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Introduction of SELEX and Important SELEX Variants

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1.1 SELEX

In 1990, from a randomly synthesized nucleic acid library composed of more than 10^{15} different sequences [1, 2], two laboratories led by Larry Gold and Jack William Szostak invented independently a technique for selection of aptamers or single-stranded functional oligonucleotides (DNA or RNA), which shows high affinity to their respective targets. As shown in Figure 1.1, the aptamers are developed by repeated selection and amplification processes [3]. This *in vitro* oligonucleotides selection scheme is termed as SELEX (Systematic Evolution of Ligands by EXponential Enrichment), and has become a general and powerful method for the discovery and isolation of nucleic acid aptamers for a rich variety of analytical applications.

Mechanistically, the SELEX process starts with a single-stranded deoxyribonucleic acid (ssDNA) library generated by solid-phase synthesis using traditional phosphoramidite method, the library comprised of random sequences at the center flanked by defined primer binding sites at each 5′ and 3′ termini. The variety of the ssDNA library relies on the length of the random region. Around 10^{15} different sequences are contained in an initial ssDNA pool, which makes it appropriate for the existence of sequences specific for a target. Actually, the entire aptamer sequence library has only a short fraction that binds effectively to the target, which suggests that aptamer screening can be eventually accomplished within a short period of time [4]. It is noteworthy that long random sequences can provide higher structural complexity, which is vital in isolating aptamers with high affinity [5]. In an early stage of aptamer selection, RNA libraries are widely used due to the fact that RNA can easily fold into complex 3D structures which show higher affinity to the targets. However, RNA aptamers are much more expensive than DNA aptamers, and they are relatively difficult to be modified in SELEX cycles [6]. Furthermore, stems and loops can be generated when ssDNA folds into a 3D configuration [7]. So DNA libraries are used more frequently now.

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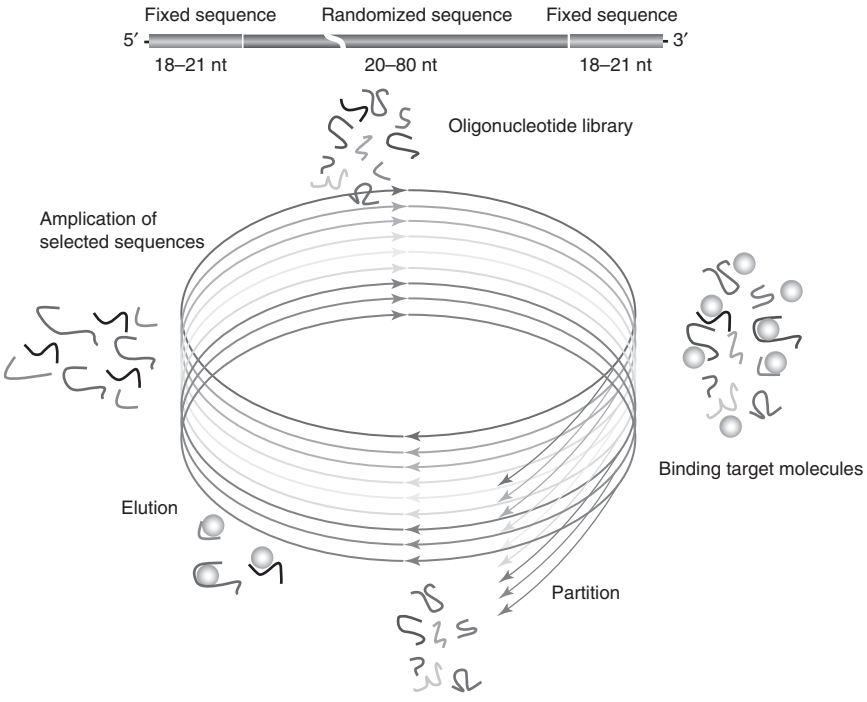

Figure 1.1 Schematic drawing of SELEX procedures. Source: Dong et al. 2013 [3]. Reprinted with permission of Taylor and Francis.

The essential steps of a typical SELEX process include binding, partitioning/eluting, amplification, and identification. Normally, due to limited resolution and efficiency, many cycles of SELEX rounds need to be carried out to obtain a good result. In the first step, a random DNA or RNA library is incubated with the target to ensure definite binding of some oligonucleotides with the target, while others will be removed in the following steps. In the partitioning/eluting step, unbound oligonucleotides can be removed on the basis of the different molecular weights of nucleotide-target complexes and nucleic acids. For example, a chromatographic column packed with the target-immobilized beads can be applied for the separation of oligonucleotides binding with the target molecules. Besides chromatography, other methods can also be used to separate unbound, weakly bound, or elute bound oligonucleotides, for example, filtration, heating, the change of ionic strength or pH, the addition of denaturing substances such as urea, sodium dodecyl sulfate (SDS), or ethylenediaminetetraacetic acid [8–10]. In this step, target molecules are interacted with either free nucleic acid or separable nucleic acid immobilized on a certain substrate [11]. However, it is difficult to elute strongly bound oligonucleotides from targets, which may restrict the isolation of the aptamers with extremely high affinity. Because of this, high affinity of aptamers are commonly obtained by SELEX with free-form target molecules [12, 13]. In some cases, it is difficult to remove unbound oligonucleotides from the free target-oligonucleotide

complex. So, low-affinity targets are appropriate for SELEX with free-form target molecules. After partitioning/eluting, the next step is to amplify the bound oligonucleotides by PCR (polymerase chain reaction) with primers for aptamer screening. The amplification will generate a new population of oligonucleotides for the next round of SELEX. Commonly, 10–20 amplifications are needed [14]. After repeated cycles of selection and amplification, the nucleotide pool becomes enriched, while the affinity between nucleotide and aptamers becomes higher because low- or no-affinity oligonucleotides are removed in previous cycles. The progress of SELEX can be monitored by the quantification of target-bound oligonucleotides among the pools of incubated nucleotides at each round of SELEX [15]. The selection is stopped when oligonucleotides bound to the target are fully dominant in the pool of oligonucleotides or significant enhancement of target-bound oligonucleotides cannot be observed during two or three successive SELEX rounds. These selected oligonucleotides are subject to amplification. Subsequently, the sequences of individually selected oligonucleotides are identified by cloning and sequencing of the selected clones, and the number of different aptamer sequences screened by the SELEX process depends on the stringency of the selection conditions and target characteristics thereof.

For the discovery of aptamer as a novel molecular recognition biochemical element, conventional SELEX is inherently an *in vitro* screening protocol of elegant simplicity, independent of animal or cell lines, which applies three principles of evolution – heredity, variation, and selection pressure [16]. Various aptamers, e.g. tetracycline aptamer [17] and thrombin aptamer [4], were successfully developed with iterative experimental rounds of incubation with ssDNA or RNA library, partitioning from the unbound, amplification of the binders, affinity characterization, and sequence identification, respectively.

However, the efficiency of conventional SELEX in the discovery of aptamers is somewhat low in terms of its cost-effectiveness, unsatisfactory specificity, limited partition capability, the necessity of a foreknowable targetability, difficult predictability, and inadequate stability or cross-linking capability, etc. Hence, different upgraded SELEX variants, i.e. negative SELEX (counter SELEX, subtractive SELEX), one-round SELEX, capillary electrophoresis (CE)-SELEX, microfluidic-SELEX, cell-SELEX, HTS-SELEX or *in silico*-SELEX, post-SELEX or *in chemico*-SELEX, auto-SELEX, primer-free SELEX, genomic SELEX, photo-SELEX, qPCR-SELEX, and so on were developed to further enhance the selection efficiency or analytical functionality, accordingly.

1.2 Negative SELEX and Its Analogs

In order to ensure the specificity of the aptamer recognition with target analytes solely, negative SELEX (counter SELEX, or subtractive SELEX), was frequently applied to remove nonspecific binding or erroneous recognition with structurally similar compounds of target analytes, and this is especially the case when target molecules of low abundance are in complex matrices, such as cell lysates, whole blood, or other body fluids.

To our knowledge, the first negative SELEX practice was reported by Ellington and Szostak in 1992, when they successfully developed DNA aptamers against small organic dye molecules. To specifically enrich the DNA pool with aptamer candidates, a non-cognate dye precolumn was suspended over a cognate dye column, then a chemically synthesized DNA pool with an estimated complexity of 2–3 × 10^{13} different sequences were loaded onto the precolumn, and the precolumn retained most nonspecific bound sequences when washed with one column volume buffer. The selectivity in percent DNA bound after negative selection can be improved from 2.2, 1.5, 0.8 to 19.0, 7.0, 4.8, using affinity resin cibacron blue (CB), reactive green 19 (GR), and reactive blue 4 (B4) coupled to cross-linked agarose beads, respectively [18].

On the basis of a similar scheme, Takahashi et al. [19] successfully developed an isogenic cell-SELEX with a counterselection strategy to generate RNA aptamers toward cell surface protein, say, integrin alfa-V (ITGAV), a major transmembrane receptor widely expressed in almost all the cells and closely associated with human diseases such as cancers and pulmonary fibrosis. As illustrated in Figure 1.2, gene of interest (GOI) overexpressed human cell line HEK293 cells were used for positive selection, while GOI knockdown cells as mock cells by microRNA-mediated silencing were used for counterselection, a 100-fold difference in the expressing level of ITGAV between these two isogenic cells gives rise to several RNA aptamers toward ITGAV with dissociation constants ranging from 300 to 400 nM. Thus, the impediment of endogenous expression of target proteins in mocked cells or the heterogeneity of surface proteins between selection and counterselection cells was successfully overcome, as expected.

In addition, in order to discover aptamers to specific biomarkers that identify cells of interest from their homologous counterparts, SELEX with subtractive selection (subtractive SELEX) was frequently applied. Wang et al. reported a subtractive SELEX method to distinguish differentiated PC12 cells from normal PC12 cells. To subtract, randomized ssDNAs were incubated first with regular

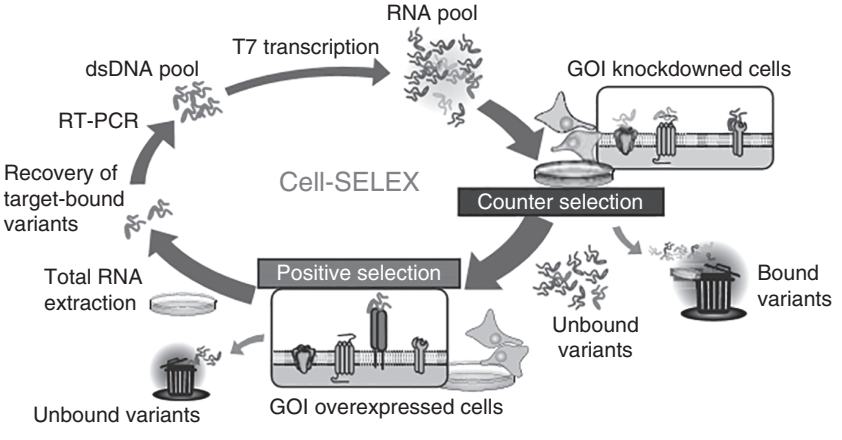

Figure 1.2 Schematic drawing of the isogenic cell-SELEX procedure. Source: Takahashi et al. 2016 [19]. Reprinted with permission of Elsevier.

PC12 cells eliminating those that recognize the common cellular components of both differentiated and undifferentiated PC12 cells; and after six rounds of cell-based selection, aptamers were found binding to differentiated PC12 cells, but not to the parental PC12 cells [20]. The subtractive SELEX presented herein is so potential that it is envisaged that the scheme can be further applied to tumor cell or stem cell research someday.

Nowadays, the negative SELEX and its analogs as aforementioned have become widely available and are rather potential for highly selective isolations of aptamers toward assorted analytes, e.g. human fatty acid–binding protein (FABP3) [21], aflatoxin (B1) [22], and prostate-specific membrane antigen (PSMA) [23], etc.

1.3 One-Round SELEX

The conventional SELEX protocol for a successful aptamer discovery generally necessitates 10+ iterative rounds of incubation, partition, PCR amplification, and sequence identification. The procedure is time-consuming and labor-intensive; hence, minimizing selection rounds to one round with different schemes can obviously enhance selection efficiency and save experimental cost.

In 2007, Nitsche et al. proposed a MonoLEX strategy to acquire high-affinity DNA aptamers binding Vaccinia virus used as a model organism for complex target structures. The approach combined a single affinity chromatography step with subsequent physical segmentation of the affinity resin and one single final exponential amplification step of bound aptamers, and binding specificity was evaluated using an aptamer-based blot assay [24].

Similarly, Arnold et al. identified two DNA aptamers with nanomolar dissociation constants using the one-round SELEX process in 2012 [25]. Kallikreine-related peptidase 6 (KLK6), an active serine protease that has been implicated in neurodegenerative disorders such as Parkinson and Alzheimer disease and certain types of cancer, was immobilized in a 96-well ELISA plate and incubated with the aptamer library overnight. Afterwards, unbound and nonspecifically bound aptamers were separated by salt solutions in a step elution gradient. The most tightly bound specific aptamers were eluted by the highest salt concentration. This fraction was then collected and characterized by competitive ELISA, fluorescence spectroscopy, and quartz crystal microbalance methods, and the specificity of the aptamers was tested on serum albumin.

Interestingly, one-round SELEX can be further applied for the generation of aptamer pairs for "sandwich" assays as in antibody- and antigen-based immunoassays. Using lysozyme as a target, a single-round SELEX based on the proximity ligation selection scheme was successfully developed by Chumphukam et al. [26] As shown in Figure 1.3, a sequence from the forward (F) library and another sequence from the reverse (R) library bind simultaneously to target molecule, allowing hybridization of the connector to both F- and R-sequences. Therefore, the 3′-end of the F-library is sufficiently close to the 5′ phosphate of the R-library, ligation of these two sequences can occur, and the aptamer pairs with nanomolar affinities can be generated after two partitioning steps thereof.

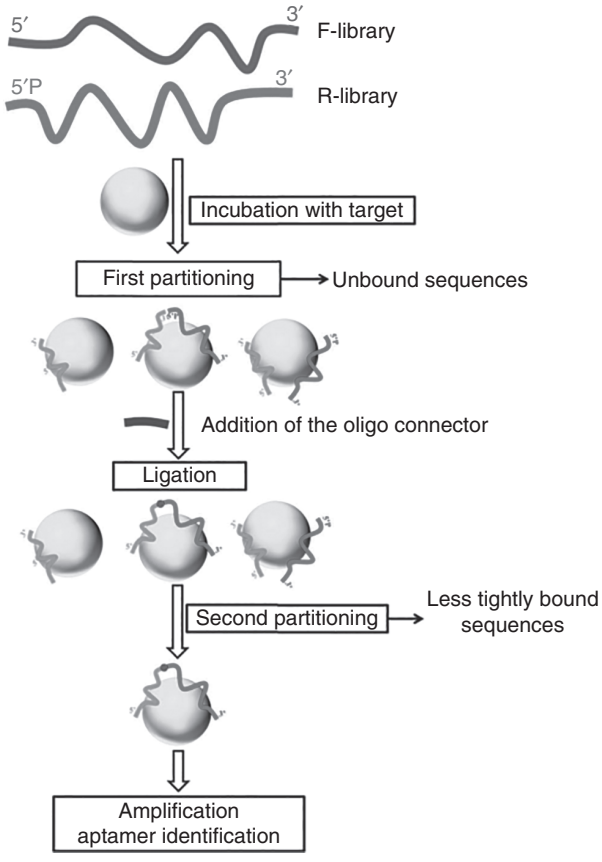

Figure 1.3 A schematic diagram of one-round proximity ligation selection (PLS). Source: Chumphukam et al. 2015 [26]. Reprinted with permission of Royal Society of Chemistry.

Although one-round SELEX can greatly enhance selection efficiency, it is commonly deemed to be inherently a violation of the basic variation principle of evolution. In addition, the selection stringency for the success of aptamer discovery cannot be easily acquired with just a single-round practice in most cases; hence, one-round SELEX for the development of highly affine aptamers still needs to extend its real-world applicability.

1.4 CE-SELEX

CE-SELEX takes advantage of CE and SELEX technology for screening aptamers efficiently. The different charge/mass ratios of the separated materials in the capillary result in different apparent migrations under high voltage, and CE-SELEX uses this mechanism to differentiate effectively between target-aptamer complexes and unbound oligonucleotides in the electric field.

CE-SELEX can greatly improve aptamer screening efficiency, and 2–4 rounds can get the targeting aptamer successfully [27]. The efficiency of the enrichment is attributed to (i) the screening is in a homogeneous solution, can eliminate disadvantages from the interaction of the stationary phase and the mobile phase, and improve the binding specificity thereof; (ii) the separation efficiency of CE is far better than the affinity chromatography or filtration method, the specific recognition sequence and nonspecific sequence can be separated completely, eliminating the elution step in the traditional SELEX method.

As shown in Figure 1.4, in a typical CE-SELEX process, the randomized nucleic acid library is firstly incubated with the target in the free solution; then the incubation mixture is injected into CE capillaries and separated under the high voltage. Nucleic acids that bind to the target show different apparent mobility compared with unbound sequences, and can be collected as different fractions. In the following steps, the binding sequences are amplified and purified for further round selection [28].

Normally, CE-SELEX with rounds of selections can achieve higher affinity aptamers, especially aptamers for relevant proteins than those using conventional selection methods. The combination of strong binding and substantial heterogeneity suggests that CE-SELEX is more successful in retaining very strong binders while eliminating weak ones [28]. The CE can separate a sample regardless of the size or sequence length of the target because binding and nonbinding sequences have obviously different migration behaviors in the capillary [29]. CE-SELEX was used to generate ssDNA aptamers for human immunodeficiency virus reverse transcriptase (HIVRT) in 2005. The ssDNA library was initially incubated with HIVRT, and cycles of HIVRT binding, PCR amplification, and purification were repeatedly performed using CE to procure enriched ssDNA libraries suitable for further rounds of selection [28].

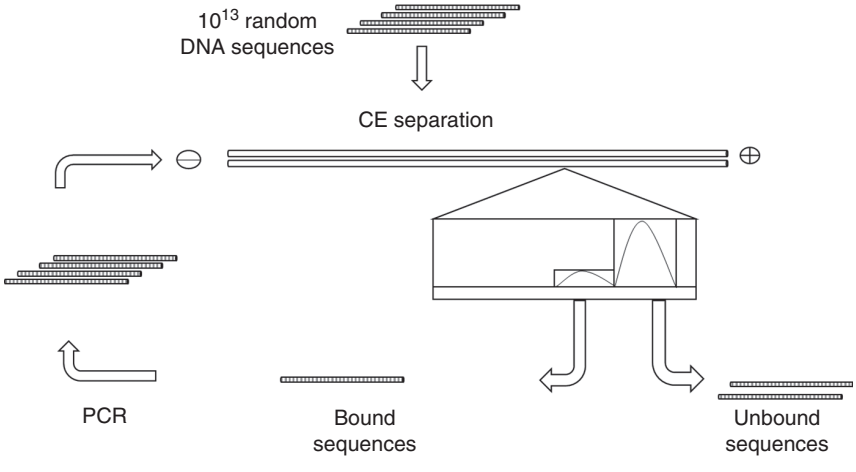

Figure 1.4 Schematic of CE-SELEX. Source: Mosing et al. 2005 [28]. Reprinted with permission of American Chemical Society.

In addition, specific aptamers against IgE [27], neuropeptide, and ricin [30] with low nanomolar dissociation constants had also been achieved successfully.

1.5 Microfluidic SELEX

Microfluidic SELEX or M-SELEX, which generally integrates magnetic microspheres with microfluidic chips, can be an extensive, efficient, and even fully automated nucleic acid aptamer screening platform. Compared to traditional SELEX processing equipment, microfluidic systems are more compact and consume less sample. Typically, a single-cycle SELEX process using a microfluidic chip takes about one hour, which is faster than the traditional SELEX process. In addition, the total sample volume consumed in each operation is only 40 μl, which is significantly less than the required sample volume in the large system (100 μl) [31]. The detailed steps about microfluidic SELEX are summarized in Figure 1.5: (a) the starting ssDNA library consists of 10^{14} unique sequences, each containing a 60-base internal randomized region flanked by two 20-base PCR primer-specific sequences; (b) the target protein is conjugated to the magnetic beads through carbodiimide coupling; (c) target-conjugated beads are incubated with the heat-treated ssDNA library; (d) aptamers that bind to the target protein are separated in the continuous-flow magnetic-activated chip-based separation (CMACS) device; (e and f) the aptamers bound on the target-coated beads are amplified via PCR and single-stranded products are generated; (g) the binding kinetics is measured for resulting aptamers.

Microfluidics technology has some unique advantages in improving the screening efficiency of conventional SELEX with the aid of magnetic beads. The microchannel device with laminar flow minimizes the negative influence of molecular diffusion on separation, and the screening process can be finished within a few rounds. Qian et al. developed a disposable micromagnetic separation (MMS) microfluidic chip and integrated ferromagnetic structures to

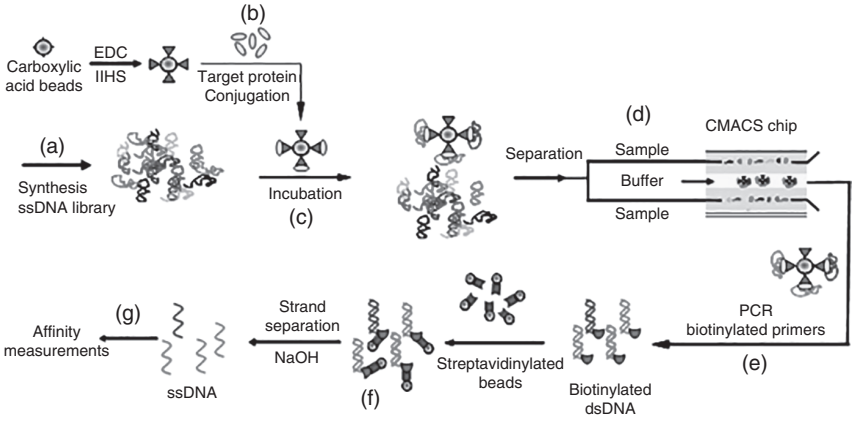

Figure 1.5 Overview of a microfluidic-SELEX process. Source: Lou et al. 2009 [31]. Reproduced with permission from PNAS.

reproducibly generate large magnetic field gradients within the microchannel to trap magnetic bead–bound aptamers efficiently [32]. The DNA aptamer for streptavidin demonstrating good affinity with nanomolar dissociation constant was obtained by three rounds of positive screening, and the specificity of nucleic acid aptamer was improved by negative selection against bovine serum albumin (BSA).

During the microfluidic SELEX process, magnetic beads play an important role. Normally, the target is fixed on the magnetic beads through covalent bonding or non-covalent coupling. Oligonucleotide molecules in the solution diffuse to the surface of the magnetic beads, and interact with the target. Then the non-bound and weak bound oligonucleotide molecules are removed by the elution. The nucleotide molecules bound to the target are separated from the magnetic beads, and the next round of screening library is obtained until good affinities are met.

An integrated microfluidic system with magnetic beads can provide a rapid automated screening approach. The scheme not only improves the efficiency of the SELEX greatly but also is effective for any type of molecular targets, and experimental data show that aptamers screened using an integrated microfluidic system are found to have an excellent affinities. The screening process firstly incubates the magnetic beads coupled to the target in the random nucleic acid library to find specific oligonucleotides; then the bead-bound oligonucleotides are captured and purified for further aptamer extraction by applying a magnetic field in the microchannel. The integrated microfluidic system generally consists of three modules: a microfluidic control module for sample incubation and transport, a bead-based ssDNA extraction module for aptamer screening, and a rapid nucleic acid amplification module. For example, C-reactive protein (CRP)-specific aptamers screened by this method are compared to those performed using laboratory-scale equipment and manual manipulations. Using a combination of magnetic beads and microfluidic transport technique, the CRP-specific aptamer from a complete random ssDNA library is successfully purified and enriched in an automated process [33].

Recently, an acousto-microfluidic SELEX method was suggested by Park et al. to obtain a prostate-specific antigen (PSA)-binding aptamer based on an acoustophoresis technique with simultaneous washing and separation in a continuous flow mode to improve selection efficiency. In addition, next-generation sequencing (NGS) was applied to accelerate the identification of the screened ssDNA pool. After eight rounds of acousto-microfluidic SELEX and following sequence analysis with NGS, seven PSA-binding ssDNA aptamer candidates were obtained and characterized with surface plasmon resonance (SPR) for affinity and specificity. The best PSA-binding aptamer showed specific binding to PSA with a dissociation constant (K_d) of 0.7 nM [34].

In summary, M-SELEX can greatly accelerate the aptamer separation using a very small number of target molecules to achieve highly stringent selection conditions. M-SELEX has become a versatile and automated method for rapid generation of aptamers nowadays, and can utilize many unique microfluidic phenomena such as laminar flow and electroosmosis to achieve unparalleled selection efficiency.

1.6 Cell-SELEX

The SELEX process is universally applicable to different classes of targets. Besides defined single targets, complex target structures or mixtures without proper knowledge of their composition are suitable for a successful aptamer selection. Using complete living cells as target molecules, cell-SELEX can be utilized to screen specific aptamers to identify potential or discover novel biomarkers on the cell surface. Generally, cell-SELEX includes two steps: firstly, the target cells are incubated with oligonucleotides and applied for positive screening; secondly, non-target cells are subject to negative screening. The advantages of cell-SELEX are listed as follows: (i) multiple aptamers targeting different receptor molecules on the target cell surface can be screened simultaneously; (ii) targeting aptamers can effectively recognize the receptor molecules on the cell surface under normal cellular growth condition; (iii) the targeting aptamers can be directly used for cell identification and cell binding studies; (iv) the targeting aptamers can identify the cell surface binding sites, which can be used as potential cell surface biomarkers [35]. Figure 1.6 illustrates the process of cell-SELEX screening.

Recently, Ara et al. successfully screened tumor cell surface antigen aptamers [36]. The results show that the relevant aptamers can be used

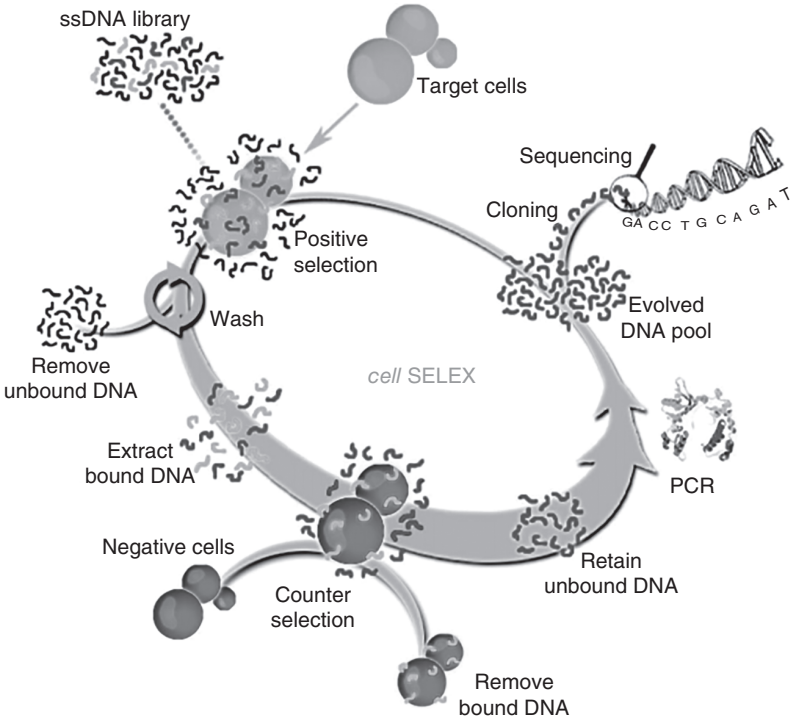

Figure 1.6 Mechanism of cell-SELEX [35]. Source: Fang and Tan 2010 [35]. Reprinted with permission of American Chemical Society.

not only for tumor molecular markers and diagnosis but also for the treatment of cancer. This technique avoids the purification process of the antigen and maintains the antigenic conformation of the cell surface. It is well known that the cell surface contains a plurality of antigens, which are all target substances. So it is particularly necessary to remove nonspecific nucleic acid strands to obtain the desired aptamers. The current cell-SELEX technique uses cells that possess no or low expression aim at target substances for negative screening, and applies control cells to remove nonspecific nucleic acid fragments from the selected secondary library [37].

Cancer-related biomarkers such as platelet-derived growth factor (PDGF), human epidermal growth factor 3 (HER3), vascular endothelial cell growth factor (VEGF), nuclear factor kappa B (NFKB), PMSA, and tenascin-C have been developed to identify cancerous cells [35]. Most of them use purified proteins as targets. Recently, it has been proved that more and more aptamers can be selected for complex targets, especially for whole cells. The selection of aptamers for living cells vs target protein-expressing cells is straightforward: cell surface proteins are endowed with their natural conformation, which is crucial for biological functions [38]. Recognition of human aptamer receptor tyrosine kinase RET is obtained with RET expression cells as targets as well. Compared with protein-based SELEX, cell-based selection can be performed on molecular signature without prior knowledge of the whole cell. When molecular recognition of cancer cells is needed, it is not necessary to know about the amount or type of proteins on the cell membrane. The selection process itself can distinguish between different types of cells, resulting in a specific type of cancer cells that can only bind to the aptamer, rather than normal cells or other types of cancer cells [39]. Moreover, it is feasible to perform whole cell selection in the presence of many receptor proteins on the surface of the cell membrane, so we can select a set of aptamer probes which can reveal the molecular characteristics of the target cancer type. This is the main advantage of cell-SELEX for cancer diagnosis and clinical analysis.

Currently, cell-SELEX is commonly utilized in aptamer selection for cancer study. A cultured precursor T-cell acute lymphoblastic leukemia (ALL) cell line, CCRF-CEM, has been used as the target. Negative selection is performed with a B-cell line derived from human Burkitt's lymphoma as a negative control by adding a Ramos selection process to exclude possible binding of DNA sequences to common molecules on the surface of leukemic cells [40]. In this work, CCRF-CEM cells are incubated with ssDNAs. The cell surface–binding sequences are eluted by heating after they are washed, and interact with excess Ramos cells afterwards. The sequences still free in the supernatant were amplified by PCR to form the starting pool and can be selected for the next round.

Several types of cancer cells have been successfully used in the cell-SELEX process to filter out novel aptamer probes. These aptamers show superior affinity and excellent specificity. However, it should be noted that the number and duration of cell-SELEX selection is longer than conventional SELEX, and cell-SELEX usually takes the risk of failure to damage fragile cells [40].

1.7 *In Silico*-SELEX

Conventional SELEX strategies after multiple *in vitro* selection rounds for the generation of aptamers are not always successful. Rationalizing random single-stranded oligonucleotide libraries via molecular simulation and tuning structural complexities by algorithm filtering are prevailing computational aptamer selection schemes which can be termed as *in silico*-SELEX, consequently.

Probably the first *in silico*-SELEX was reported by Chushak and Stone [41] when they proposed a computational method to develop RNA aptamers for codeine, gentamicin, theophylline, etc. Firstly, RNA sequences were selected from randomly generated RNA pools based on the criteria that limited the presence of sequences with abundant simple structural motifs and maximized the presence of stable low-energy structures. The Rosetta package was used for the prediction of tertiary structures of these selected RNA sequences, the structures were minimized using the AMBER force field and generalized Born implicit solvent, and then ensemble docking with a modified DOVIS package having AutoDock4 software as the docking engine and run in parallel on Linux clusters for a library of RNA molecules with lowest energy structures. RNA aptamers against codeine, gentamicin, theophylline, and other analyte molecules were selected by this high-throughput virtual screening scheme and experimentally validated, respectively.

Inherently, i*n vitro* random RNA/DNA pools are not structurally diverse and heavily favor simple topological structures such as stem–loop structures; and increasing the structural diversity of the starting oligonucleotide pool can enhance the possibility of finding novel aptamers with improved affinity thereof. In 2011, Luo et al. [42] developed two computational algorithms of random filtering and genetic filtering to generate sequences that exhibit higher structural complexity and can be used to increase the overall structural diversity of initial pools for *in vitro* selection experiments.

Nowadays, *in silico*-SELEX has found its practical utilization in biomarker analysis for tumor diagnosis and treatment. Ahirwar et al. [43] reported an *in silico* selection strategy to select a candidate RNA ERaptR4 as human estrogen receptor α (ERα) aptamer. RNA analogs of human estrogen response elements (EREs) were used to obtain aptamer-like sequences. AutoDockVina, HADDOCK, and PatchDock docking were applied to examine the likelihood of near-native RNA analogs of selected single-stranded EREs emerge as ERα aptamer. In addition, isothermal titration calorimetry (ITC) was used to validate *in silico* prediction results by thermodynamic characteristics of ERα-RNA complex. After careful analysis of the predicted intermolecular interactions in the selected ERα-RNA complex, as shown in Figure 1.7, ERaptR4 was finally identified as ERα aptamer with a binding constant of $1.02 \pm 0.1 \times 10^8$ M^{-1}. The aptamer had a good specificity confirmed through cytochemistry and solid-phase immunoassays as well. Furthermore, the aptamer could resist serum and RNase degradation in the presence of ERα, and the stability will enable

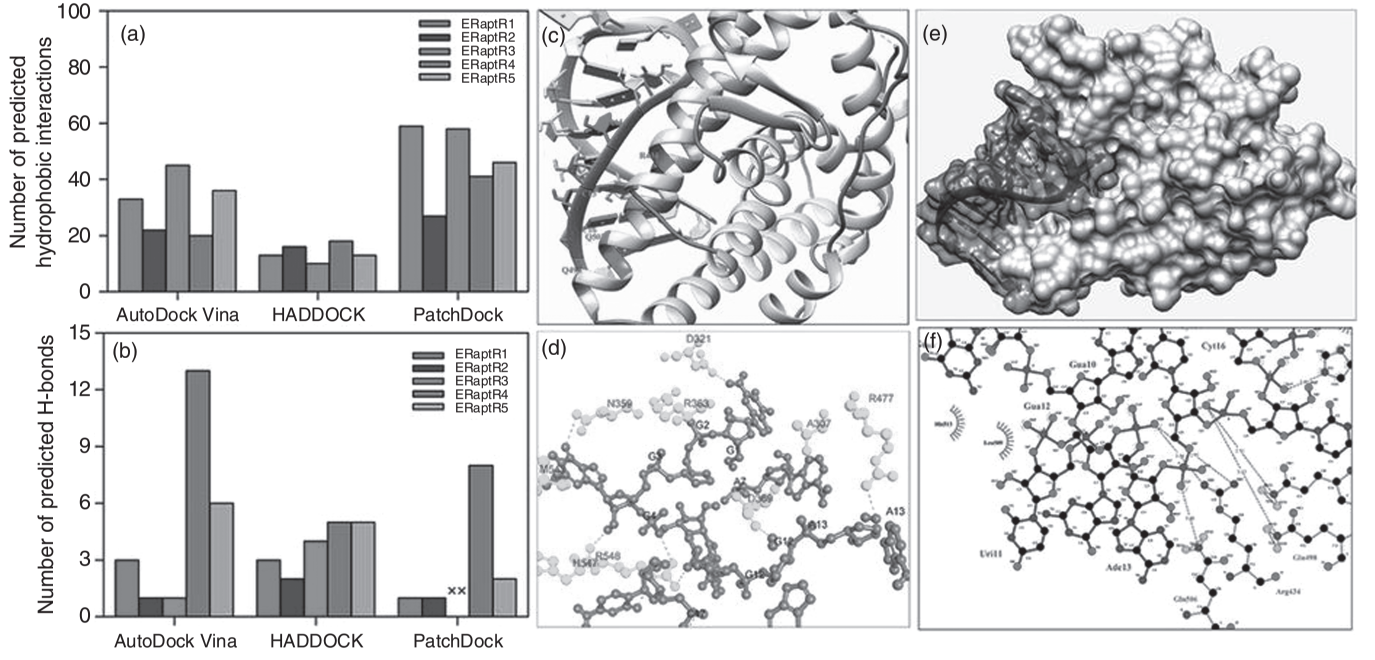

Figure 1.7 Analysis of the predicted intermolecular interactions in the selected ERα-RNA complex. (a) and (b) Numbers of the predicted hydrophobic interactions and H-bonds in complex of ERα with ERaptR1–ERaptR5. These interactions are predicted using Ligplot and Nucplot. (c) Ribbon view of the HADDOCK predicted ERα (1SJ0)-ERaptR4 complex, depicting the interacting residues and the spatial arrangement of protein chains in the vicinity of aptamer molecule. (d) H-bonding residues in the AutoDock Vina generated complex of ERα-ERaptR4. The dark gray color represents the aptamer bases while the light gray color indicates the amino acids. (e) Surface view of the PatchDock-generated ERα-ERaptR4 complex showing the relative orientations of interacting bases and amino acid chain. (f) Structural representation of H-bond and hydrophobic interactions in the ERα-ERaptR4 complex as predicted using Ligplot. H-bonds are represented by dashed lines between H-bonding atoms, whereas the hydrophobic interactions are shown by an arc with spokes radiating toward the interacting ligand atoms. Source: Ahirwar et al. 2016 [43]. Reprinted with permission of Springer Nature.

future applications of this aptamer for the detection of ERα in breast cancer and related diseases as expected.

In summary, although *in silico*-SELEX can facilitate pool rationalization and the subsequent aptamer generation, a good command of molecular simulation and algorithm implementation, and a facile accessibility to computing resources, are generally required.

1.8 Post-SELEX and *In Chemico*-SELEX

To further enhance conformational adaptability and improve *in vivo* stability to degradation and binding affinity of the selected aptamer with analyte molecule, a good command of nucleotide chemistry and organic syntheses is a must. Some frontier SELEX strategies, which can be termed as post-SELEX or *in chemico*-SELEX, were developed successfully in recent years based on either modifying nucleotides or using non-natural nucleotides.

Chemically, the 2′ hydroxyl of ribose in RNA molecule is rather reactive, especially at alkaline pH and particularly in the presence of ubiquitous endonuclease. The 2′ hydroxyl will attack and break the phosphodiester backbone, resulting in a 2′, 3′ cyclic phosphate; hence, aptamer can be modified to bridged nucleic acid (BNA)/locked nucleic acid (LNA) at the 2′, 4′-position, or substituted with —F, —OCH_3 or —NH_2 groups for hydroxyl at the 2′ position of the ribose. In addition, the phosphodiester backbone of the aptamer can be derivatized as well.

2′,4′-BNA/LNA with high nuclease resistance and low cytotoxicity were independently developed by Imanishi et al. [44] at Osaka University and Wengel et al. [45] at the University of Southern Denmark in the late 1990s, and *in vitro* selection of BNA/LNA aptamers is feasible owing to the discovery of polymerases available for BNA/LNA-containing oligonucleotide syntheses and genetic engineering of these polymerases in recent years. Using CE-SELEX, high-affinity thrombin-binding aptamers (TBAs) were obtained from DNA-based libraries containing 2′-*O*,4′-*C*-methylene-bridged/linked ribonucleotides in the 5′-primer region. After around 10 rounds of selection, as shown in Figure 1.8, 40 sequences were identified, and the binding affinity (K_d) of the best aptamer was 18 nM [46].

Oncostatin M (OSM) is a multifunctional member of the interleukin-6 cytokine family and has been implicated as a powerful proinflammatory mediator and may represent a potentially important, novel therapeutic opportunity for treatment of established rheumatoid arthritis. Andrew Rhodes et al. [47] isolated an RNA aptamer ADR58 for human OSM with a high affinity of 8 nM successfully, the pyrimidine positions in ADR58 all contain a 2′ fluoro group on the ribose ring, and this modification stabilizes the aptamer with respect to ribonuclease activity. To better ensure aptamer stability, each individual purine position was sequentially substituted with a 2′ O-methyl purine residue. In addition, truncated ADR58 from 71 bases in length to 33 bases with the same OSM affinity was terminated at the 3′ end with a 3′-3′ thymidine cap to further increase resistance to nuclease attack.

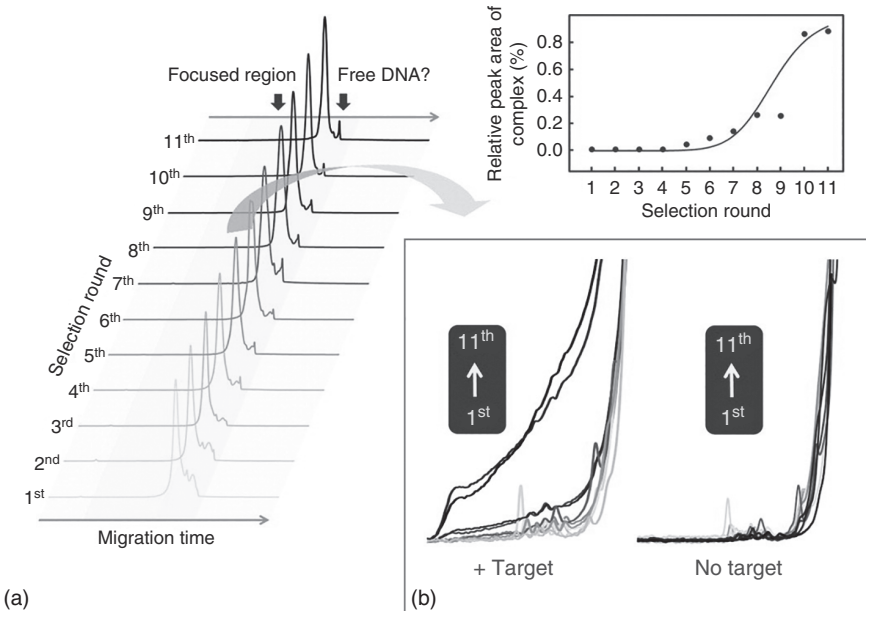

Figure 1.8 Process of active species enrichment in selection rounds. (a) Capillary electrograms for library E of each round with human thrombin (left graphic). All electrograms recorded fluorescent intensity of 5′-labeled 6-FAM vs migration time. Saturation curve of library enrichment for TBA acquisition (right graphic). (b) Enlarged and overlapped view of each round of capillary electrograms with or without human thrombin. Source: Kuwahara and Obika 2013 [46]. Reprinted with permission of Taylor and Francis.

An oligonucleotide library with phosphorothioate backbone thio-substituted at dA positions was synthesized and used for the selection of enhanced nuclease-resistant DNA aptamer to target NF-IL6, a basic leucine zipper transcription factor involved in the induction of acute-phase responsive and cytokine gene promoters in response to inflammation. An individual monothiophosphate 66-mer cloned from the 10th selection round gave an observed binding constant of <2 nM, and, as shown in Figure 1.9, thiophosphorylation of the family A 66-mer at only the dA sites (except for the primers) results in a duplex that is more resistant to DNase I degradation than the unmodified 66-mer [48].

In order to increase both chemical and structural diversity of the DNA molecules for aptamer selection, non-natural nucleotides as developed by Benner [49] and Hirao [50] were introduced into various SELEX protocols. The potential of this genetic alphabet expansion becomes a powerful tool for creating highly functional nucleic acids such as analytical aptamers and medicinal pharmaceuticals.

Zhang et al. [51] developed aptamers against cells overexpressing glypican 3 (GPC3), a probable biomarker of hepatocellular carcinoma (HCC), from an artificially expanded six-letter genetic information system (AEGIS) with two non-natural nucleobases (2-amino-8H-imidazo[1,2-*a*]-[1,3,5]triazin-4-one, Z and 6-amino-5-nitropyridin-2-one, P) as shown in Figure 1.10. With counterselection against non-engineered cells, eight AEGIS-containing

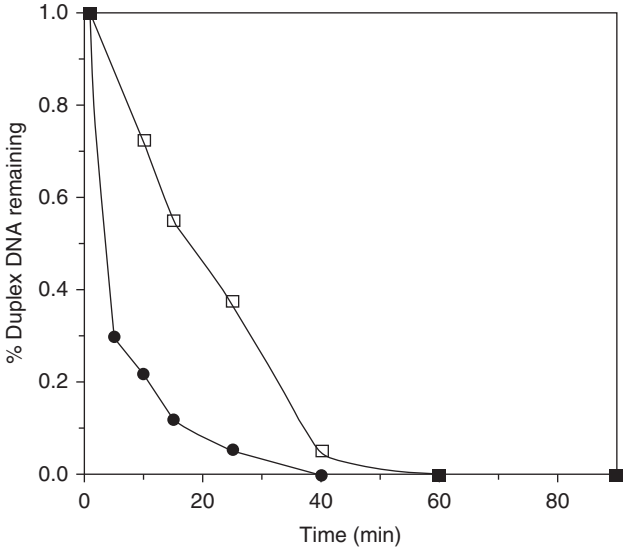

Figure 1.9 Relative sensitivity value of family A 66-mers to degradation by DNase I. Unmodified, phosphoryl duplex (●) and monothiophosphorylated at nonprime dA sites only (□) with 0.0 and 1.0 at time 0 and after 60 minutes, respectively (■). Source: King et al. 1998 [48]. Reprinted with permission of American Chemical Society.

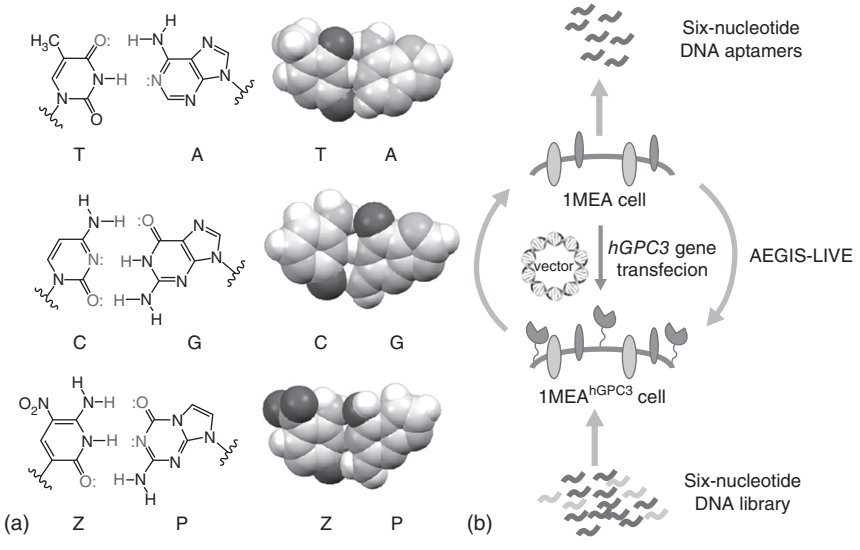

Figure 1.10 Chemical structures of the six nucleotides and AEGIS-LIVE (laboratory *in vitro* evolution). (a) Molecular structures (left) and space-filling models (right) of C:G, T:A, and Z:P pairs showing their similarity (PDB ID: 4RHD). (b) Engineering hGPC3-overexpressing cells and AEGIS-LIVE procedure. Source: Zhang et al. 2016 [51]. Reprinted with permission of John Wiley and Sons.

aptamers were recovered, among which five bound selectively to GPC3-overexpressing cells.

Similarly, Kimoto et al. [52] generated high-affinity DNA aptamers using an expanded genetic alphabet of four natural nucleotides and an unpaired artificial nucleotide with the hydrophobic base 7-(2-thienyl)imidazo[4,5-b]pyridine(Ds). Selection experiments against two human target proteins, VEGF-165 and interferon-γ (IFN-γ), yielded DNA aptamers that bind with K_d values of 0.65 pM and 0.038 nM, respectively, affinities that are >100-fold improved over those of aptamers containing only natural bases.

1.9 Auto-SELEX

The automated SELEX process refers to the selection cycles without any direct manual intervention steps, and is designed to provide high flexibility and versatility in the selection of buffers, reagents to meet rigorous selection criteria [53].

In 2001, the Beckman-Coulter Biomek 2000 automated workstation was used to screen aptamers of lysozyme. The workstation included a mechanical control station, thermal cycler, magnetic bead automatic separator, multiscreen vacuum filter, enzyme cooler, and an automatic pipetting tool. The screening process involved immobilizing the biotinylated target protein on the magnetic beads by the interaction of streptavidin with biotin. The specific binding sequence of separation, reverse transcription polymerase chain reaction (RT-PCR) amplification and transcription were all automated by the programmed procedure. The final sequence was cloned into the vector for sequencing and identification. By this automated screening workbench, the author only needs less than two days to complete 12 rounds of screening [54]. Automated selection saves time and gets good results [55]. Using the automated SELEX method, RNA aptamers to the mirror-image configuration (d-peptide) of substance P have been identified [53]. The anti-lysozyme aptamer is also selected [55]. To date, the auto-SELEX method has been applied for various targets, ranging from small organic molecules to supramolecular structures [1, 6, 56] and organisms [57, 58].

1.10 Primer-Free SELEX

Standard phylogenetic evolution of ligands by SELEX schemes requires libraries containing two primers on both sides of the central random domain, which amplifies the target binding sequence by PCR or RT-PCR. However, these primer sequences may cause nonspecific binding, and result in a large number of binding sequences or interfering with specifically binding random sequences.

The scientists started to develop the primer-free SELEX method that eliminates the primer sequence from the target binding process, thereby eliminating the interference caused by the primer sequence. The method allows the primers to be regenerated and eliminated after selection. It is fast, simple, and does not require any chemical modification [59].

Wen et al. develop a primer-free genome SELEX method to avoid the interference from the interaction of the primer and the oligomers during the amplification process [60]. The method removes the primer from the genomic library before screening, and incubates the library with the target molecule. The gene fragments are screened by hybridization-extension thermal cycling reaction to carry out PCR amplification. This primer-free genomic SELEX is a new platform technology. The primer-free SELEX significantly simplifies SELEX procedures and eliminates the primitive interference problem, and becomes a good method for identifying potentially bio-important nucleic acid sequences of target molecules while reducing human workload [58].

1.11 Genomic SELEX

Genomic SELEX is based on the generation of RNA species that are derived from a library of an organism's entire genomic DNA *in vitro*. The generated RNA pool will undergo successive rounds of association with a given RNA-binding protein, partitioning, and reamplification. This method does not require isolation of non-coding RNAs (ncRNAs) from an organism or cell.

RNA sequences that are stringently bound by the protein partner will be enriched. Once the sequence of the bound RNAs is determined, this method will be used to search for matches in the genome, and predicted genomic regions that can be tested for the expression of unknown ncRNAs. Genomic SELEX has been successfully applied to select mRNA (messenger RNA)-binding protein partners [61, 62].

Valentin-Hansen et al. applied genomic SELEX to identify new Hfq-binding RNAs from *Escherichia coli*. A representative library of the *E. coli* genome is constructed from random 50–500 bp genomic DNA fragments to which defined linkers, one of these containing a T7 RNA polymerase promoter, is attached in the course of the initial library generation step [63]. These fragments are *in vitro* transcribed with T7 RNA polymerase, incubated with Hfq, and selected for Hfq binding on filters. Taking the standard SELEX procedure [64], the retained RNA is converted to complementary DNA (cDNA) and subjected to additional reamplification and selection rounds, which finally result in a pool of RNAs that bind Hfq with K_d values of 5–50 nM. Subsequently, specific Hfq interaction of the enriched RNAs is determined *in vivo* using a yeast three-hybrid screen [65]. Preliminary results suggest that these experiments identified a number of novel Hfq-binding RNAs, including antisense RNAs and candidate ncRNAs from intergenic regions.

However, the studies that focused on ncRNAs have not been published for any organism yet. The combination of genomic-SELEX technology and computer-aided design (CAD) will be used to select ncRNAs in future. Genomic-SELEX would clearly have its strength in finding ncRNAs that are overlooked by methods that require ncRNA genes to be expressed at a certain level. This method can be used in prokaryotes because of their small genome sizes. Since functional ncRNAs are mostly encoded by intergenic regions in

microbes, the DNA original fragments will be loaded by amplifying this portion of the genome specifically, which constitutes below 10% of the entire genome in microbes.

1.12 Photo-SELEX

In photo-SELEX, base moiety in a library oligonucleotide sequence can be replaced by an optically active compound, such as 5-iodouracil and 5-bromouracil; then the sequence is incubated with the target molecule. Induction of target molecule by light irradiation increases the specificity of its covalent cross-linking, which can increase the specificity and affinity of the selected aptamers. The 5-bromouracil chromophore has absorption at 310 nm, while native chromophores of nucleic acids and proteins show very weak or no absorption [66].

Bromodeoxyuridine (BrdU) photoaptamers are rapidly emerging as specific protein capture reagents in protein microarray technologies. A mathematical model for the kinetic analysis of photoaptamer-protein reaction has been presented by Koch et al. [67]. The model is based on specific aptamer/protein binding followed by laser excitation that can lead to either covalent cross-linking of the photoaptamer and protein in the complex or irreversible photodamage to the aptamer. The models are used to characterize the photocross-linking between three photoaptamers and their cognate protein targets (human basic fibroblast growth factor and HIV MN envelope glycoprotein). The cross-linking reaction yields, laser energy dose, and target protein concentration are used to analyze the affinity constants and cross-link reaction rates. The binding dissociation constants derived from the cross-linking data are in good accordance with independent measurements.

Photo-SELEX can obtain an aptamer with higher affinity compared to conventional SELEX. If a good photo-SELEX result is expected, the target molecule must be a protein molecule with photocross-linking functional groups such as aromatic or sulfur-containing amino acid residues.

1.13 qPCR-SELEX

With the development of PCR instruments, aptamers can be quantified by quantitative PCR techniques in real time with aptamer-specific primers after screening with SELEX. This quantitation scheme, which is termed as qPCR-SELEX, can not only be used as an additional monitoring tool to determine the enrichment of bound aptamers for sequence selection but also detect the presence of the corresponding target substance and its amount indirectly. Furthermore, the contamination of the amplified aptamer pool with by-products can be prevented by prior determination of bound aptamers during SELEX rounds.

qPCR-SELEX combines the affinity of the aptamers with the amplification of the PCR reaction, and can obtain a significant increase in the detection capability

for target substance. Besides, due to the high specificity of the aptamers binding to the target substance, the accuracy of the testing can be ensured. In 2013, on the basis of a SYBR Green I real-time PCR technology and cell-SELEX, Avci-Adali et al. used a qPCR-SELEX strategy to determine the accurate aptamer amount on cells after the incubations. The method is highly sensitive and allows the detection of very small quantities of aptamers in cell lysate samples. The lower detection limit is 20 fg [68].

1.14 Perspectives

SELEX and its assorted updates are now evolved rapidly for various analytical or medicinal applications. Aptamers with comparable or even superior affinities of antibodies and improved stabilities are successfully developed on the basis of frontier technologies of random library rationalizing, unbound fractionating, affinity characterization, and sequence identification, accordingly. For instance, Xianbin Yang et al. successfully developed phosphorothioate (PS-) or phosphorodithioate (PS2-) oligonucleotide thioaptamers as potential diagnostic reagents or therapeutics in recent years [69–71].

However, the effort for a successful SELEX is still painstaking. In an effort to overcome conventional SELEX limitations, AM Biotech (see www.am-biotech.com) developed a patented selection technology that eliminates the need to enzymatically replicate the binding aptamer sequences during selection. This exponentially expands the chemical diversity available for target interaction and also enables the X-Aptamer Selection Kit (Figure 1.11), which significantly

Figure 1.11 X-aptamer selection kit developed by AM Biotech. Source: With permission from AM Biotech.

eases the work required for selection. In addition, a universal tool (FCE-SELEX) for the development of aptamers was recently reported by Luo et al. [72], by integrating fraction collection with facile oil sealing to avoid contamination while amplifying the bound DNA–target complex. In a single CE-SELEX round of selection, a streptavidin-binding aptamer with an affinity of 30.8 nM was generated.

It is noteworthy that high-throughput sequencing with bioinformatics is becoming a routine tool for aptamer discovery nowadays [73], and aptamer microarrays [74] have been incorporated into traditional SELEX protocols for unparalleledly efficient sequence characterization and identification as well. The readers are suggested to read Chapter 13 for more information on SELEX with NGS and microarry-based SELEX thereof.

Although aptamers are mostly *in vitro* selected for a definite target, their analytical utilization for non-target profiling and broad-spectrum or class-specific determination remain very limited. Antibodies with good affinities and specificities are still predominant recognition elements for analysis. More theoretical investigations and experimental practices are therefore needed to foresee or interpret aptamer configuration and adaptive folding mechanism at the molecular level, which will necessitate the evolution of SELEX and its variants in the future.

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