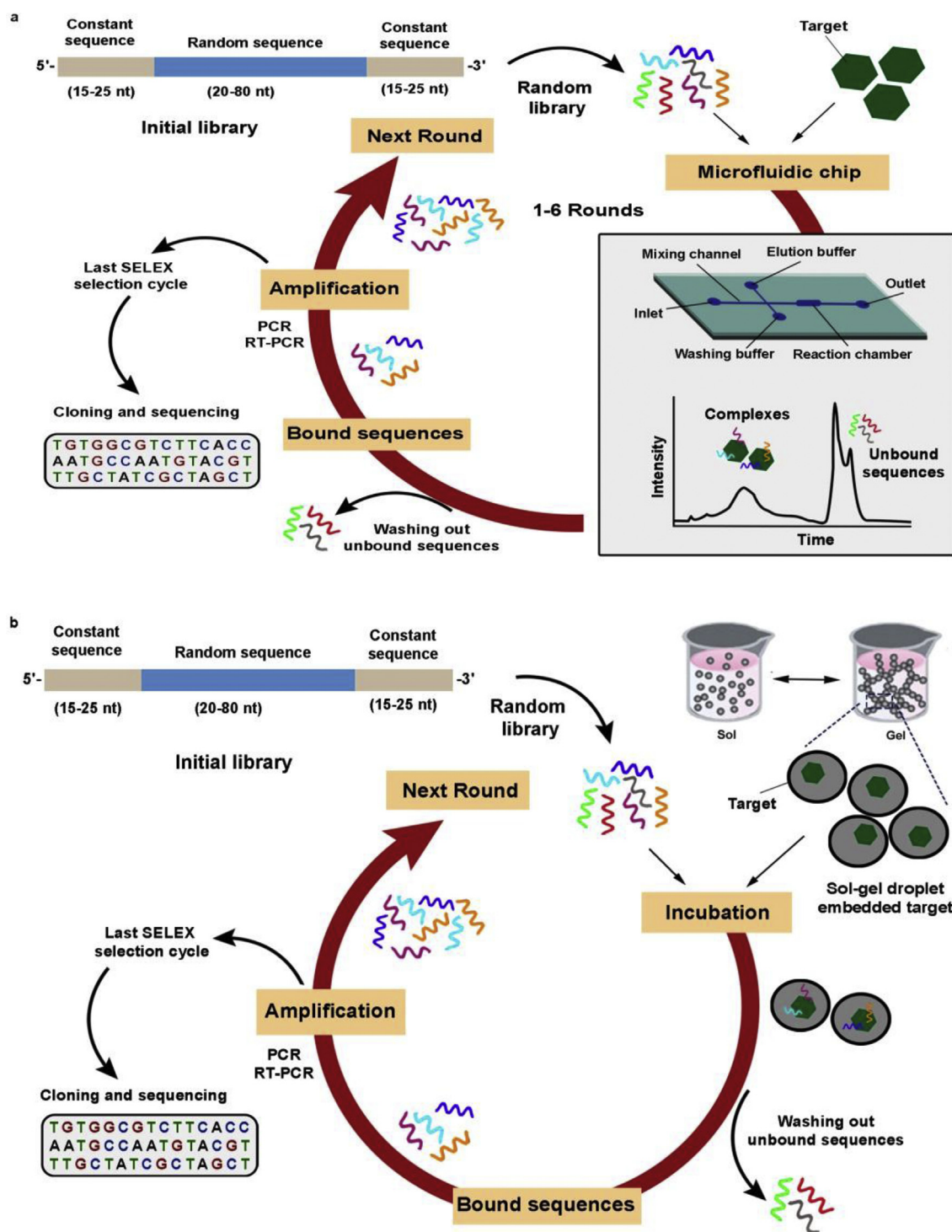


Fig. 3. Schematic representation of A) microfluidic SELEX: a random library of aptamer is incubated with target molecule-magnetic beads conjugates. In the next step, a continuous-laminar-flow, magnetically-activated, cheap-based separation (CMACS) device is exploited to isolate bound oligonucleotides from unbound ones. Under the effect of magnetic field, bound oligonucleotides to magnetic bead-target complex move at the center of chip and are eluted through middle outlet whereas unbound oligonucleotides are eluted through the side waste outlet. Then, the bound sequences are amplified by PCR and used for the next round of selection. B) Sol-gel based microfluidic SELEX: Sol-gel matrices contain nanoscale pores and microscale channels. Nanoscale pores act as a support to retain target molecules whereas aptamers can freely move through microscale channels until reaching the entrapped target molecules. In sol-gel method, affinity tags are added to reaction mixture during synthesis of matrices.

fluorescent protein allophycocyanin (APC) through a smart microarray-based SELEX called Closed Loop Aptameric Directed Evolution (CLADE). In this method, after first round of selection, the calculation of aptamer fitness is carried out according to the aptamer binding intensity and thereby the frequency distribution

of the first round bound aptamers is estimated. Based on *in vitro* score distribution, some of these sequences are selected and then mutated *in silico* to generate a new sequence set. This new set of sequence is synthesized *in vitro*, and employed for the next round of selection. A highly increased binding affinity to target is obtained

at the final set [114].

A single microarray experiment is performed within less than one day and data is analyzed within 1–2 days. There is no need for PCR, cloning and sequencing since known sequences are located in predefined spots. In contrast to other SELEX methods, in microarray-based SELEX, sequences are covalently linked to the surface so, highly stringent conditions can be imposed to isolate sequences with high binding affinity [115].

Microarray-based SELEX method suffers from a major drawback that an array containing maximum 10^6 sequences can be synthesized on a slide while in other SELEX methods, an initial library as large as 10^{15} sequences can be implemented. Recent studies have proposed that using partially structured pool of starting library instead of a random RNA/DNA library can increase the degree of structural complexity of library sequences that in turn, can enhance the possibility of isolating aptamers with improved activity and affinity [116]. In this regard, Davis et al. used a mixture of fully random and partially structured library to select high-affinity aptamers against guanosine triphosphate GTP molecule. The experiment demonstrated that aptamers originated from partially structured library (containing centrally located stable stem-loop) had the highest affinity for GTP molecule [117].

3.13. Capture-SELEX

In most SELEX methods, immobilization of target molecule on a solid matrix is inevitable. Such methodological approach is not applicable to all aptamer targets such as very small target molecules without suitable functional groups. Adding functional groups to such molecules may change their conformation that in turn affects aptamer binding features. The capture-SELEX introduces a substitution method via immobilization of oligonucleotide library on a solid matrix rather than target immobilization. In this method, the starting library is different from that of conventional methods. The library of capture-SELEX is designed based on the structure-switching mechanism described by Nutiu and Li in 2005 [118]. Based on this mechanism, each aptamer has the ability to switch structures from a duplex structure (upon hybridization with an antisense DNA) to a complex one (upon binding to a target molecule). Special DNA library used in this strategy contains a central fix sequence flanked by two different-sized random sequences, each further flanked by specific primer binding sites (PBSs). The central constant sequence is complementary to a 5'-end biotinylated antisense oligonucleotide (capture oligo) so that act as a docking sequence allowing immobilization of the oligonucleotide library on avidin-coated magnetic beads following DNA hybridization. During selection process, those sequences that can bind the target molecules undergo conformational changes leading to switching from duplex formation (oligonucleotide-capture oligo duplex) to complex one (oligonucleotide-target complex). This structure switching can result in target-dependent release of oligonucleotides from the matrix. The released sequences are collected using a magnet stand, amplified by PCR and used for the following round of selection. Fluorescent label attachment to oligonucleotides during PCR enables the quantification of sequences in the SELEX steps to evaluate the progress of selection process. This contributes to monitoring of target-binding oligonucleotides enrichment. It is noteworthy to mention that some oligonucleotides of capture-SELEX library, which can bind to the target, do not undergo conformational changes, are not released and therefore are not selected. Capture-SELEX is able to isolate aptamers against two or more different target molecules in one SELEX process. Accordingly, this method was successfully employed to isolate DNA aptamer against a mixture of pharmaceuticals (kanamycin A, sulfacarbamide, sulfamethoxazole and sotalol hydrochloride). By this way, an aptamer

with high affinity (85.6 nM) to kanamycin was obtained [119].

3.14. In silico SELEX

In silico SELEX method is a new method for the discovery and optimization of aptamers. This technique has provided a practical computational solution for three key obstacles in aptamer selection including 1) design of an enhanced starting library, 2) enrichment of sequences, and 3) identification of high-affinity aptamers.

Recently, *in silico* SELEX approach was used to search a sequence space containing 4^{13} sequences for new aptamers against theophylline. As a result, six novel aptamers with similar or higher affinity than the isolated aptamers by original SELEX against theophylline were obtained [120].

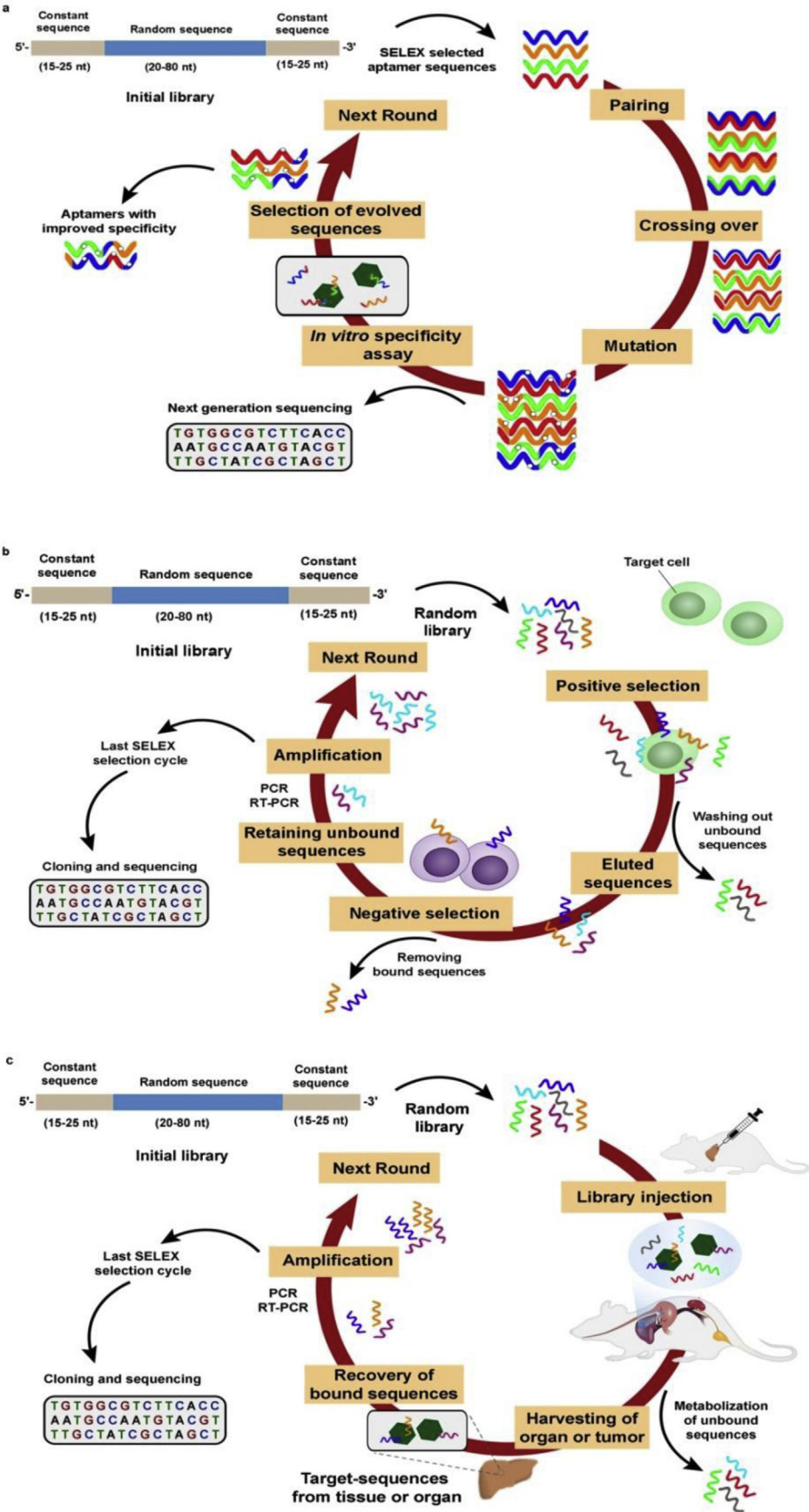
Briefly, *in silico* SELEX is performed in two steps (Fig. 4A):

- Step1 screening of sequences based on their secondary structure. The analysis of aptamer pool revealed that high-affinity aptamers are thermodynamically different from the random sequences. For instance, free energies of secondary structure formation of GTP binding aptamers are significantly lower than those of random sequences with the same length [121]. Therefore, minimizing the presence of sequences with simple stem-loop structures or slightly branched structures and maximizing the presence of aptamers with stable low-energy structures at the same time, seems beneficial. This kind of sequences can be selected to form an enhanced initiating library for the next step.
- Step2 screening the initiating library using computational docking tools. There are different docking tools in order to identify the sequences that bind target molecule. One of these tools is a modified version of the DOVIS package that was employed by Zhang et al. for high-throughput virtual screening of RNA library [122]. Based on data from docking analysis (e.g. free energy of binding and dissociation constant of aptamers), the most versatile aptamer is selected and used for further studies.

3.15. Atomic force microscopy (AFM) SELEX

Atomic force microscopy is a method that can precisely examine the affinity, interaction forces and recognition features at the molecular level [123]. The AFM possesses a flexible cantilever that benefits from a sharp tip at its end detecting even weak forces between sample surface and cantilever. Vertically or laterally displacement of the cantilever sharp tip with respect to the sample surface is achieved by the cantilever holder or piezoceramics stage, holding the sample. When the sharp tip comes into contact or near contact with the sample surface, a deflection of the cantilever occurs that is registered by a laser beam reflected off the back of the cantilever. Upon deflection of the cantilever, a topographic image of the sample can be created via raster-scanned in x-y direction. On the other hand, when the cantilever is displaced in z direction, i.e. at first it moves downward so that it contacts the sample and then moves upward until does not come into contact with sample, the so-called force curves is generated [124,125].

Because AFM method can dynamically detect the affinity force and adhesion between sample surface and cantilever, an efficient method has been developed to select aptamers that bind to their targets with very high affinity called AFM-SELEX method. In a successful experiment, DNA aptamer to thrombin with very high affinity ($K_D = 200$ pM) was isolated within three rounds of selection.



In the mentioned study, streptavidin molecules were immobilized on the cantilever tip and then the biotinylated ssDNA library was mounted on the cantilever. Thrombin as target molecule was also immobilized on the gold chip. When the ssDNA-soaked cantilever tip comes into contact with the gold chip, the DNA aptamers against thrombin bind to immobilized thrombin on the gold chip. The DNA aptamer with high affinity for thrombin can break the streptavidin-biotin interaction and leave the DNA-thrombin complex on the gold chip. The remaining DNA is eluted, amplified by PCR and used for the next round of selection [126]. Very recently, high affinity aptamers against human serum albumin (HSA) were isolated using AFM-SELEX wherein the cantilever was modified by HSA as target molecule and ssDNA aptamers were immobilized on the gold chip by means of a DNA duplex composed of 35 mer of adenines (A35) and 10 mer of thymines (T10). The modified cantilever with HSA is brought into contact with the immobilized ssDNA. The hydrogen bonds between A35-T10 duplex are cleaved when the interactive force of aptamer-HSA is larger than that of A35-T10. The selected aptamers were amplified and used for the next round of SELEX [127].

Although AFM-SELEX method is able to isolate high affinity aptamers only within 3–4 rounds of SELEX, the following disadvantages limit its usage: the need for expensive equipment, immobilization of the target and aptamers, non-equilibrium binding and relatively small size of starting library [75].

3.16. High fidelity (Hi-Fi) SELEX

Although powerful methods of SELEX such as microfluidic SELEX, capillary electrophoresis SELEX and atomic force microscopy SELEX have improved the isolation of high-affinity aptamers in fewer rounds of selection compared to traditional methods (magnetic bead-based SELEX and nitrocellulose filtration SELEX), such methods are not available in ordinary molecular biology laboratory. High fidelity SELEX is a recently developed method which does not require sophisticated instrument and also enables users to obtain high affinity aptamers within fewer rounds of selection [128]. The potential of this method is ascribed to various modifications including: 1) annealing complementary sequences to fixed region of each sequence of library to generate a competent starting library. Using such a competent library can eliminate the different manners by which common sequences can interfere with the fold and function of aptamers that are located within the random region of library; 2) passivating the surface against non-specific adsorption of oligonucleotides. It has been demonstrated that dendritic polyglycol chains can passivate the surface against the non-specific adsorption of oligonucleotides [129] that, in turn, leads to an increase in partitioning efficiency (PE); 3) using digital PCR (dPCR) to ensure that all selected sequences exist in their fully complementary duplexed form.

Hi-Fi SELEX platform was employed to isolate ssDNA aptamers against human α -thrombin. A competent library containing about 10^{14} sequences was added to human α -thrombin which was immobilized on the adsorption-passivating polyglycol coated-surface. The isolated aptamers were amplified by dPCR and used for the next rounds. After only three rounds of SELEX, aptamers with high affinity against human α -thrombin (K_D about 2.6 nM)

were obtained [128].

3.17. Cell SELEX

All the aforementioned SELEX methods are on the basis of performing *in vitro* SELEX against purified target molecules and especially applicable to purified proteins that possess stable conformation. Nonetheless, when the target molecules are present in a modified state or their binding domain is masked in a physiological context, the ligand-based SELEX methods are not capable of recognizing the natural structure of target ligand [130]. For instance, although the isolated RNA aptamers against the recombinant ectodomain of EGFRvIII expressed in *Escherichia coli* bound *in vitro* to purified EGFRvIII, their binding to the full-length EGFRvIII protein expressed on the eukaryotic cells surface was not observed [131].

The disadvantages of selected aptamers against non-native targets necessitate the employment of alternative methods. One of these methods is cell SELEX process wherein whole living cells are considered as target for aptamers isolation. Therefore, using cell SELEX can be culminated in generating aptamers that distinguish tumor cells from normal cells. Some of the advantages of cell SELEX are as follows: 1) unlike the ligand-SELEX, the prior knowledge of target conformation is not necessary; 2) since the cell surface is complex and encompasses countless molecules, aptamers can be isolated against many different targets; 3) molecules on the cell surface are in their native state and thus displaying their natural conformation and distribution; and 4) using cell SELEX obviates the requirement for purification methods of target molecule that can disrupt its native conformation [132].

In general, cell SELEX process is composed of three major steps that are shown in Fig. 4B. In brief, the target cells are incubated with the starting library for a specified period of time and at a specified temperature. Then unbound aptamers are removed and bound aptamers are eluted. Finally, the eluted aptamers are amplified by PCR and used for the next round of selection. However, to increase the specificity of the aptamers, the target-free cells (negative cells) are used in the so-called counter (negative) selection step. In this step, the negative cells are incubated with the eluted aptamers and unbound sequences are isolated and amplified by PCR. It is expected that aptamers with specificity for a large number of non-specific targets at the cell surface to be removed during negative selection [132]. Hence, cell SELEX often contains the positive selection against the target cells along with negative selection against closely related non-target cells [133].

As mentioned above, previous knowledge of the target molecules is not necessary in cell SELEX method and this feature makes cell SELEX an attractive strategy to generate aptamers that can recognize and bind to different cell populations including lymphocytic leukemia [133], liver cancer cells [134], small cell lung cancer [135], gastric cancer cells [136] and mouse dendritic cells [137]. Some examples of isolated aptamers against respective targets using cell SELEX are summarized in Table 3.

3.18. In vivo SELEX

In vivo SELEX is a method that is employed to generate aptamers

Fig. 4. Schematic representation of A) *in-silico* SELEX: in this method, the sequences are selected based on their secondary structure. The sequences that possess simple stem-loop or slightly branched structures are disposed. The more complex structures are selected and their tertiary structure is generated. Using computational docking tools, molecule ligands that can bind to selected sequences are identified. B) Cell SELEX: whole living cells are considered as target in the cell SELEX method. In these cases, the cell surface proteins and even some unknown structures of the cells can be targeted by aptamers. The library is incubated with the living cells. The unbound sequences are washed out and the bound ones are eluted and amplified for the next round. The cells without target molecules are used for the negative selection. C) *in vivo* SELEX: in this method an experimental animal is used instead of an *in vitro* cell culture system or purified molecules. The aptamer library is injected into the animal. The target organ or tumor tissue is harvested and bound sequences are extracted. The obtained sequences are amplified by PCR and applied for the next cycle of selection.

Table 3

Examples of isolated aptamers by cell SELEX method.

Aptamer	Cell Type	Target	Ref.
GBI-10 (ssDNA)	Glioblastoma cell line U251	tenascin-C	[150]
Aptamer III.1 (ssDNA)	Endothelial cell line YPEN-1	pigpen	[234]
TD05 (ssDNA)	Burkitt's lymphoma cell line Ramos	Ig μ heavy chain	[235]
Sgc8 (ssDNA)	T cell acute lymphoblastic leukemia CCRF-CEM	PTK-7	[236]
A07 (RNA)	Chinese hamster ovary (CHO) cells expressing TGF- β type III receptor	TGF β RIII	[237]
EP166 (ssDNA)	HepG2 cell line expressing EpCAM	EpCAM	[238]
Apt02 (RNA)	HEK293 expressing integrin alpha V (ITGAV)	ITGAV	[239]

in living organism instead of using *in vitro* cell culture or recombinant protein. This method is different from other *in vitro* SELEX methods in a few ways. First, *in vitro* SELEX methods select aptamers against targets that may or may not be localized to specific cells or tissues whereas *in vivo* selection is performed through localization in specific cells or tissues. Second, in *in vitro* selection aptamers are bound to purified target proteins and may not bind to the same protein in its natural environment, an obstacle that is bypassed by *in vivo* selection. Third, *in vivo* SELEX benefits from a built-in negative selection where aptamers with affinity for normal cells or organs are removed with consecutive rounds (Fig. 4C). In addition, this technique encompasses aptamers properties including bioavailability, circulation time and tissue distribution and therefore reduces the need for post-selection pharmacologic optimization [138]. *In vivo* SELEX was first applied to select the replication-competent HIV-1 viruses from the initial library where CD4⁺ T cells were transfected with an infectious library of HIV-1 genomes containing random mutations [139]. A similar study was done to probe the sequence space in the single-stranded purine-rich regions of HIV-1 genome. The obtained results demonstrated that two out of three strongly conserved regions were critical for virus replication [140]. *In vivo* SELEX method has the ability to isolate aptamers capable of localization inside the tumor cells and bind to tumor-related targets. In one study, Jing Mi et al. inoculated a randomized library of 2'-fluoropyrimidine-modified RNA into implanted human xenograft-bearing nude mice and after 12 rounds of *in vivo* SELEX, RNA aptamers against protein DHX9 (also known as RNA helicase A) was obtained [138]. The advantages and disadvantages of mentioned methods are summarized in Table 4.

4. Applications of aptamers

4.1. Biomarker discovery

The discovery of novel biomarkers not only contributes to a better understanding of disease processes, but also provides clinical data for early detection and appropriate treatment. Although proteomic methods such as two-dimensional gel electrophoresis (2D-GE), differential imaging gel electrophoresis (DIGE) and mass spectrometry (MS) are extensively used to discover new biomarkers, the identification of the disease-related membrane proteins remains a great challenge [141,142]. One common limitation of these technologies is that even small operational differences during sample preparation including cell/tissue lysis, isolation and purification steps may lead to remarkable deviations in the results [143]. In addition, since biomarkers can be used for downstream purposes such as targeted drug delivery and imaging, the requirement for the development of molecular biomarker-recognizing probes seems essential. In this respect, antibodies and aptamers have been most commonly used as biological recognition elements. Apart from that, the presence of a detector or transducer is essential not only for the signal generation upon target-probe interaction but also for the signal amplification [144]. The unique properties of

nanomaterials such as composition-dependent optical property, high surface/volume ratio and easy tuning of surface properties [145] make the nanomaterials suitable to be incorporated into affinity probes to form smart biosensing tools. Carbon-based nanomaterials (e.g. graphene, carbon nanotubes and fullerene), plasmonic materials (e.g. gold and silver nanoparticles) and photoluminescence nanoparticles (e.g. quantum dots) are the most popular nanoparticles to be functionalized with affinity probes. Among them, carbon-based nanomaterials have extensively been exploited (about 45% of studies) followed by gold nanoparticles-based materials [146]. Key modification techniques used to functionalize the surface of these nanomaterials with antibodies or aptamers have been mainly classified as thiol-noble metal surface interaction, streptavidin-biotin interaction, π - π stacking interaction and NHS-EDC carbodiimide reaction. To confirm the presence and activity of the probes coupled with the nanomaterials, characterization studies are performed. Ultraviolet-visible absorption spectroscopy, Circular Dichroism (CD), Dynamic Light Scattering (DLS) and Gel Electrophoresis (GE) are the most common characterization techniques that have been applied to validate the nanoparticle biomodification. Protein-based antibodies and oligonucleotide-based aptamers are two groups of probes that have been widely used for nanoparticle surface biomodification. However, significant batch to batch variation, immunogenicity, limited chemical modifications are some shortcomings of antibodies [146]. Another major obstacle of using antibodies is the presentation of membrane proteins to the immune system in their native conformation which is challenging. In most cases, the immunization approaches that have been used for many membrane targets have failed. Hence, while the number of human transmembrane proteins reaches 13000, only 400 antibodies have been identified against cell surface biomarkers [147,148]. In comparison with the antibodies, aptamers can be chemically synthesized with negligible batch to batch variation and can be modified at any desired nucleotide position for downstream applications such as sensing, imaging, targeted drug delivery and therapeutics. Considering that aptamers can discriminate diseased cells from normal cells on the basis of molecular differences of unknown membrane proteins at the diseased cells surface in comparison with normal cells, the application of aptamers for new biomarker discovery is not beyond expectation. Moreover, the discovery of tumor new biomarkers can accelerate the early diagnosis of cancer especially protein biomarkers since they reflect the genotype and phenotype of a particular abnormality. The unwanted mutation in cancer cells can result in the expression of new proteins in tumor cells and/or over-expression or under-expression of certain proteins. These changes can disturb the ratio of proteins in the tumor cells context. Therefore, not only the discovery of new proteins is necessary but the quantification of expression levels of various proteins should also be considered. The aptamers are capable of discovering the tumor new biomarkers without the need for prior knowledge of tumor biomarker. The cell-SELEX method is a strategy for discovery of unknown tumor biomarkers [135]. Using cell-SELEX, a group of

Table 4
Advantages and disadvantages of some SELEX methods.

SELEX methods	advantages	limitations
Nitrocellulose filter binding	Relative ease of performance No need for special equipment Equilibrium, in solution aptamer–target binding Medium-throughput binding assay Aptamer isolation for multiple targets in parallel	Merely applicable to targets that can be captured by filter Non-selective binding of aptamer to filter Large number of selection rounds is necessary
Bead-based SELEX	Applicable to most targets Aptamer isolation for multiple targets in parallel Ease of fine-tuning of selection stringency	Target immobilization required Density-dependent Cooperative binding
Microcolumn bead-based SELEX	Minimizing the amount of target and aptamer Reducing dead volume and non-specific binding	Target immobilization required Need for automated devices Density-dependent Cooperative binding
Particle display	Few number of selection rounds required Using FACS process for aptamer screening FACS-based Quantitative and reproducible determination of aptamer affinity threshold	Need for FACS instrument
Capillary Electrophoresis SELEX	Rapid partitioning of bound aptamers from unbound ones Equilibrium, in solution aptamer–target binding Small amount of analyte required	Capillary electrophoresis instrument required Relatively small size of starting library (~10 ¹² sequences) Restricted to targets that cause a shift in aptamer electrophoretic mobility pattern
Micro Free Flow Electrophoresis	Rapid isolation of aptamer (2–4 rounds) Ability of continuously Introducing, separating and collecting analytes	Need for sophisticated devices Restricted to targets that cause a shift in aptamer electrophoretic mobility pattern
Microfluidic SELEX	Very small amount of reagent needed Rapid isolation of aptamer (1–6 rounds) Aptamer isolation for multiple targets in parallel	Encapsulation or immobilization of target required Fabrication of fluidic and electronic instruments and flow pumps required Diversion of flow direction upon microbubbles Formation Possibility of cross-contamination
Microarray-based SELEX	Large-scale analysis Equilibrium binding with in-solution target and immobilized aptamer	Small size of starting library (10 ⁶ sequences) Fabrication of microarray chip with different sequences required Microarray scanner is required to measure binding
In-silico SELEX	Rapid process of finding the desired aptamer High-throughput virtual screening of the library using computational docking tools	Small size of starting library Requires sophisticated computational methods and programs
Atomic Force Microscopy (AFM) SELEX	Few rounds of selection (3–4 rounds)	Need for expensive equipment (AFM system) Immobilization of both target and aptamers required Non-equilibrium binding Relatively small size of starting library
High fidelity (Hi-Fi) SELEX	Inhibition of non-specific retention of aptamers No need for sophisticated instruments Few rounds of selection (2–3 rounds) Increased partition efficiency	Target immobilization and surface passivation are required
Cell SELEX	No need for prior knowledge of target Aptamer isolation to native state of target Biomarker discovery Bypassing purification methods of target molecule	Restricted to cell surface molecules Non-specific uptake of aptamers by dead cells Isolation of aptamer(s) against unknown and/or unintended targets
<i>In vivo</i> SELEX	Isolation of <i>in vivo</i> functional aptamers No need for prior knowledge of target Aptamer isolation to native state of target Built-in negative selection Aptamer isolation of high bioavailability, circulation time and tissue distribution Minimizing the need for post-selection pharmacologic optimization	Small size of starting library (10 ⁴ sequences) A large number of selection rounds is usually required (up to 14 rounds) Isolation of aptamer(s) against unknown and/or unintended targets

aptamers that could recognize human T-cell acute lymphoblastic leukemia cells (CCRF-CEM) was isolated. The selected aptamers were able to discriminate the target cell line from other cancer cell lines (e.g., Ramos, a Burkitt's lymphoma cell line). Subsequently, one of the isolated aptamers (sgc8) was exploited as a capture probe to determine its target molecule from the CCRF-CEM cell lysate. The lysate was subjected to the biotinylated sgc8 aptamer and streptavidin-coated magnetic beads. Following analysis of the collected molecules by mass spectrometry, protein tyrosine kinase 7 (PTK7) was identified [133,149]. A similar strategy could identify tenascin-C (an extracellular matrix protein) as a glioblastoma tumor marker using U251 cell line. U251 cell line derived from glioblastoma was considered as the target for cell SELEX. The aptamer GBI-10 was isolated against U251 and it was demonstrated that it could specifically bind to respective cell line. The affinity

purification and mass spectrometric analysis indicated that GBI-10 aptamer could recognize an extracellular matrix protein called tenascin-C at the cell surface [150]. In another study, NB4 AML cell line was used as cell population of target to isolate DNA aptamer. After eight rounds of selection, aptamers with low K_D value were obtained that specifically bound to NB4 cell line. In addition, the aptamer–target protein complexes were extracted and analyzed using mass spectrometry. The MS data was used to identify possible protein candidate and consequently the only cell surface protein identified was sialic acid-binding Ig-like lectin 5 (Siglec-5) [151].

These reports highlight that aptamers not only enable us to discover tumor biomarkers that contribute to distinguish between cancerous and normal healthy cells but they can also help to recognize the tumor cells stages in carcinogenesis process. For example, in one study, highly specific aptamers against 44 different

protein biomarkers were able to discern among stage I–III lung cancer [152]. Therefore, the determination of tumor stage using aptamers makes it more possible to evaluate the cancer progression to prescribe the best treatment options. A new class of ssDNA aptamers called Slow Off-rate Modified Aptamers (SOMAmers) consists of chemically-modified nucleotides (e.g. adding benzyl, naphthyl, tryptamino or isobutyl on deoxyuridine nucleotide) to mimic amino acid side chains. Such modifications increase aptamers chemical diversity and thus improving interaction capacity and binding affinity of aptamers [153]. SOMAmers are able to discover disease-related biomarkers including lung cancer [152], malignant mesothelioma [154], age-related neurodegenerative disease [155] and Alzheimer disease [156]. Aptamer-facilitated biomarker discovery (AptaBID) system is another method applied for biomarkers discovery. In this method, the enriched aptamer pools is constructed against target cells *via* cell SELEX and then the isolation of candidate proteins is done based on the binding activity of the aptamer pools. Finally, the candidate proteins are identified by mass spectrometry analysis. A good example of such a system is the discovery of six and three biomarkers for immature and mature dendritic cells, respectively [157].

4.2. Aptamer for diagnosis and detection

The power of diagnostic methods of cancers is influenced by both the specificity of detecting probes and the sensitivity of the detection. The conjugation of aptamer molecules as detecting probes with various transduction platforms such as optical assays, electrochemical assays and mass difference assays can remarkably increase their sensitivity [158]. Another strategy to increase the sensitivity of detection is the application of multivalent binding instead of the single-aptamer binding. The large surface area and variable sizes of nanomaterials provide the required multivalent ligand scaffolds for multivalent binding. The attachment of up to 80 fluorescently labeled aptamers sgc-8 (an aptamer against hematopoietic cancer cells) on one Au-Ag nanorod resulted in a 26-fold higher affinity and over 300-fold higher fluorescence signal [159]. The detection of diagnostic markers including circulating tumor cells (CTCs) [160,161], secreted cancer cell biomarkers, growth factors, cytokines and hormones [162,163] contributes to the cancer diagnosis. Since the amount of CTCs and secreted tumor biomarkers is little, the high-affinity aptamers should be exploited in the form of aptamer-conjugated nanoparticles (ACNPs). The ACNPs are mainly composed of highly specific aptamers conjugated to silica-coated magnetic and fluorophore-doped silica nanoparticles to extract and detect the target tumor cells. Accordingly, when four different aptamers were used in the ACNPs complex, the theoretical limit of detection (LOD) of 6.6 cells was obtained [164]. In a more creative method *via* dual-aptamer target binding strategy and with no need for complicated instruments, the limit of detection of tumor cells decreased up to 10 cells per ml. The principle of experiment was based on colorimetric assay wherein mucin-1 (MUC-1) binding aptamer was immobilized on platinum gold nanoparticle (PtAu) while VEGF binding aptamer was conjugated to magnetic beads (MB). Aptamer of MUC-1 modified on PtAu served as sensing probe and VEGF aptamer conjugated to magnetic bead acted as capture probe. In the presence of the tumor cells (MCF-7 in this case), after the addition of substrate, the color change occurred which was distinguished by naked eyes [165].

Unlike antibodies, aptamers can be generated against toxic and non-immunogenic targets such as metal ions [166], small organic molecules [167] and organic dyes [168]. Such a large pool of aptamers has attracted an increasing number of attentions in development of novel aptamer-based sensors to detect a wide range of target molecules. For example, among many efforts that

have been made to increase the sensitivity of aptamer-based cocaine detection methods, one of the most sensitive methods is based on unique near infrared (NIR) fluorescence [169], NIR absorption [170] and Raman light scattering [171] properties of single-walled carbon nanotubes (SWNT). In this regard, a label-free cocaine detection method on the basis of immobilization of cocaine-binding aptamer on the surface of single-walled carbon nanotubes was developed [24].

4.3. Imaging applications

Unique features of aptamers including excellent tumor penetration, superior signal to noise ratio and sub-centimeter resolution in solid tumors [172], offer the conjugation of aptamers with nanomaterials and fluorescent molecules as a suitable alternative to traditional contrast agents. Such conjugates can be used to visualize, trace and monitor the tumor-related molecules that affect tumor behavior and tumor response to therapy. Nanoparticle-aptamer conjugates along with colorimetric systems where color change happens following aggregation of nanoparticle-aptamer complexes is an example of aptamer applicability for tumor imaging [173]. In another instance, when aptamer AS1411 against nucleolin (a protein that is commonly expressed on the cancer cells membrane) was conjugated with a multimodal nanoparticle, it could detect and provide images of cancer cells [174]. In addition to cell imaging, aptamers can be applied to explore cell signaling, cell to cell communication and cellular interactions with the microenvironment. In one experiment, platelet derived growth factor (PDGF) binding aptamer containing a pair of fluorescent dyes was employed to quantitatively detect the PDGF that was secreted from mesenchymal stem cells in real time. Aptamer binding to PDGF induced aptamer conformational changes that resulted in closer proximity of two dyes to each other, producing an optical signal [175]. Depending on the molecule conjugated with aptamer, the resulting aptamer-based molecular probes can be used in magnetic resonance (MR) imaging, CT imaging and ultrasound imaging. It has been indicated that the conjugation of prostate surface membrane antigen (PSMA) binding aptamer with gadolinium-containing compounds and superparamagnetic iron oxide nanoparticles (SPIONs) can be successfully used for MR imaging-based molecular imaging [176]. The high atomic number of gold nanoparticles makes them proper particles for CT imaging. Anti-PSMA aptamers-gold nanoparticle conjugates have also been used in CT imaging [177].

4.4. Molecular and cellular profiling

Molecular profiling technology is used to monitor a large number of transcripts, proteins, metabolites or other substances in a biological sample of interest. The molecular profiling studies can be done at three levels: DNA analysis to indicate the mutated, deleted and amplified genes, RNA and protein analysis to determine differences in transcription, translation and post-translational modification of proteins. Although the application of tumor biomarkers such as prostate-specific antigen (PSA) [178] and carcinoembryonic antigen (CEA) [179] can contribute to early diagnosis and prognosis of disease, it is known that the outward manifestation of the cancer phenotype is resulted from interacting pathways of cancer cell and its environment. To capture a more precise picture of the molecular state of a tumor, the researchers have shown a tendency toward molecular profiling of cancer cells. It is important to consider that even the originated tumor cells from the same source obtain various molecular characteristics during each stage of disease. Therefore, the determination of tumor profiling of each patient can contribute to prescribe suitable treatment regimen for

the same patient and avoid the excessive application of ineffective treatments. For example, the standard treatment regimen for breast cancer is commonly determined based on morphological evaluation (grade and stage of tumor) and immune-phenotyping analysis (estrogen receptor and HER-2/neu expression) [180]. However, the estrogen receptor positive breast cancer cells can, in turn be classified into two distinct populations with different survival profile according to the determination of molecular profiling [181]. The development of molecular profiling algorithm for patients with breast cancer decreased the number of patients placed in the high-risk population and accordingly the requirement for unnecessary chemotherapy was obviated. On the contrary, the molecular profiling demonstrated that a large population of patients placed in low-risk group *via* pathology classification method was found to have poor prognosis [182]. Molecular profiling aptamer technology is a good candidate for the following reasons: 1) multiple aptamers can be isolated against a wide variety of cell surface molecules; 2) the specificity of isolated aptamers can be increased using negative selection against healthy cells; and 3) the aptamers can be easily modified for intended applications [183].

4.5. Aptamers in therapy

The first aptamer approved by Food and Drug Administration (FDA) for the treatment of age-related macular degeneration was a modified RNA aptamer to VEGF called Macugen (Pegaptanib sodium) [184]. VEGF signaling causes the growth of abnormal new blood vessels in the eyes that finally leads to blood leakage and loss of vision. Dominant isoform of VEGF (the isoform containing 165 amino acids called VEGF₁₆₅) was chosen as the target for selection of anti-VEGF aptamer. The efforts to isolate aptamer against VEGF were made by three separate groups. In the earliest study, in the 1994, the isolated RNA aptamers could block the actions of VEGF *in vitro*, promising the potential utilization of an aptamer-based approach to angiogenesis inhibition [185]. To increase the resistance of the obtained aptamer to nuclease digestion, 2'-NH₂-pyrimidines were incorporated into aptamers as well as 2'-O-methyl was substituted for the 2'-hydroxyl of purines. Adding phosphorothioate-linked polydeoxythymidine caps at both aptamer termini was another modification to improve aptamer half-life and binding affinity for VEGF [39]. Using 2'-F-pyrimidine-substituted nucleotides during SELEX process instead of 2'-NH₂-pyrimidine-substituted nucleotides even further increased anti-VEGF aptamer affinity and finally three high-affinity aptamers to VEGF were characterized. Although all three aptamers indicated high affinity for VEGF₁₆₅, one of the aptamers (named t44-OMe) was the most effective at vascular leakage inhibition from dermal microvessels upon VEGF injection into guinea pigs. The t44-OMe was further modified *via* addition of 40-kDa polyethylene glycol and 3'-3'-terminal deoxythymidine, which was selected for further investigations. This aptamer was designated as NX1838 [186] and then EYE001 [187] in a limited period of time until was known as pegaptanib [188]. The direct injection of the aptamer into the vitreous cavity and its binding to VEGF can block VEGF signaling. In clinical trials, following Macugen administration, stable or improved vision in more than 80% of patients was observed [189]. In addition, therapeutic aptamers can be considered as promising drug candidates to block coagulation. Although heparin is the most common anticoagulant drug, its pharmacokinetics is challenging to control. In contrast, an anti-coagulation factor IXa RNA aptamer containing a cholesterol moiety coupled with an antidote (a 2'-O-methyl RNA oligonucleotide) showed a controlled and transient anticoagulation activity and successfully passed phase I clinical trial [190]. The serious side effects of conventional treatment approaches of cancer such as radiotherapy and chemotherapy, make

the invention of alternative options inevitable. To minimize the side effects, targeted therapy has created new horizons due to increase in drug delivery to tumor cells on one side and decrease in exposure of non-target cells to the radio and chemotherapeutic agents on the other side. Antibody-based therapy is one of the strategies to accomplish this goal. Nevertheless, the advantages of aptamers over antibodies including smaller size, more stable structure, the easier synthesis and lower immunogenicity confer the aptamers superiority compared with antibodies. The specific aptamers against tumor cell surface biomarkers have culminated in the utilization of aptamers for targeted delivery of therapeutic agents. So far, aptamers have been conjugated to nanoparticles, anti-tumor drugs, si-RNA and mi-RNA. Therapeutic agents can be covalently or non-covalently conjugated to aptamers. For example, doxorubicin (Dox), a chemotherapeutic agent that is widely used for the treatment of various types of cancers, demonstrated an enhanced therapeutic efficacy and reduced toxicity in the form of Dox-aptamer scg8c complex in contrast to Dox alone [191]. The Dox-aptamer complex not only retained the properties of aptamer scg8c including high affinity and efficient internalization by target cells, but also as a result of the presence of an acid-labile linker, Dox-aptamer conjugates could be cleaved inside the acidic environment of tumor cells. In one more creative method, a photo-switchable hybrid-aptameric nanoconstruct (HyApNc) as a delivery vehicle was designed. This molecular carrier was consisted of a lipid-functionalized anti-cMet aptamer (lipid chain modified aptamer) and a doxorubicin-carrying domain (a GC-rich hairpin motif containing 2',6'-dimethylazobenzene). It was demonstrated that lipidation could protect aptamer from enzymatic degradation and thereby increases its serum half-life. In addition, multiple doxorubicin molecules are intercalated into GC-rich hairpin combined with dimethylazobenzene (DMAB). The DMAB-modified motif is destabilized after UV-light irradiation leading to doxorubicin release. Therefore, HyApNc method can selectively target and deliver anti-cancer drug into cMet-expressing cells and release the payload under light irradiation [192]. Over the past decade, various nanoparticles have been introduced to increase both the half-life and capacity of drug payload of aptamer-based drug delivery. Common characteristics of nanoparticles such as biocompatibility, drug-loading capacity and suitable biodistribution along with physical and chemical properties of specific each nanoparticle, offer them as an attractive tool in tumor therapy setting. Many studies have focused on the exceptional photothermal properties of nanoparticles such as graphene oxide (GO) and gold nanoparticles (AuNPs) to promote the efficiency of tumor therapy. In an experiment, targeted chemotherapy and photothermal therapy was integrated in a multifunctional drug delivery platform. The MUC-1-binding aptamers-GO-AuNPs composites were loaded with doxorubicin and targeted MUC-1 expressing tumor cells. The method took advantage of synergistic effects of chemotherapy and photothermal therapy, simultaneously [193]. Another promising nanoparticles are PEG-PLGA nanoparticles that have been emerged as drug delivery vehicles. These polymeric nanoparticles consist of PLGA as a hydrophobic core, that encapsulates drug and a PEG as a shell, which prolongs half-life of drug [194]. Therefore, encapsulation of cytotoxic drug with PEG-PLGA nanoparticles functionalized with aptamers can target tumor cells. In this respect, gemcitabine (GEM)-loaded copolymers containing PEG-PLGA were conjugated to aptamers AS1411 to form Apts-GEM-NPs. The complex significantly improved the cytotoxic effects of GEM on nucleolin over-expressing non-small cell lung cancer [195]. Aside from drug delivery potential of aptamers, they can be regarded as carriers for miRNA and/or siRNA. siRNA and miRNA inhibit protein synthesis through degradation of coding mRNA or inhibition of translation initiation [196]. Although a large number of studies have focused on

the therapeutic potential of this class of molecules, their safe and efficient delivery to the desired cell type, tissue or organ remains a major obstacle. To circumvent this hindrance, aptamers have been offered as targeted delivery tools of short therapeutic RNA (siRNA or miRNA) [197]. An evidence for this claim is to target PSMA-expressing prostate cancer cell lines by PSMA-Plk1 chimeras containing RNA aptamer portion (against PSMA) and the modified siRNA moiety (complementarity to Plk1 RNA). Such chimera not only induced the cell death in tumor cell lines *in vitro* but also substantially decreased tumor size when was injected intratumorally in a xenograft mouse model of prostate cancer [198]. The main advantage of the PSMA-Plk1 chimera dates back to its specificity to desired tumor cells that results from the aptamer specificity to PSMA-expressing tumor cells and siRNA potential in silencing cancer-specific transcripts.

5. Conclusion and future perspectives

Remarkable strides have been taken in SELEX technology since its discovery in 1990. The reason for focusing on SELEX progress is stemmed from advantages of aptamers over antibodies including more simple synthesis, lower cost of production, easier modification with functional groups, lower batch-to-batch variations, lower immunogenicity, smaller size, better penetration into the target and higher biological stability. Accordingly, aptamers have been proposed as promising molecular probes that can rival and even replace antibodies. Since these molecules can bind to their targets with high affinity and specificity, they have been introduced as prognostic, diagnostic and therapeutic tools. Nonetheless, there are several concerns related to aptamer technology including: 1) the isolation of specific aptamers for target by diminishing false positive or negative selection. In this regard, the natural properties of respective target (e.g. protein versus whole cell) and the type of selection method are two key parameters. Using time-consuming SELEX method is usually accompanied by low rate of successful isolation. For instance, using SOMAmer can improve the success rate of aptamer production from less than 30% to more than 50% [199]. In addition, aptamer selection can be accelerated by methods that allow simultaneous measurement of affinity and specificity of thousands of candidate sequences in parallel. For example, Cho et al. developed a Quantitative Parallel Aptamer Selection System (QPASS) method, which incorporated microfluidic selection, NGS and *in situ*-synthesized aptamer arrays. Such technique could successfully generate an aptamer against human cancer biomarker angiopoietin-2 (Ang2) within four rounds with about 400% higher affinity compared to the similar aptamer isolated using nitrocellulose membranes method [105]. 2) The selection of cancer-relevant targets for aptamer generation is another challenge that should be considered. Although using tumor cells lines containing certain biomarkers that are highly expressed in pathologic conditions is a useful trick, the immortal cultured cells are different from tumor tissue *in vivo*. To target more reliable tumor-relevant biomarkers *in vivo* SELEX can be applied. The selection of cell/tissue-related biomarker can be useful for treatment of non-cancerous diseases as well. As an example, Macugen (as an anti-angiogenic drug) is one of the most pioneer aptamers that was approved by FDA and is currently used in the treatment of age-related macular degeneration. 3) Biostability of aptamer is a challenge for *in vivo* applications of the isolated aptamers. As mentioned earlier, a range of modifications can be employed to improve aptamer resistance to enzyme-mediated degradation and blood residence time. Some modifications such as 2'-substitutions on the sugar ring, using locked/unlocked nucleic acids and replacement of phosphodiester linkage with phosphorothioate, methylphosphonate and triazole have increased the aptamer resistance to enzyme digestion. On the

other hand, it has been demonstrated that incorporation of PEG into aptamers is an attractive strategy to increase aptamer half-life in blood stream. 4) The most serious threat to aptamer commercialization is that whether aptamer-based therapies are able to become effective and FDA-approved treatment options. Apart from Macugen approved in 2004, a considerable number of aptamer have completed phase II clinical trial (Table 1). However, clinical trial results are promising and it is expected that the progress in aptamer field is a harbinger of the appearance of newer aptamers for new biomarker discovery, earlier diagnosis of diseases and more effective drug delivery so that they would be brought into the market as therapeutic tools.

Conflicts of interest

The authors declare no conflict of interest.

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