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Three decades of nucleic acid aptamer technologies: Lessons learned, progress and opportunities on aptamer development

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ABSTRACT

Aptamers are short single-stranded nucleic acid sequences capable of binding to target molecules in a way similar to antibodies. Due to various advantages such as prolonged shelf life, low batch to batch variation, low/ no immunogenicity, freedom to incorporate chemical modification for enhanced stability and targeting capacity, aptamers quickly found their potential in diverse applications ranging from therapy, drug delivery, diagnosis, and functional genomics to bio-sensing. Aptamers are generated by a process called SELEX. However, the current overall success rate of SELEX is far from being satisfactory, and still presents a major obstacle for aptamer-based research and application. The need for an efficient selection strategy consisting of defined procedures to deal with a wide variety of targets is significantly important. In this work, by analyzing key aspects of SELEX including initial library design, target preparation, PCR optimization, and single strand DNA separation, we provide a comprehensive analysis of individual steps to facilitate researchers intending to develop personalized protocols to address many of the obstacles in SELEX. In addition, this review provides suggestions and opinions for future aptamer development procedures to address the concerns on key SELEX steps, and post-SELEX modifications.

1. Introduction

First introduced in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990), aptamers are short synthetic single-stranded (ss) nucleic acid sequences that can bind to a broad range of targets including metal ions, chemical compounds, proteins, cells and whole micro-organisms. As a class of affinity ligands, aptamers display some advantages over traditional antibodies such as prolonged shelf life, low batch to batch variation, low/no immunogenicity, and the flexibility to incorporate chemical modifications for enhanced stability and targeting affinity (Wang et al., 2015). Consequently, over the past few decades, aptamers quickly found their way in diverse applications ranging from therapy, drug delivery, diagnosis, and functional genomics to bio-sensing (AlShamaileh et al., 2017; Khan et al., 2018; Shigdar et al., 2013a; Tapsin et al., 2018).

Aptamers are generally developed in vitro by a very defined iterative procedure known as Systematic Evolution of Ligands by Exponential enrichment (SELEX) (Tuerk and Gold, 1990). The originally reported SELEX procedures is, however, time and labor consuming. Over the past three decades, by adaption and integration of progresses in material sciences and analytical techniques, various SELEX variations have been reported in an attempt to either reduce processing time (e.g. CE-SELEX

(Yang and Bowser, 2013), Flu-Mag SELEX (Stoltenburg et al., 2005), *in silico* SELEX (Rabal et al., 2016)), generate aptamers with novel designs and functions (e.g. µFFE-SELEX (Jing and Bowser, 2011), Capture-SELEX (Istamboulie et al., 2017)), or increase the process throughput (e.g. Sweep-CE-SELEX (Okhonin et al., 2004)). Today, some groups can reduce the time for aptamer development from months required for conventional SELEX to several hours (Lokesh et al., 2017; Martin et al., 2015; Zhuo et al., 2017). Furthermore, a recently developed computer supported *in silico* assay promises to predict an aptamer structure even before SELEX is conducted (Ahirwar et al., 2016). Stemming from this, aptamers such as the hydrophobic SOMAmers (Eid et al., 2015), thioaptamers (Volk and Lokesh, 2017), and X-Aptamers (Lokesh et al., 2017) have been developed. NOX-A12, a nuclease resistant L-nucleic acid aptamer (Vater and Klussmann, 2015), recently entered Phase 1/2 clinical trial for multiple oncology indications (ClinicalTrials.gov Identifier: NCT03168139). Progress in nucleic acid chemistry and selection strategies significantly contribute to the commercialization and clinical translation of aptamers. However, it is worth mentioning that the number of aptamers showing strong binding capacity and specificity is still limited (Baird, 2010). Improving the current SELEX procedure and developing more aptamers with high binding capacity and specificity remains a major hurdle for aptamer related basic and applied studies, and the knowledge accumulated over the last three decades would be of significant benefit in developing the successful individual SELEX procedure.

SELEX is a typical multidisciplinary procedure related to different fields such as molecular biology, nucleic acid chemistry, material science, and bioinformatics to develop affinity ligands against a vast variety of targets (Blind and Blank, 2015). As such, a standard SELEX protocol suitable for all experimental settings may not exist (Svobodova et al., 2012). To identify aptamers for a specific target, one needs to design a SELEX protocol with various factors in consideration such as time- and cost-efficiency of the chosen SELEX protocol, type and modification of initial libraries, accessibility to instruments etc. Indeed, to manipulate the art of SELEX, it is necessary to comprehensively study each of the key steps of SELEX (Fig. 1) and associated techniques. Recent SELEX review articles generally provide readers with an overview of SELEX strategies or aptamer related applications, in most cases, detailed illustration and comparison of key techniques of SELEX variations are overlooked. In this review, instead of describing individual SELEX procedures, by focusing on key steps of SELEX, and via critical discussion and analysis of knowledge and lessons collected during the past three decades, we evaluate the current SELEX strategies and provide an insight on promising SELEX associated techniques to broad researchers intending to develop tailored SELEX protocols for different application settings. In addition, current strategies for post-SELEX modifications are also discussed.

2. SELEX library design

The initial nucleic acid (NA) library provides a crucial starting point for SELEX. Herein, we summarize and discuss the fundamental aspects of NA library design such as the type of library, the primer binding site, chemical modification, the presence of fixed sequences, and randomization strategies, to present a comprehensive picture of SELEX library design.
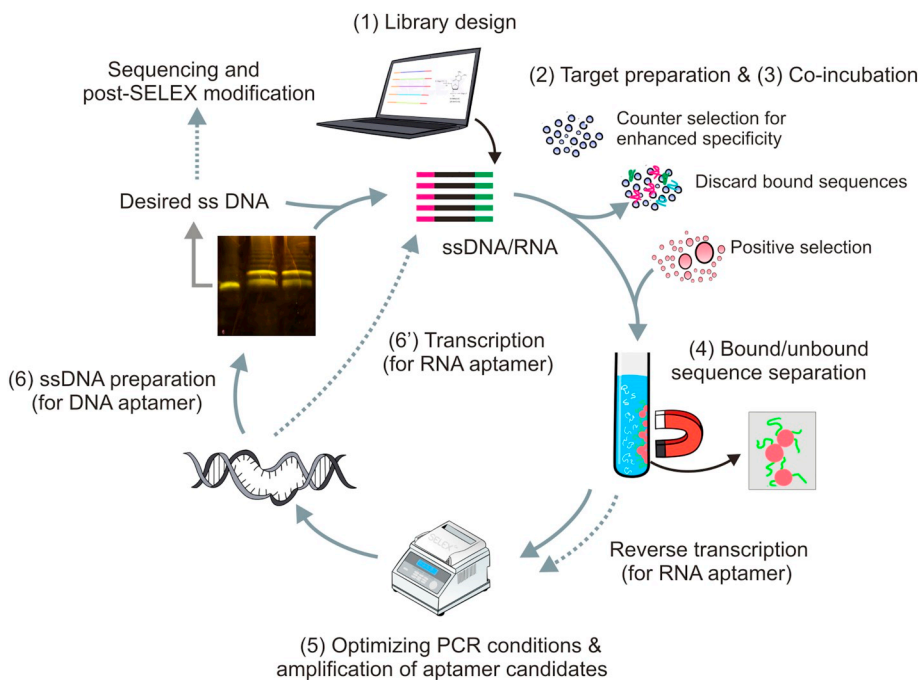
2.1. DNA or RNA?

Native DNA and RNA based libraries are used in majority of the currently performed SELEX procedures, although the use of artificial nucleic acids (see Section 2.3.3.2) has also been emerged as a tool for aptamer development with improved properties (Sefah et al., 2014). Despite the fact that a DNA library had been successfully employed for

SELEX since very early stages of aptamer development (Ellington and Szostak, 1992), RNA libraries were preferentially used in the majority of the early SELEX protocols - based on the belief that RNA could fold into more functional motifs and result in higher affinity binders (Cheng et al., 2001). However, accumulating evidences suggest that ssDNA exhibits comparable propensity for forming intricate tertiary structures with that of RNA. This finding is important for aptamer related work. Evidently, in terms of aptamer development and subsequent application, DNA aptamers show advantages over their RNA counterparts (Lakhin et al., 2013). Firstly, DNA is more chemically and biologically stable, which makes both selection and application easier. This is especially true when microarray platforms are applied for high throughput SELEX; secondly, DNA-based SELEX is more cost and time-effective, as an extra reverse transcription step essential for RNA SELEX is not required; thirdly, from the perspective of commercialization, DNA is easier to synthesize and is more robust than RNA in terms of shelf life (Lakhin et al., 2013). Accordingly, DNA libraries are being used more frequently in recent SELEX studies. By 2007, DNA represented half of the performed SELEX libraries. And more than 85% of the reported aptamers in 2013 were developed using DNA. Today, DNA aptamers represent almost all of the products provided by commercial aptamer developers (Darmostuk et al., 2015). As a special variation of SELEX, genomic SELEX is very different from common SELEX by employing genomic DNA (double strand), genome encoded RNA, and transcriptomic total RNA as starting libraries (Cheung et al., 2013). Genomic SELEX is mainly for functional studies aiming to identify regulatory domains in a genome such as DNA sequences recognized by transcription factors, RNA fragments recognized by splicing factors, RNA polymerases, ribosomes or other components of the gene expression machinery (Vorobyeva et al., 2018). Since RNA especially non-coding RNA plays an important role in gene regulation, RNA represents the majority of the libraries used in genomic SELEX (Cheung et al., 2013). A critical feature of a genome library is the restricted sequence information. As reported, the human genome encodes 3×10^9 bp with a plethora of redundant and repeat sequences (Hood and Rowen, 2013). As a result, a typical genomic SELEX library displays substantially lower sequence diversity as compared with randomized NA libraries (10^8 times lower than a standard SELEX library with 40 nt random sequences). Although low sequence diversity is problematic for normal SELEX, it presents an advantage for genomic SELEX. This is because the binding sequences are endogenously included in the pool, a dramatic decrease in the diversity of the starting library would favor the successful identification of targets.

2.2. Constant region of SELEX libraries

2.2.1. Primer binding sites
2.2.1.1. How primer-binding sites affect the SELEX process. A classic SELEX library contains a randomized region, flanked by two fixed primer-binding sequences on both ends to enable amplification of the enriched sequences via PCR (Fig.2) (Wu and Kwon, 2016). This type of library format has been used in most published SELEX studies. Ideally, the developed aptamer sequences should bind their targets through tertiary structures formed only by nucleotides from the random region. However, during the SELEX procedure, the interaction between the constant primer binding sites and sequences in the random region is inevitable (Tolle et al., 2014). Previous studies confirmed this phenomenon where the primer-binding sites participate in the functional secondary structures of developed aptamers (Shui et al., 2012). Considering the significant number of primer binding sites in a SELEX library, they would critically influence which aptamers are selected from the random pool. This is because, theoretically, functional motifs whose structure exploit the constant primer binding regions may be more prevalent in the enriched pool and predominate during the

Fig. 1. An illustration of the key steps of a typical SELEX protocol. SELEX is a comprehensive procedure consisting of a very defined iterative procedure. A typical SELEX procedure includes library design, target preparation, counter selection, positive selection, bound/unbound sequence separation, reverse transcription (for RNA aptamer), PCR amplification, single strand DNA separation (for DNA aptamer) and transcription (for RNA aptamer).

selection process. Following this logic, primer-binding sites would engage in many of the developed aptamers and cannot be simply cut off to minimize the length of the sequence during aptamer truncation and additional minimization studies.

However, according to an analysis of > 2000 sequences from the Aptamer Database, although a number of outliers exist, for the majority of aptamers, their folding secondary structure was independent of the flanking primer-binding sequences. In other words, such constant regions do not seem to participate in aptamer binding structures (Cowperthwaite and Ellington, 2008). One explanation is that, in a certain NA library, the functional sequences and structures for target binding will only rarely require the arbitrary sequence provided by a constant primer-binding region (Cowperthwaite and Ellington, 2008).

The structures formed by random region alone should be much more complex than structures created by the interaction between primer-binding sites and random sequences (Cowperthwaite and Ellington, 2008). However, this statement does have drawbacks. Firstly, although for an individual sequence the chance of creating an information-rich structure by the interaction between the primer-binding site and the internal random sequence is low, considering the large amount of such constant sequences in a given pool, the contribution of constant primer binding sites to the creation of information-rich structures is hard to estimate. Secondly, there is accumulating evidence indicating that pre-designed constant sequences could significantly improve the binding propensity of SELEX libraries – suggesting the effect of the constant region in a SELEX library should not be disregarded. Another
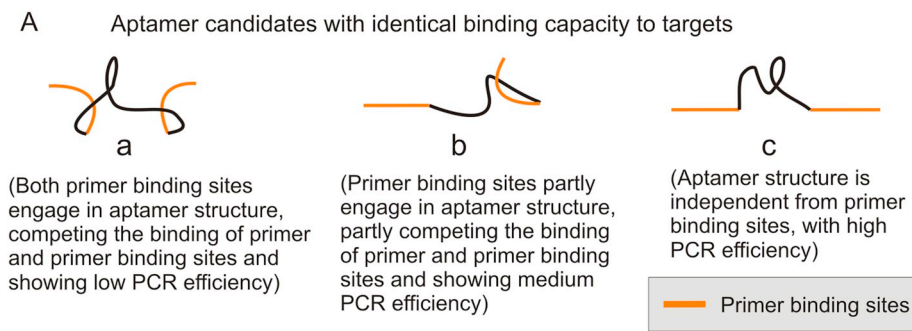
Fig. 2. The interaction between primer binding sites and random sequences of SELEX library causes PCR bias and affects the progress of SELEX. (A) Sequences with the random region displaying interaction with the primer binding sites (sequence a and b) show a selective disadvantage for primer binding and subsequent PCR amplification. (B) Even with identical binding capacity to targets, sequences displaying PCR disadvantage with random region displaying interaction with the primer binding sites (compete for the binding of primers to primer binding sites as shown in b and c) are gradually eliminated from the library pool with the progress of SELEX.
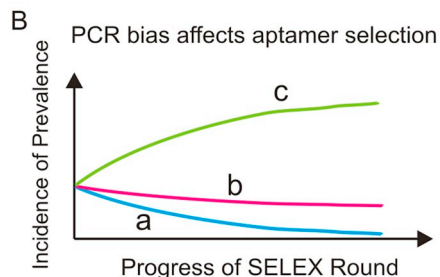
explanation claims that the primer-binding sites may only be able to participate in a limited number of structural motifs because they are located at the end of the library sequences (Cowperthwaite and Ellington, 2008). However, there is no evidence to support this explanation, hence the effect of the 5'- and 3'-end locations of constant regions and their interaction with the random region of libraries need to be further studied.

The observed negligible participation of primer-binding sites on aptamer structures is more likely resulting from the evolution process of SELEX (Fig. 2). As an essential part of SELEX, PCR amplification starts by the binding of primers to the constant primer binding sites. Sequences with the random region displaying strong interaction with the constant primer binding sites (compete for the binding of primer to primer binding sites) may show a selective disadvantage for primer binding and subsequent amplification (Coleman and Huang, 2005). Over multiple rounds of selection and amplification, this selection disadvantage is likely to surpass any selective advantages of functional motifs formed by the random regions (Fig. 2). In contrast, sequences with the random region that do not display interactions with the constant primer binding sites (Fig. 2Ac) are more likely to dominate the PCR product pool due to their adaptation to the PCR program (does not affect primer and primer-binding site recognition) (Coleman and Huang, 2005). Based on this explanation, the selected aptamers actively avoid interference with the primer-binding site. The fact that primer binding site does not present prominently in overall aptamer structures and that it is impossible to adequately predict the interaction between primer binding sites and random regions in a library (including up to 10^{15} sequences species) make the design of primer binding site of library pools simplified. Theoretically, any primer sequences that are generally suitable for PCR would be appropriate for SELEX screenings.

2.2.1.2. Minimizing primer-binding sites. The absence of primer binding sites on selected aptamers does not mean these sites do not affect the SELEX process. As shown in Fig. 2, it can detrimentally affect aptamer selection by reducing the number of functional motifs encoded in random regions. Strategies have been made to minimize the detrimental effect caused by primer-binding sites. Optimization of the length of the random region represents the mostly employed approach. Theoretically, with four natural bases, the maximum number of available spaces in a given library can be reached by random regions with a length of 28 nt (Pobanz and Luptak, 2016). And libraries with random sequence as short as 15 nt had been successfully used in a previous study (Kupakuwana et al., 2011). Indeed, utilizing short aptamer libraries has various advantages including (1) the convenience and low cost of its chemical synthesis; (2) the reduced possibility of PCR by-products formation; and (3) facilitate subsequent sequence truncation and applications. However, as demonstrated by a comparative study using a variety of pool lengths, aptamers selected from libraries with short random sequence pools relied much more heavily on primer binding sites for structure and function than aptamers developed from longer library pools (Lozupone et al., 2003). This is understandable, to be compatible with common PCR polymerase, SELEX primers are designed generally with around 20 nt. The disproportional structure displayed by pools with short random regions certainly impose heavier impact on the SELEX procedure. Therefore, libraries with random region of 30–50 nt are mostly preferred (Cowperthwaite and Ellington, 2008).

The ultimate strategy to eradicate the effect of primer-binding site is to simply delete it by using primer-free (or minimal-primer) libraries (Fig. 3) (Pan and Clawson, 2009; Tsao et al., 2017). However, employing primer-free libraries do present additional complications. Firstly, the additional costs and time by introducing restriction and ligation enzymes has to be considered. Secondly, as one of the most inefficient molecular biological reactions (generally 30-50%), the introduction of a ligation step could result in a significant loss of potential binders and compromise the advantage of implementing such strategy

(Kuo, 2011). Lastly, additional steps associated with purification and separation surely introduce uncertainties and complexities to the already complex SELEX procedure. Consequently, in reality, this strategy is hard to be advantageous over conventional methods and rarely employed in current SELEX practice.

In the case of genomic SELEX, however, primer-free library is strongly recommended. As mentioned in Section 2.1, genomic SELEX using genome encoded RNA or DNA as libraries focus on identifying regulatory domains rather than developing target-binding aptamers. Differing from normal SELEX in which the blockage of a certain amount of random sequences generally does not affect the overall experiment, annealing of primer binding sites with functional regions within random fragments of a genomic library could lead to inappropriate selection. Shtatland et al. conducted genomic SELEX using *E. coli* genome fragments, and demonstrated that by interacting with random regions, the fixed primer binding sites of the library resulted in a large number of experimental artifacts, approximately 90% of the generated sequences were represented by artifacts rather than sequences presented on the genome (Shtatland et al., 2000). In addition, it should be noted that even when primer-free strategy is applied, as illustrated in Fig. 3, PCR is unavoidable, which is enabled by ligating primer binding sites to the ends of genomic sequence fragments after each round of incubation and enrichment. As discussed previously, any sequences of the random region displaying strong interaction with the constant primer binding sites may detrimentally affect PCR amplification. If it happens that the random sequences show strong interaction with the ligated primer binding sites during PCR, it may influence the success of SELEX. To resolve this problem, it is suggested to conduct parallel primer binding site experiment (during ligation steps, introducing more than one set of primer binding sites to the random region) (Zimmermann et al., 2010). At the same time, via employing next generation sequencing (NGS) technique to (1) minimize the SELEX rounds and (2) compare information collected from different primer binding site groups to identify potential sequences for functional study.

2.2.2. Constant regions rather than primer binding sites

Apart from the primer-binding site, other types of constant regions can also be observed in published SELEX libraries. One example is the partially randomized (doped) library or segmented library (Santosh and Yadava, 2014). A feature of this type of library is not total randomization, but a limited variation of nucleotides within a known sequence. This is because the purpose of this type of library is to determine and improve the binding regions of existing aptamers or for functional analysis of endogenous oligonucleotide by introducing carefully designed constant regions (Santosh and Yadava, 2014). In another case, libraries used in Capture SELEX (Istamboulie et al., 2017) provide an excellent example in which a constant region is utilized for a purpose rather than enhancing the binding capacity of libraries (Fig. 4). Originally designed to develop aptamers against small molecules (e.g. ATP, GTP, metal ions) that could not be immobilized, this type of SELEX featured by immobilizing the library sequences to a solid phase via docking the constant sequences (Fig. 4). When library sequences bind to target molecules, the interaction between library sequence and the target molecule can result in the 3-D formation change of the docking structure of some library sequences and results in sequences dissociation. Since only sequences undergoing structural shift upon target binding could be released from the solid phase, this method is suitable to develop structure-switching aptamers ready for engineering analytical sensors, such as fluorescent beacons for the detection of target molecules.

Importantly, for both of these partially randomized library pools, extra care should be given to primer- binding site design so that the primer-binding site does not conflict with the folding or accessibility of the known constant regions.

A. a typical method for minimal primer library design
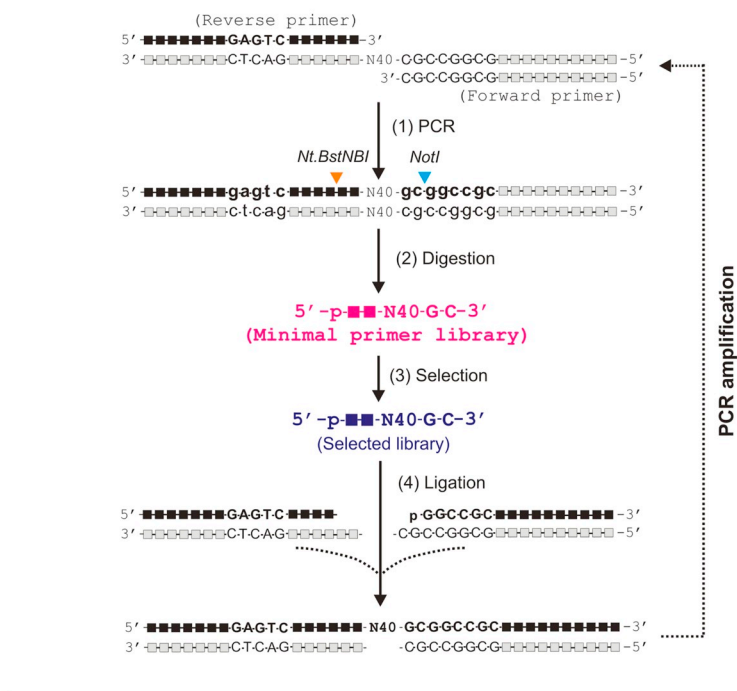
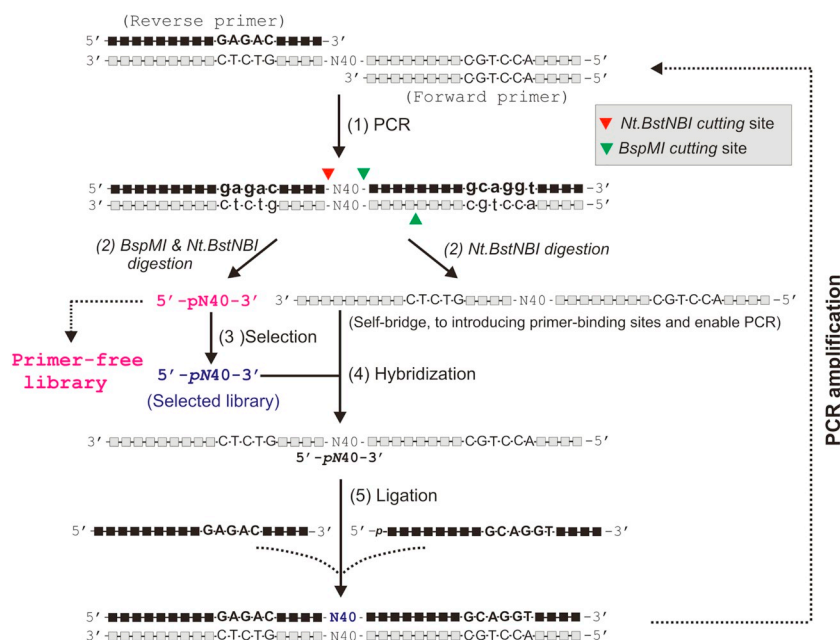**B. a typical method for primer-free library design**

Fig. 3. Illustration of typical methods for primer-free library and minimal primer library design. The design of both types of libraries takes risk of introducing extra uncertainties during restriction enzyme digestion, ligation and PCR amplification.

2.3. Introduction of functional motifs for high quality libraries

The overall success rate of developing high affinity aptamers is generally lower than their antibody counterparts (Mercier et al., 2017). One reason lies in the nature of its structure. The binding sites of an antibody are formed by six segments of variable structures composing 110-130 amino acids of 20 different species (Schroeder Jr. and Cavacini, 2010). In contrast, a typical aptamer consists of 10 – 40 nucleotides of 4 nucleotide species. The structure variations created by aptamers are much less than antibodies. Also, unlike nucleotides, different amino acids in antibodies display a much greater variety of

physiochemical properties such as charge, solubility, and pKa, which are all critical factors for target binding. In this section, we focus on strategies made in recent years aiming at expanding functional motifs and introducing protein-like properties to aptamers.

2.3.1. A simple way to increase the functional motif of initial libraries

Theoretically, during NA library synthesis, the maximal sequence diversity can be achieved by equally inputting the four types of nucleotides and thus increasing the probability of selecting high affinity aptamers. However, it was found that diversity only may not be sufficient to define a good library (Thiel et al., 2011). In three independent
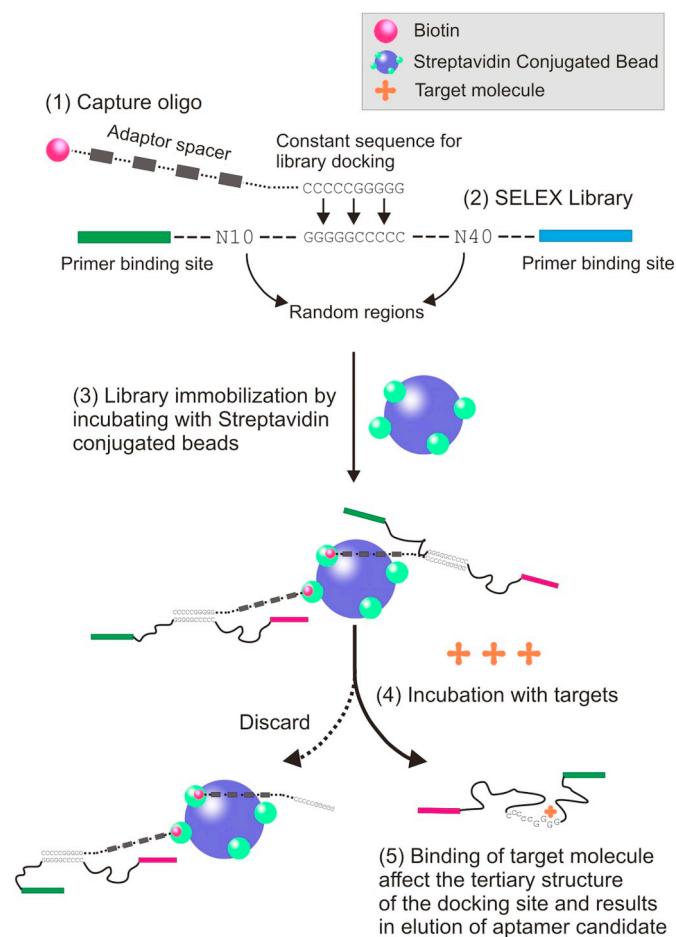
Fig. 4. Capture-SELEX provides an example to immobilizing SELEX library to avoid non-specific binding. Capture-SELEX is featured by immobilizing the library sequences to solid phase via docking of the constant structure (step 1,2,3). During target/library incubation (step 4), aptamer candidates displaying structural change of the docking structure can be eluted (5) to incubation solution.

cases, smoothly-randomized initial libraries were used for aptamer development, and it was found that the selection progress is accompanied by a steady decrease in the overall minimum free energy of the libraries and an accumulation of pyrimidine-rich sequences (Thiel et al., 2011; Takahashi et al., 2016). These findings suggest that a bias of the GC region may create libraries with improved structural stability and gain selective advantages during SELEX progress. This hypothesis was supported by a computer-based analysis of the structure distribution of the 40 nt random region of a library (Gevertz et al., 2005). As reported, a slight shift to G and C (30% each) led to the predominant formation of structures with more stems and more complex 3-D structures when compared to an A and T shift. In fact, it has long been recorded in genomic studies that genomic RNA sequences from archaea, bacterial to eukaryote all display a GC enrichment pattern, implying the capacity of this pattern to result in more complex structures for various biological functions (Mann and Chen, 2010). As a result, instead of using smoothly-randomized libraries, slight GC biased libraries with G: C: A: T proportion of 3:3:2:2 was suggested (Vorobyeva et al., 2018). In another case, when an RNA library was studied, Knight et al. proved a combination of 25% U, 15% C, 20% A, and 40% G could maximize the probability of identifying aptamers targeting targets to as high as 99% even when the size of initial library was lowered to 6.23×10^9 species (Knight et al., 2005), 10,000 times lower diversity than a standard SELEX library.

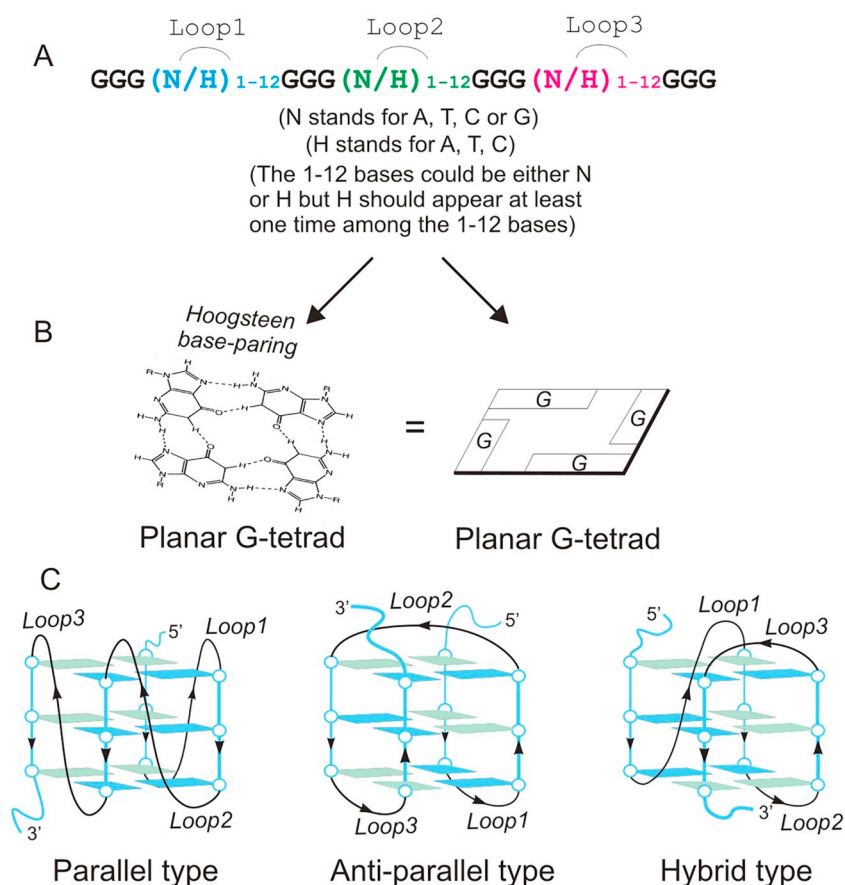### 2.3.2. Computer based library design

Inspired by the fact that library quality can be improved by simply adjusting base composition, a series of computer-based library design have been performed, aiming at providing initial libraries with optimized structural patterns for enhanced binding potential (Chushak and Stone, 2009; Kim et al., 2007; Luo et al., 2010). Indeed, as a type of genetic entity, NA libraries display a combinatorial diversity and complexity that pose many questions that are well suited to computational analysis, which is further powered by the rapidly developed NGS technology and bioinformatics approaches for functional NA motif identification.

2.3.2.1. Patterned SELEX libraries. Like proteins, aptamers require a secondary structure to bind their targets. As reported, with 4^n (equals to the number of nucleobases) species of oligonucleotides, a typical NA library contains up to 10^{15} sequences, this makes up an enormous, highly diverse library for aptamer selection (Wu and Kwon, 2016). With such a library, one would expect that aptamers can be developed for any target imaginable. Unfortunately, the success rate of SELEX is merely 30% (Famulok and Mayer, 2014; Zhuo et al., 2017), confounded by many sequences in a library displaying simple structural motifs and unstable structures, both of which would be unlikely to form high quality aptamers. By introducing designed patterns favoring information-rich structures into the random region of a library, the occurrence of functional secondary structure could be considerably increased, and therefore represents a practical way to design high efficiency SELEX libraries. During the past decade, a couple of computational approaches have been applied in this area. Moreover, effective NA distribution patterns and general disciplines for new library design have been suggested.

2.3.2.1.1. G-quadruplex. The first exploited pattern is the well-described G-quadruplex structure, a kind of high-order structure formed by G-rich sequences (Fig. 5) (Ishizuka et al., 2017). In a way similar to antibodies, G-quadruplex structures couple a common scaffold to varying loop motifs that act in target recognition (Edwards et al., 2015). Besides, the four-stranded structures of G-quadruplex allow enhanced hydrophobic binding to various types of targets such as nucleolin, hemin, and light-up fluorogens (Trachman et al., 2017; Warner et al., 2014). Unsurprisingly G-quadruplex motifs have been observed in a significant proportion of published aptamers (Marusic et al., 2013; McManus and Li, 2013; Varizhuk et al., 2016). Developing libraries with a pre-designed G-quadruplex structure is likely to increase the quality of initial libraries.

Interestingly, it was found that G-quadruplex structures in general show correlation with certain biological functions (Varizhuk et al., 2016). For example, by investigating the interaction of 12 intramolecular G-quadruplex structures in different cancer cell lines, Chang et al reported that the intramolecular parallel G-quadruplex appears to be essential for cancer-selective anti-proliferative activity (Chang et al., 2013). This observation is consistent with another study in which the G-rich libraries with structural features of G-quadruplex strongly inhibit cancer cell growth while sparing non-malignant cells (Choi et al., 2010). This implies that the general G-quadruplex scaffolds can be used to develop aptamers with particular biological functions.

In an early study, to obtain G-quadruplex enriched libraries, Zhu et al. designed three 81 nt ssDNA libraries containing up to 45% guanine bases within the 45 nt random region to evolve hemin-binding DNA aptamers (Zhu et al., 2012). Recently, Simon et al. improved this work by introducing a pre-designed G-quadruplex pattern in the random regions (McManus and Li, 2013). In this latter study, a two-layered G-quadruplex patterned library was generated by interspersing different random-sequence domains for loop formation. Via Circular dichroism analysis, the author confirmed that libraries with a minimum of three and maximum five such interspersed sequence domains are sufficient to form stable quadruplex structures featuring all three types of known quadruplexes (parallel, antiparallel, or mixed-type hybrid) for

Fig. 5. Schematic representation of the formation and structural features of G-quadruplex. (A) G-quadruplexes are formed by guanine tracts interspersed with three loops and (B) stabilised through stacked, Hoogsteen base-paired, planar G-tetrads. (C) The change of sequences in loop regions or change of loop lengths could resulted in changes of G-quadruplex topology among the three typical types.

SELEX (Fig. 5). Surprisingly, being a semi-randomized library, introducing pre-designed G-quadruplex pattern to the random region of SELEX libraries seems not to compromise their sequence diversity. As shown in the study conducted by Bugaut et al., small changes of the loop length or sequence in loop regions of certain oligonucleotides could result in dramatic changes from one stable quadruplex topology to another (Fig. 5) (Bugaut and Balasubramanian, 2008; Miyoshi et al., 2005). Theoretically, a total of 26 possible loop orientation topologies could be formed by quadruplexes containing only three loops (Webba da Silva, 2007). Consequently, the introduced G-quadruplex region significantly increases the structural complexity and diversity, and therefore potential functional motifs necessary for successful SELEX.

However, it should be noted that although G-quadruplex libraries can increase the chance of obtaining aptamers, this type of library tends to bind to a lot of targets, especially hydrophobic molecules, which raises concerns of non-specific binding (Mcrae et al., 2017). Another challenge for G-quadruplex library is the fact that these structures are relatively hard to amplify by PCR enzymes, owing to the tendency of polymerases to stall at G-tetrad sites (Ruggiero and Richter, 2018). As reported, in the presence of K^+, the melting temperature of a G-quadruplex structure could be as high as 99 °C (Stevens et al., 2015), inaccessible for currently available polymerases. Therefore, sequences containing G-quadruplex structures may display amplification disadvantages during PCR reactions of the SELEX selection (Ruggiero and Richter, 2018). Efforts have been made to solve this problem as reported by Stevens and colleagues. Although Mg^{2+} favours G-quadruplex conformation, the combination of K^+ and Mg^{2+}, as present in PCR buffer, appears to have a more significant stabilising impact on G-quadruplex structure (Stevens et al., 2015). As a result, excluding K^+ in the buffer but retaining Mg^{2+} required for the polymerase activity represents a practical method to amplify G-quadruplex libraries. To further minimise the detrimental effect of G-quadruplex on PCR reaction,

strategies such as employing strict selection conditions, high efficiency DNA polymerases, and minimizing PCR rounds by new technologies (i.e. high-resolution capillary electrophoresis) are also recommended.

2.3.2.1.2. Other types of patterned libraries for SELEX. Apart from G-quadruplex, computational approaches were used to design other types of patterned libraries including a DNA library with enriched stem-loop structures by alternating purine and pyrimidine nucleotides (Ruff et al., 2010). This study demonstrated a considerable enrichment of stem-loop structures in the patterned library as compared to a random library via the sequencing of library pools consecutively selected for 10 SELEX rounds. Aptamers targeting streptavidin, immunoglobulin E (IgE), and vascular endothelial growth factor (VEGF) were developed using this library (Ruff et al., 2010). This alternating purine and pyrimidine patterning method was further improved by Martin et al. by using UNAFold software to increase the structural complexity of a DNA library (Martin et al., 2015). First of all, a set of criteria were defined to create libraries with maximized proportion of potential binders: (1) the first nucleotide of each sequence must pair with the final nucleotide; (2) the total number of unpaired bases must be between 10 and 30 for a 50 nt long sequence; (3) there must be at least two stretches of 4-unpaired nucleotides. Then, random sequences were automatically input and eventually 50000 sequences that fit all these criteria were selected. As a result, with merely 50000 sequences incorporated onto a microarray chip, effective aptamers were selected via a single round selection. Although only thrombin was tested in this experiment, it highlights the high value of applying a computational method for library design. Importantly, although patterned pool appears to be one approach that can be readily implemented in selections for rare/complex functions (McManus and Li, 2013; Pobanz and Luptak, 2016; Ruff et al., 2010), structure-promoting patterned library itself displays less sequence diversity than ordinary libraries. It is important to conduct careful investigations to design such libraries to avoid decreased diversity and

specificity. As demonstrated by Pobanz and colleagues (Pobanz and Luptak, 2016), over-engineering the starting pools may be detrimental to the success of selection.

2.3.2.2. Smart library and in silico SELEX - future aptamer development. The incorporation of NGS and SELEX was firstly practiced in Genome-SELEX to identify endogenous ligands from genomic DNAs (Jolma et al., 2010; Roulet et al., 2002). Nowadays, the NGS technique coupled with computational algorithms enables the comprehensive identification of rare functional motifs in selected aptamers and quantification of their abundance. Since such functional motifs can be used as one descriptor for the target (Beier et al., 2014), Beier and colleagues introduced a novel randomization strategy to adjust the shape of the random region of a library to fit the structure of a certain molecule or a class of molecule. However, the developed target-specific NA library requires an extensive structural characterization of not only targets but also SELEX libraries, and therefore is too complicated to be generalized to various molecular targets. Even so, the idea of identification of common motifs from published aptamers and introducing such particular sequence or structural elements to libraries have great potential in improving the selection efficacy of libraries. Soon, the search of common motifs of aptamers against particular targets or a group of targets was extended to searching for common motifs present in aptamers targeting a vast variety of targets (Hamada, 2018). Upon recognizing a five-way junction structure as one of the most structured elements and involves in a variety of different functional roles, Luo et al. developed a computational method, named Random Filtering to generate starting DNA libraries with increased structural diversity (Luo et al., 2010). Firstly, by analyzing all of the secondary structures of a library, only sequences containing higher complexity five-way junction structures are isolated, and then, for each sequence, a set of mutant versions is generated with all four possible nucleotides at all positions not involved in base pairing. Random Filtering thus pre-enriches the starting library with highly-structured five-way junction motifs. During subsequent studies, it was found that this type of library provides complex spatial structures favorable for the selection of aptamers against targets difficult for aptamer identification with traditional NA libraries (Luo et al., 2010). Instead of simply increasing the number of five-way junctions in library pools, Genetic Filtering strategy was designed as an improved edition of Random Filtering, allowing researchers to develop library pools with desired junction structure distribution. For example, to develop libraries with 20% 1-way (1J), 2-way (2J), 3-way (3J), 4-way (4J), and 5-way (5J) junctions (Luo et al., 2010). Similar to the Random Filtering method, Genetic Filtering strategy exhibits higher structural complexity and can be used to increase the overall structural diversity of initial pools.

The aptamer affinity maturation technique represents another promising computational method for aptamer development and affinity enhancement. Evidently, although an aptamer identified by classical SELEX may not always be the best aptamer with high affinity and specificity, however, the best possible aptamer sequence can be identified from the selected aptamer family. Based on this hypothesis and existing aptamer sequence information, Julian and colleagues designed a novel library via Resample software. After a single round of micro-array-based selection, the binding affinity of the aptamer pool was improved by an order of magnitude (Kinghorn et al., 2016).

Recently, the effort aiming at developing optimised libraries has resulted in the development of computer based *in silico* selection (Rabal et al., 2016). Indeed, with the ever-growing aptamer database and rapidly progressing 3D modeling of existing aptamers, together with structural characterization of target molecules and sophisticated docking programs such as RosettaDock, 3dRPC, FTDock, GRAMM and PatchDock (Marze et al., 2018), it is possible to model and predict the target-aptamer interaction without physically conducting SELEX. However, three obstacles weigh heavily against the reliability of *in silico*

selection. Firstly, the ability to predict *de novo* target-aptamer interactions relies strictly on well-characterized aptamers. This is not really practical given the fact that current aptamer databases are still limited; secondly, most of the suggested docking programs model protein–protein interactions (Ruff et al., 2010), their validation and accuracy in predicting target-aptamer interaction is questionable. Lastly, the highly specialized selection conditions such as metal ions, temperature, and different bio-fluids make it difficult to mimic and therefore pose extra challenges to the reliability of *in silico* selection. Even so, computer based structural characterization and 2D structure clustering could greatly expand our knowledge about aptamer-target interaction and represent very promising strategies for future aptamer development.

2.3.3. Modified and synthetic genetic polymers expand the functional motif of SELEX libraries

Although aptamers are a relatively new research field, applying chemically modified genetic polymers (also known as xeno-nucleic acids or chemically modified unnatural nucleotides) on antisense oligonucleotides (AONs) for enhanced stability and minimized immunogenicity has been practiced for more than ten years before aptamers were introduced (Shen and Corey, 2018; Veedu and Wengel, 2009). Indeed, apart from desired physiochemical properties, chemically modified genetic polymers provide extra chemical spaces to expand the functional motif of SELEX libraries and therefore represent an excellent platform for novel SELEX library design. As demonstrated by a recent comparative study, chemically modified SELEX libraries, especially libraries containing hydrophobic characteristics, can generate aptamers with an affinity of nM or even pM for proteins that had repeatedly failed using conventional DNA libraries (Rohloff et al., 2014). Consequently, chemically modified libraries are currently being applied in most of the aptamer clinical studies (Yoon and Rossi, 2017) and by various leading groups such as Sullenger's group at Duke University (2′-Fluoro, 2'-O-methyl and 2'-OH nucleosides) (Blind and Blank, 2015), AM Biotech at UT Houston (Phosphorodithioate, 2'-O-methyl and X-modified dU) (Lokesh et al., 2017) and Somalogics (SOMAmers modified nucleosides) (Rohloff et al., 2014).

2.3.3.1. Existing chemically modified genetic polymers for library design. With a large bank of existing toolkits, chemical modifications can be introduced into different parts of nucleic acids such as the base (e.g. 5-methyluridine, pseudouridine, and dihydrouridine), sugar (e.g. 2′-O-methyl, 2′-Fluoro, 2′-FANA (2'-fluoroarabino nucleic acid) and LNA (locked nucleic acid), or backbone (e.g., morpholino, peptide nucleic acid, phosphorothioate and boranophosphate), though it is the sugar, especially the 2′-OH position, that has been most extensively exploited (Ni et al., 2017; Pinheiro et al., 2012; Veedu and Wengel, 2010). In fact, the only approved aptamer-based drug, Macugen, is modified in this manner, by 2′-fluoro and 2'-O-methyl substitutions (Wang et al., 2017a, 2017b). Another promising modification candidates rising in recent years is the slow off-rate (e.g., low dissociation rate constants) modified aptamers (SOMAmers) (Rohloff et al., 2014). SOMAmers are DNA aptamers containing 2′-deoxyuridine nucleotides that have modifications on the C5 position of the base. With such a modification, the uracil could be added with a variety of residues (e.g., benzyl, 2-napthyl or 3-indolyl-carboxamide) that mimic and even exceed the structural diversity displayed by amino acids. Since its first report in 2010 (Gold et al., 2010), SOMAmers targeting more than 3000 different human proteins ranging from growth factors, cytokines, enzymes, hormones, and receptors have been developed (Eid et al., 2015). Moreover, the SOMAscan™ proteomic assay based on SOMAmers is now commercially available. Recently, the structural study of SOMAmers and corresponding protein targets, once again, confirmed that the introduction of hydrophobic moieties to libraries contributes significantly to the interaction between aptamers and their corresponding targets (Davies et al., 2012; Gelinas et al., 2014). Indeed, the ability to incorporate hydrophobic moieties by chemical
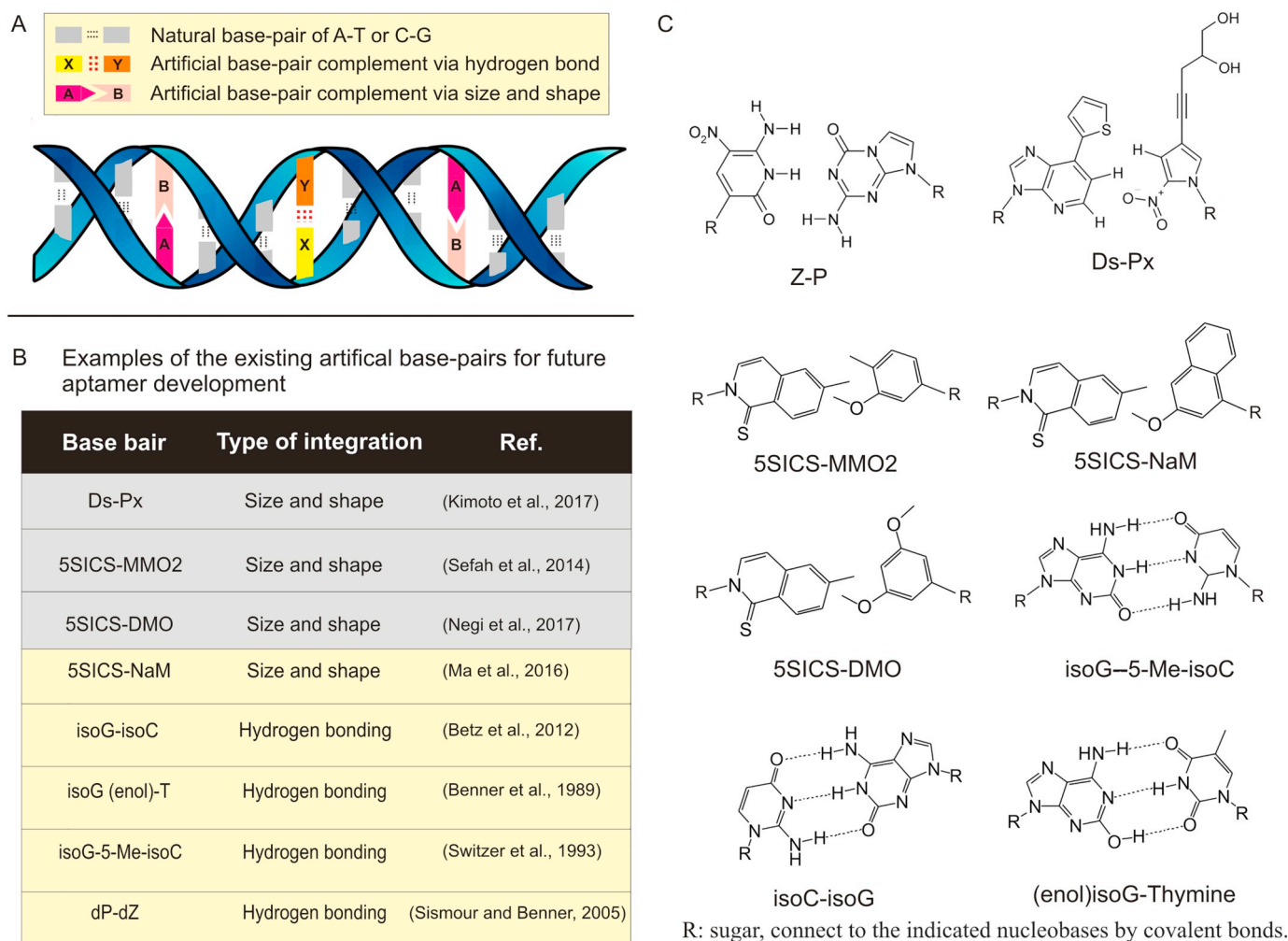
Fig. 6. The artificial base-pairs. (A) Schematic fig. of two types of artificial base-pairs; (B) Examples of existing artificial base-pairs for future aptamer development. (C) Molecular formula of artificial base-pairs.

modifications solves a longstanding weakness of aptamer technology.

2.3.3.2. Synthetic genetic polymers – new tools for future aptamer development. In the past decade, the arduous journey in search for additional base pairs (Fig. 6) have greatly expanded the understanding of biological evolution and shook our common belief of the origin of life (Chaput et al., 2012; Herdewijn and Marliere, 2009). From the view point of aptamer development, by introducing artificial base pairs apart from A-T, C-G, together with existing chemical modifications, the functional motifs of a library could be significantly expanded.

The idea of introducing an extra artificial base pair was firstly practiced by Sefah et al. in their AEGIS-SELEX (artificially expanded genetic information system - SELEX) (Sefah et al., 2014). In this study, the starting library is composed of the four naturally occurring nucleotides and two artificial nucleotides, 2-amino-8-(10-β-D-2-deoxyribofuranosyl)-imidazo[1,2-a]-1,3,5-triazin-4(8H)-one (P) and 6-amino-5-nitro-3-(10-β-D-20-deoxyribofur-anosyl)-2(1H)-pyridone (Z). With established strategies and enzymatic tools to manipulate such artificial oligo nucleic acids for PCR amplification, cloning, and sequencing, new horizons for developing aptamers with unprecedented properties was realized (Sefah et al., 2014). Recently, Morales et al. developed 7-(2-thienyl) imidazo [4, 5-β] pyridine (Ds) and 2-nitro-4- propynylpyrrole (Px) base pairs (Kimoto et al., 2017). Differing from any previously reported base pair, this base pair depends on shape complementarity rather than hydrogen bonding (Fig. 6). This invention is biologically significant as it proves that hydrogen bonding is not a definite

requirement for replication. Importantly, the strong hydrophobic property of Ds makes it SELEX-friendly. Based on this technology, a new type of SELEX library containing several artificial Ds-Px base pairs was developed by Kimoto and colleagues (Kimoto et al., 2016). The selected VEGF-165 and INF-c aptamers displayed 100-fold higher binding affinity as compared to the non-modified analogs (Okamoto et al., 2016). As a result, Ds-Px base pair was recently used by several leading aptamer groups such as TagCyx Biotechnologies - Ichiro Hirao's group (Hirao and Kimoto, 2012). Importantly, a couple of artificial base pairs either employing hydrogen bonding or shape complementarity have been reported during the past decade (Benner et al., 1989; Betz et al., 2012; Ma et al., 2016; Negi et al., 2017; Sismour and Benner, 2005; Switzer et al., 1993) (Fig. 6), they are expected to play important roles in future aptamer development.

2.3.3.3. Spiegelmers – exploiting the bio-stable feature of mirror-image oligonucleotides. Normal RNA or DNA aptamers consisting of native nucleic acids are highly susceptible to the omnipresence of nuclease enzymes in virtually all biological fluids. The serum half-life of which could be as short as several seconds (Zhou and Rossi, 2016). To endow better drug-like properties to aptamers, various techniques have been introduced. As mentioned above, the serum stability of aptamers could be significantly enhanced by employing chemically-modified SELEX libraries. However, this method comes with a problem of incompatibility in enzymatic enrichment of selected aptamers, and increased selection cost (as well as other obstacles detailed in Section

2.3.3.3). Post-SELEX modification via exchanging native nucleic acids of an aptamer with chemically modified bases represents a more popular method for enhanced aptamer physico-chemical properties including high stability (Gao et al., 2016a). However, in many cases, this stabilization process cannot be used to replace native bases to chemically-modified bases which may change the conformation of aptamers, necessary for target binding (Gao et al., 2016a). The mirror-image aptamers, firstly introduced by Klussmann and colleagues represent an alternative option in this respect (Klussmann et al., 1996). Also known as spiegelmer, mirror-image aptamers are built from non-natural L-nucleotides (mirror-image of natural D-RNA or DNA) that is not recognized by the ubiquitous plasma nucleases (Eulberg and Klussmann, 2003). Like any other enzyme in the human body, the naturally occurring nucleases responsible for the degradation of oligonucleotides consist of chiral building blocks (made of L-amino acids). Their catalysing function is strictly stereo-dependent, only native nucleic acids composed of D-nucleotides could be recognized and processed (Eulberg and Klussmann, 2003). Therefore, unlike normal aptamers, L-oligonucleotide made spiegelmers could escape from nuclease recognition rendering high bio-stability. In an early study, after a 60h incubation of an adenosine binding spiegelmer with serum, no evidence of degradation was observed (Klussmann et al., 1996). Having obvious stability advantages, various spiegelmers against pharmacologically attractive targets were reported during the past decades (Hoehlig et al., 2013; Kulkarni et al., 2007; Menne et al., 2017), and three of them (emapticap pegol, olaptesed pegol, and lexaptepid pegol) entered in clinical trials (Vater and Klussmann, 2015). Notably, all of them were tested for systematic administration for cancer treatment, unlike other aptamer related clinical trials, which are normally designed for local administration (such as Macugen). Spiegelmer technique holds great potential in expanding the current aptamer-based clinical applications.

However, being a type of aptamer made of non-natural nucleotides, spiegelmers do have disadvantages. First, spiegelmers cannot be directly obtained by employing the ordinary SELEX process (Vater and Klussmann, 2015). L-nucleotide cannot be recognized and processed by native enzymes involved in SELEX such as PCR polymerase and transcriptase/reverse transcriptase (Vater and Klussmann, 2015). As demonstrated in Fig. 7, the current spiegelmer identification relies on the development of conventional, D-config.d aptamers against a synthetic non-natural L- config.d target that is the mirror-image of the biological target of interest. Then, by synthesizing the identified aptamers with non-natural L-nucleotides, spiegelmers against the target of interests could be developed. According to the rules of symmetry, the developed L-config.d spiegelmers should be able to bind to the natural target with identical binding properties as the conventional D-aptamers bind to the

mirror-image target used for SELEX (Klussmann et al., 1996). Secondly, unlike conventional aptamers which can be developed theoretically to any targets, spiegelmers can only be developed for targets with chiral properties such as protein and peptides. Thirdly, an inherent complication of developing mirror-image spiegelmers is the requirement of synthesizing the non-naturally L- config.d form of the target of interest. If the SELEX as shown in Fig. 7 was carried out against a natural target, the developed spiegelmers would only recognize an unnatural L-configurated target. Spiegelmers are normally developed by targeting low molecular weight peptides such as epitopes or domains of the whole proteins (Kulkarni et al., 2007). In this case, to make sure that the developed spiegelmers can recognize the peptide segment in the context of the whole protein, the targeted epitopes or domains are required to be (1) physically stable; (2) located on the surface of the protein; (3) functional, or at least close to a functional site (when biologically functional spiegelmers are required) (Vater and Klussmann, 2015). Lastly, although displaying extraordinary stability, spiegelmers show an identical binding property with their D-config.d aptamers counterparts, additional efforts may be required to improve its chemical space for enhanced binding properties.

2.3.3.4. Challenges and solutions for the application of modified and synthetic genetic polymers. The expansion of the genetic code does bring along limitations. For example, for the Ds-Px base pair-based platform, the locations of the Ds nucleotides must be fixed, and a tagging system is required to keep track of their location and number in a sequence (Wang et al., 2017a). This means that the sequence is not fully randomized, thus limiting the diversity of the starting libraries. Yet the biggest challenge comes from the compatibility of existing enzymes to unnatural nucleotides (Lapa et al., 2016). Of note, many of the currently applied chemical modifications are based on strategies developed for *in vivo* application of AONs. Different from the application of AON, which does not rely on auxiliary enzymes, the incorporation of modified nucleotides especially non-hydrogen bonding base pairs into SELEX library comes at the cost of enzyme fidelity and processivity, making critical steps of SELEX such as RNA transcription, PCR amplification, and sequencing difficult (Lipi et al., 2016; Sefah et al., 2014). This is one of the reasons why such a promising strategy has not yet become routine in ordinary laboratories. To increase the compatibility of enzymes to different types of chemical modifications, various mutant versions of enzymes have been developed. By a method similar to SELEX, Pinheiro and colleagues developed a system termed self-tagging strategy to develop DNA polymerases compatible with certain chemical modifications (Pinheiro et al., 2014). Firstly, a library of mutated polymerases was constructed and then by reacting with chemically modified nucleotides, the best polymerase for a particular

(2) Synthesizing unnatural mirror-image of the interested target

(1) Target of interest (natural configuration)

L-aptamer binds the target of interest

Cannot bind unnatural mirror-image target

Resist to nuclease

Nuclease

SELEX

(3) SELEX using natural enzymes and natural D-configuration libraries

(5) Synthesizing L-configuration of the identified aptamer

(4) D-aptamer binding the unnatural mirror-image target

Fig. 7. An illustration of *in vitro* Spiegelmer selection. The mirror-image of the natural L-configuration target was synthesised using unnatural D-configuration materials. The SELEX is then carried out using natural enzymes and D-configuration natural nucleic acid libraries against the D-target. After several rounds of SELEX, an aptamer displaying binding to the D-target was identified. To produce an aptamer being able to bind the natural L-configuration target, the mirror-image of the identified D-aptamer was synthesised using unnatural L-nucleic acid, which displaying resistance to nuclease attack.

type of modification was selected. Subsequently, this method was used to develop polymerases compatible with various modifications such as cyclohexenyl nucleic acid, LNA, altritol nucleic acid, arabinose nucleic acid, and 2'-Fluoro arabinose nucleic acid (Pinheiro et al., 2012). In a recent study, via fine-tuning the modified bases and mutated DNA polymerase, even Ds–Px pair, the non-hydrogen base pair can undergo a hundred cycles of PCR with 97% fidelity (Yamashige et al., 2012). With all these efforts, nowadays, a number of mutant polymerase variants, such as Phusion, KOD XL, and Deep Vent DNA polymerase have become commercially available (Lapa et al., 2016). To be sure, with the development of more and more novel enzymes, the problem of enzymatic compatibility of synthetic nucleotide polymers will be solved soon.

Minimizing the rounds required for SELEX presents obvious advantages to address the problem of enzymatic compatibility. As discussed previously, the rapid progress in techniques such as Capillary electrophoresis, Micro-assay system, and NGS platform has enabled the development of a low round or even single round SELEX (Non-SELEX) protocols (Hirose et al., 2017). However, the limiting factor for single-round SELEX (or SELEX with minimal rounds) is that it typically requires sophisticated equipment not commonly available for regular laboratories.

A new type of SELEX procedure termed Click-SELEX provides a very creative solution to bypass the necessity of employing enzyme mutants for SELEX involving the application of chemically modified/ synthetic libraries (Pfeiffer et al., 2018; Tolle et al., 2016) (Fig. 8). As illustrated in Fig. 8, by using a 5-modified, commercially available pyrimidine nucleoside-triphosphate (e.g. by replacing the canonical thymidine building block with C5-ethynyl-2'-deoxyuridine, EdU) which can be further derivatized with bio-orthogonal chemistry, Mayer G. et al. designed a versatile platform allowing the introduction of additional chemical entities to DNA libraries (Tolle et al., 2016). Importantly, because the modification is only transiently present during the selection cycle, this method enables a high degree of compatibility with the enzymatic step of the selection processes. What's more, with a broad acceptance of diverse substrates, this method can be used to introduce large chemical entities into libraries that cannot be achieved by current techniques, thus endowing aptamers with more antibody-like properties.

To summarize this section, incorporating modified nucleotides into SELEX libraries definitely brings along substantial benefits. It helps to expand the diversity of the NA libraries for more versatility in binding with different target molecules with nM or even pM affinity. Meanwhile, the modified nucleotides also enabled improved bioavailability and safety profile, making the developed aptamers ready for various therapeutic and diagnostic applications.

3. Target preparation and its importance for bound/unbound sequence separation

3.1. Immobilization of target molecules is predominant for target preparation

A successful SELEX heavily relies on the efficient separation of library sequences bound and unbound to targets, to allow the evolution of the best fitted sequences with the progress of SELEX (Spill et al., 2016). In the first SELEX report, the separation of free sequences and target-bound sequences was conducted by specific immobilization of protein molecules via a nitrocellulose membrane (Tuerk and Gold, 1990). Firstly, the protein target and library were incubated in solution and then the mixture underwent a nitrocellulose filtration, during which proteins and sequences binding to proteins were immobilized on
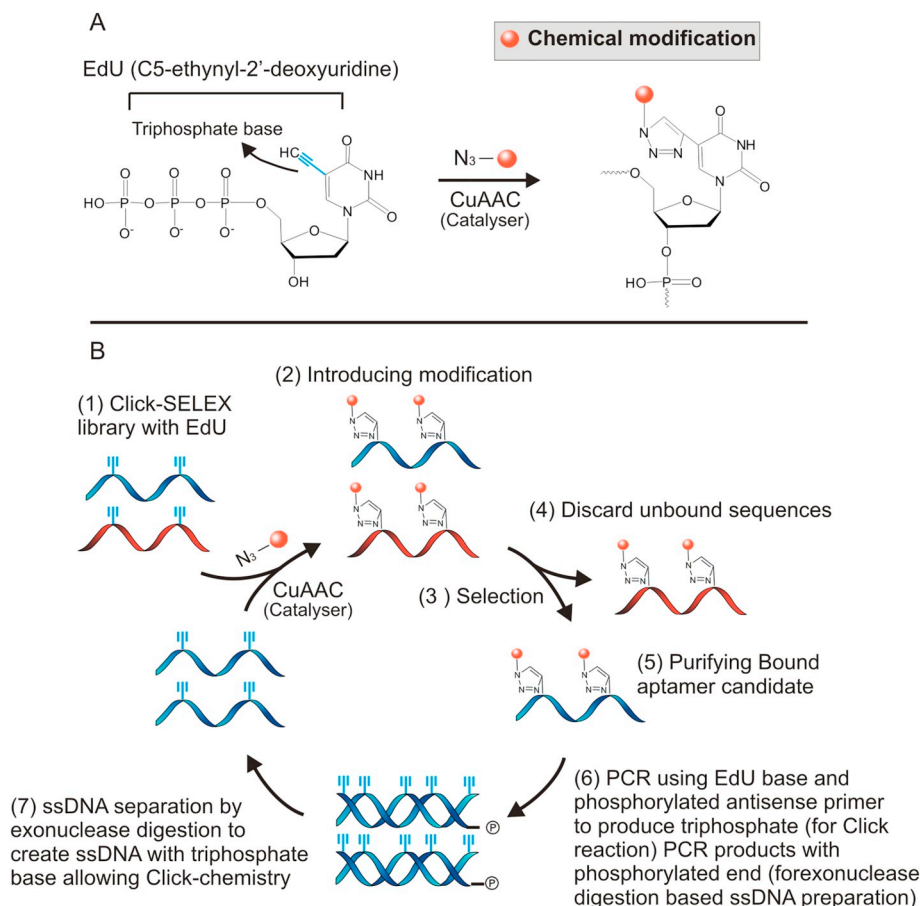

Fig. 8. Click-SELEX allows amplification of chemically modified SELEX libraries using normal PCR polymerase. The initial library is designed with 5'-modified nucleoside-triphosphate, which can be further functionalized with an azide-bearing molecule via Click chemistry (CuAAC) (A & B1,2); After incubation with the target molecule, the bound sequences are purified (B3,4) and amplified by normal PCR enzyme using the alkyne-modified triphosphate instead of thymidine (B5). Following PCR, the introduced chemical modification will be removed and the nucleoside-triphosphate moiety will be reintroduced. Then, the single stranded DNA is prepared by exonuclease digestion of the 5'phosphorylated antisense strand (B6). The chemical modification of interest can be reintroduced by CuAAC and forward to the next round of selection.

the nitrocellulose membrane and free sequences simply pass through the membrane. Because the targets were primarily proteins in the early stage of SELEX, this nitrocellulose membrane based bound/unbound sequence separation was used in several of the subsequent works. However, this method has some limitations. It only works with proteins (the non-specific affinity of nitrocellulose to amino acids is central to this technique) and is incapable of binding small peptides. In addition, because of the significant non-specific binding of free nucleic acids to the large surface area of the nitrocellulose membrane, the enrichment of target-binding aptamers could be significantly hindered, typically up to 20 selection rounds are required (Szeto and Craighead, 2014).

To facilitate easy handling and stringent washing conditions for effective bound/unbound sequence separation, prior to target/library incubation, current SELEX protocols commonly immobilize targets through solid substrates including magnetic particles (Bruno, 1997), sepharose (Soldevilla et al., 2017), agarose-based resin (Kowalska et al., 2014), microfluidic chips (Olsen et al., 2017) or glass coverslips (Lauridsen et al., 2012). Among these solid phases, the magnetic beads are perhaps the most employed. First introduced in 1997, by immobilizing targets onto magnetic beads, the separation of target-aptamer complex and unbound sequences could be achieved by a magnetic separator (Bruno, 1997). Importantly, by employing this bead based method, the selection process could be monitored by not only fluorescence based method (by introducing fluorescent tags such as FITC at 5'-end of the forward primer, to create sub-libraries with fluorescent label or introducing other molecules such as biotin to the 5'-end of forward primer and detect these molecules by fluorescent labelled antibodies), but also the more accurate calibration plot (FluMag-SELEX) (Lauridsen et al., 2012), flow cytometry (Wang et al., 2009) or ELISA (Wang et al., 2015) based strategies. In fact, research of magnetic utilization in SELEX has led to the development of various SELEX strategies including the Magnetic-Assisted Rapid Aptamer Selection (MARAS) and a couple of automatic SELEX platforms such as the microfluidic-based SELEX platform M-SELEX (Lou et al., 2009) and continuous-flow magnetic activated chip-based SELEX (CMACS-SELEX) (Blind and Blank, 2015).

The efficiency of this solid phase immobilization method could be affected significantly by two issues. Firstly, during incubation, some of the library sequences might unwantedly bind to the immobilized matrices (Darmostuk et al., 2015). To solve this problem, negative SELEX or counter-SELEX are commonly conducted by incubating libraries with matrices itself or matrices conjugated with a negative control target before incubating libraries with matrices coupled targets (Mercier et al., 2017). However, although rarely discussed, relying on negative or counter SELEX to preclude unwanted aptamers is rarely as effective as expected. As illustrated by Fig. 9, in the case of matrices/library binding via a structure-dependent mode, negative or counter SELEX do effectively eliminate non-specific binding. However, apart from structure-dependent mode, library sequences bind to matrices also follow a structure-independent mode, for example, sequences could be non-specifically captured by matrices surface or nonspecific electrostatic absorption (Szeto and Craighead, 2014). In these circumstances, negative or counter selection will be ineffective since all sequences in a library could bind to matrices with equal opportunity. Another problem of solid phase immobilization lies in the chemical reactions required to anchor the targets onto the matrices. In many cases, the immobilized target could show a structure very different from its native state (Chen et al., 2017). As a result, the binding affinity of the resulting aptamers to the native state of the target may be compromised.

3.2. Electrophoresis allows target/library incubation in a matrices-free condition

The first problem of the target immobilization method, i.e., non-specific binding introduced by matrices is a critical problem for successful SELEX. Being a classical method for protein- bound and

-unbound oligonucleotides separation, electrophoretic mobility shift assay (EMSA) presents a separation strategy with minimized non-specific oligonucleotide binding (Wang and Reed, 2012). This method starts by incubating protein targets with libraries in solution. Then the mixture is added to native PAGE or Agarose gel for electrophoresis separation. The principle of this strategy is that the mobility of oligonucleotide–target complexes and unbound free oligonucleotides are affected by their molecular weight. The oligonucleotides bound to the target have a bigger overall molecular weight than free oligonucleotides and show a slower movement. The gel blocks containing oligonucleotide/protein complexes are then collected and the aptamer sequences could be eluted by various reported methods. In line with our experience, the non-specific capture of library sequences by the gel under the electrophoresis condition is very unlikely (data not shown), which makes it both simple and promising. However, this method faces a couple of challenges. Firstly, this method is only suitable for protein targets; and secondly, very different with what happens in a denature gel, the mobility of protein, protein-aptamer complex and free library sequences are decided by not only the molecular weight, but also by the electronic charge and shape of individual molecules. It should be noticed that protein targets will surely undergo different conformational change after binding to different oligonucleotide sequences and results in a low gel resolution (Zhang et al., 2017). In addition, during gel electrophoresis, it is difficult to control some of the crucial selection conditions such as temperature, salt concentration, etc. (McKeague, 2017). Consequently, this method is not commonly used.

Powered by UV-LEDIF detection and high-resolution separation techniques, Capillary Electrophoresis-SELEX (CE-SELEX) is able to eliminate many of the disadvantages displayed by EMSA-SELEX via a gel free electrophoresis strategy (Yang and Bowser, 2013). Similar to the gel mobility method, in a CE-SELEX procedure, the targets and initial libraries are incubated in solution. After the incubation, rather than employing any solid-phase, the bound/unbound sequence separation is conducted continuously in solution under strictly controlled conditions. By performing the whole selection in solution, this method increases the number and types of viable targets (including targets smaller than the aptamer itself) for aptamer development (Yang and Bowser, 2013). Apart from great separation resolution, CE-SELEX offers flexibility to manipulate the selection stringency by varying target concentrations, separation parameters, and collection window timing (Riley et al., 2015). By employing such a strategy, one can not only effectively separate bound/unbound sequences, but also, with proper modification, separate bound sequences with different properties. Consequently, various smart aptamers with predefined Kd, k_{off}, k_{on} have been developed by novel CE-SELEX strategies such as ECEEM-SELEX (Drabovich et al., 2005) and Sweep-CE-SELEX (Okhonin et al., 2004). This not only extends the knowledge of aptamer–target complex interactions, but also is extremely useful for the development of smart drugs for therapy and diagnostics. As a result, even though the relatively limited library input allowed for a typical CE-SELEX (10^{12} for CE-SELEX versus 10^{16} for common SELEX), the high-resolution separation of CE-SELEX greatly reduces the selection rounds required for aptamer development (Yang and Bowser, 2013). In fact, CE-SELEX based single-round selection has been reported by various groups in recent years (Eaton et al., 2015; Yang and Bowser, 2013). Importantly, as a promising single/limited-round SELEX platform, the requirement for non-natural nucleic acid compatible enzymatic reactions in CE-SELEX could be considerably reduced, making it very suitable for chemically modified/synthetic non-natural nucleic acid-based libraries.

3.3. Immobilizing library sequences offers minimal non-specific binding

Although solid matrices mediated target immobilization tend to incur non-specific binding of library sequences, it could be a completely different story when the library sequences rather than target molecules were attached to solid matrices. The previously discussed Capture
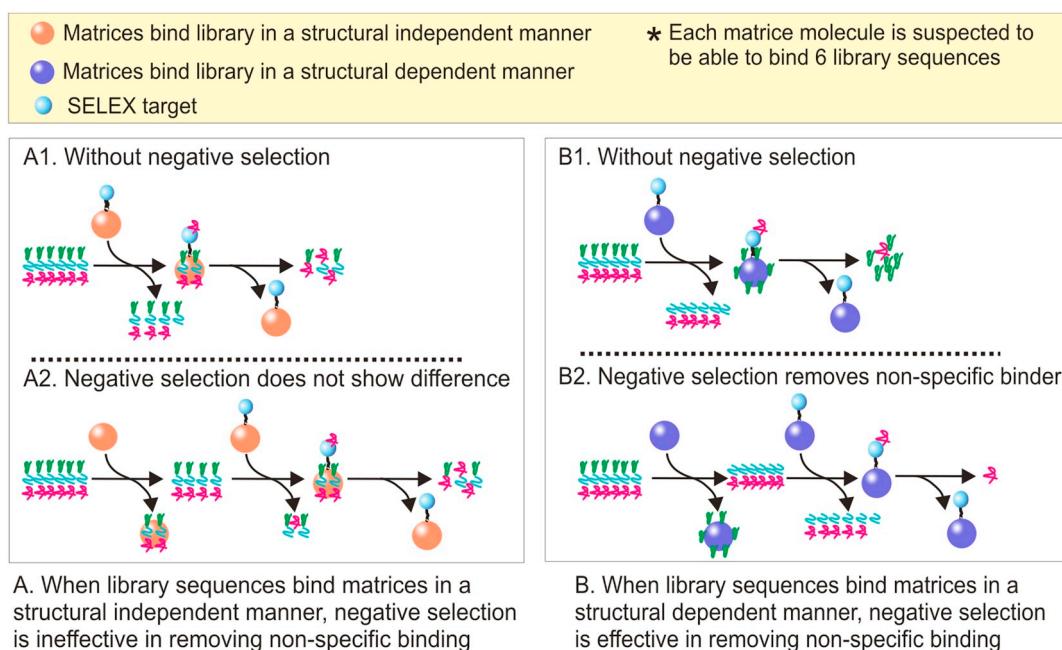
Fig. 9. Negative selection is effective only when library sequences bind matrices in a structural dependent manner. (A) When library sequences bind matrices in a structural independent manner, the conducted negative selection only minimized the library size and dose not decrease non-specific binding; in contrast, (B) when library sequences bind matrices in a structural dependent manner, negative selection can effectively remove non-specific binding of library sequences to matrices.

SELEX (Fig. 4) is an example in this aspect (Istamboulie et al., 2017). As discussed previously, Capture SELEX is characterized by immobilizing the library sequences to a solid phase. Since only sequences interact with targets and undergo structural changes could be eluted from the solid matrices, this method offers a great idea to eliminate the non-specific binding during the SELEX process. Nano-selection introduced by Martin and colleagues is another creative idea to minimize non-specific binding of library sequences to targets (Peng et al., 2007). Simply put, a library of small beads is created with each of the bead conjugating oligonucleotide with a single sequence. Then the library of beads is flowed over the immobilized target molecules on a glass cover slip. After washing, beads containing target-specific aptamers are identified via an atomic force microscope (AFM)/fluorescence microscope, and extracted from the cover slip via an AFM tip in a contact mode. Conjugating a single sequence on each bead is the key advantage of this type of SELEX. Firstly, differing from free oligonucleotides, the chance of non-specific binding of beads (plus attached oligonucleotide sequences) to target matrices is very low; besides, the conjugating library sequences on big-sized beads allows an accurate identification and extraction of the sequence of interest from the matrices and ensures a single round-SELEX. However, in this method, only one copy of an oligonucleotide sequence is conjugated to each bead, which poses a challenge for PCR amplification and subsequent sequencing. A loss of sequence information is almost unavoidable. In addition, the equipment used in this method makes it difficult to be generalized.

Recently, this protocol was significantly improved by the introduction of Non-SELEX Bead-Based Selection (Lam et al., 2015; Lokesh et al., 2017), one of the successful aptamer development platforms in the market. In this method, multiple copies of the same sequence are conjugated to individual beads to create a library of 10^9 sequence species in total. By immobilizing targets on magnetic beads rather than cover slip, the selection is simplified. Importantly, facilitated by chemically modified libraries with an improved formula, this method allows researchers to develop aptamers targeting small molecules or proteins with high binding affinities. And different from the Nano-selection where only one sequence is conjugated to each bead, with up to 10^5 identical sequences in the same bead, it is unlikely to lose the sequence information during PCR amplification and sequencing

(Table 1).

3.4. Washing buffer can be modified to develop aptamers with predesigned properties

For protein targeted aptamer selection, washing solution has been explored for effective separation of not only bound/unbound sequences, but also the separation of sequences with preferred binding properties. In 2013, the Giangrande group reported a method to elute aptamers binding to cell surface membrane via a 0.5 M NaCl solution (Hernandez et al., 2013). The principle of this method is based on the ability of NaCl to break electrostatic interaction, which is believed to be the main interaction between aptamer and protein targets (Hernandez et al., 2013). Following this idea, in the same year, Arnold et al. developed a simplified one-round SELEX to generate aptamers against Kalikreine-related peptidase 6 (KLK6) with predesigned binding affinity (Arnold et al., 2012). Firstly, proteins were immobilized on a 96-well plate and incubated with library sequences. Afterwards, unbound and non-specifically bound aptamers were washed off by NaCl solutions (gradient from 0.1M to 1M). According to this study, the most tightly bound aptamers were eluted by the highest salt concentration, thus allowing selection of aptamers with different binding properties. This is a very straightforward and inexpensive approach. However, the success of this method relies entirely on the prerequisite that the strongest binder has the most stable structure. To the best of our knowledge, this is not recorded by previous references. Surprisingly, strong denaturing detergents such as Urea and even DNase I have also been employed as elution buffer to remove non-specific or loose binding sequences after target/library incubation (Liu et al., 2012). Although effective aptamers have been developed via such methods, further studies are needed to assess if stronger binders necessarily better resist urea mediated denaturing or DNase digestion. If it is confirmed to be true, such methods would be of great value for the simple yet powerful concept it advocates.

Table 1
Current strategies for bound/unbound sequences separation.

Methods	Advantages	Disadvantages
Incubation of target & library in matrices-free condition		
Nitrocellulose membrane filtration	•Easy handling (Szeto and Craighead, 2014)	•Only for protein (depending to membrane size is incapable of binding small peptides (Tuerk and Gold, 1990) •low efficiency due to non-specific binding of nucleic acids to membrane (Szeto and Craighead, 2014)
Electrophoretic mobility shift assay (EMSA)	•Easy handling •Accessible to regular laboratory •Enable real-time monitoring selection process (Wu and Kwon, 2016) •Minimized non-specific binding (Wang and Reed, 2012)	•Only suitable for protein targets (Zhang et al., 2017) •Low resolution resulted from employment of native gel electrophoresis (Zhang et al., 2017) •Difficult to maintain stable selection conditions such as temperature, salt concentration (Wu and Kwon, 2016)
Capillary Electrophoresis	•High-separation resolution enabling single-round selection (Yang and Bowser, 2013) • Suitable for a broad range of targets, especially small molecules (Yang and Bowser, 2013) •Developing smart aptamers with predefined properties (Kd, k_{off}, k_{on}) by manipulating separation parameters (Riley et al., 2015) •Minimized non-specific binding (Eaton et al., 2015)	•Need sophisticated apparatus •Relatively limited library input (Ruff et al., 2012) •Difficult to optimize separation conditions for some proteins (Riley et al., 2015).
Immobilizing targets on solid phase & application of well-designed wash buffer		
Beads, plates or cover slips	•Easy handling (Bruno, 1997) •Suitable for a broad range of targets (Paul et al., 2009) •Accessible to regular laboratory •Enable real-time monitoring selection process (Lauridsen et al., 2012) •Enabling high-throughput selection (Lou et al., 2009)	•Non-specific binding to the immobilized matrix (Darmostuk et al., 2015) •Method employed for target immobilization could affect the native structure of targets (Chen et al., 2017).
Elute sequences with preferred properties using salt, DNase, etc.	•Simple and inexpensive (Hernandez et al., 2013) •Developing aptamers with preferred binding properties (Arnold et al., 2012)	•The reliability needs to be further investigated
Immobilizing library sequences on solid phase		
Capture SELEX	•Suitable to develop aptamers against small molecules that could not be immobilized (Istamboulie et al., 2017) •Minimized non-specific binding and selection rounds (Istamboulie et al., 2017) •Select aptamers undergoing structural-switching (Spiga et al., 2015).	•Only aptamers undergo structural-switching could be developed (Lauridsen et al., 2018) •Introducing extra variations by docking sequences (Istamboulie et al., 2017).
Nanoselection	•Minimized non-specific binding •Single round aptamer selection (Peng et al., 2007) •Aptamer candidates can be physically identified and collected via an atomic force microscope (AFM) and AFM tips (Peng et al., 2007).	•Immobilizing target molecules on glass cover slips affect the native structure of targets (Lauridsen et al., 2012) •With only one copy of oligonucleotide sequence is conjugated to each bead, loss of sequence information by PCR bias is unavoidable •Need sophisticated apparatus (Peng et al., 2007)
Non-SELEX Bead-Based Selection	•Minimized non-specific binding (Lam et al., 2015) •Single round aptamer selection (Lam et al., 2015) • Minimized the loss of sequence information by attaching multiple copies of same sequence on each bead (Lam et al., 2015)	•Immobilizing target molecules on beads may affect the native structure of targets (Lauridsen et al., 2012)

3.5. Whole cell as protein target carrier – a method to provide native state proteins for SELEX

Different to all previously described SELEX methods, which are based on the knowledge of the target for aptamer selection, in Cell-SELEX, the whole cells, either eukaryotic or prokaryotic cells, are used as targets (Civit et al., 2018). By employing proper negative controls, Cell-SELEX allows researchers to generate aptamers not only to recognize a particular type of cell, but also distinguish cells in different states (e.g., healthy cells and cells under diseases). Consequently, in recent years, Cell-SELEX has emerged as a promising strategy to identify novel biomarkers or unknown proteins of cells under study (Chen et al., 2016) (Fig. 10). Recently, a couple of aptamers developed via Cell-SELEX were used successfully in various applications such as disease diagnosis in clinical settings and microbe detection in food sciences (Song et al., 2017). Importantly, with proper modification, we believe this method can also be manipulated to develop aptamers against subcellular organelles such as exosomes and microvesicles to

tackle various challenges present in current biological fields.

Apart from recognizing whole cells, Cell-SELEX can also be employed to develop aptamers targeting specifically to proteins in their native states. As reported, protein targeted aptamers represent the majority of the currently available aptamer sequences and have been used to develop devices or drug delivery systems for various theranostic applications (Liu et al., 2011). Unfortunately, it has long been observed that aptamers selected from pure protein molecules may not be able to bind to the same proteins in their endogenous state (e.g. in cells) (Mallikaratchy, 2017). By using cells as the supplier of protein targets, this problem can be solved. In 2001, Hicke et al. introduced a hybrid SELEX that combines the advantage of Cell-SELEX and purified protein-based SELEX (Hicke et al., 2001). Firstly, tenascin-C-bearing cells were used as targets to select aptamers against tenascin-C in its native condition. After several rounds, the selection was performed using purified tenascin-C protein to improve the specificity of selected aptamers. Later, this protocol was improved by Ohuchi and colleagues by introducing cells displaying recombinant proteins as SELEX targets
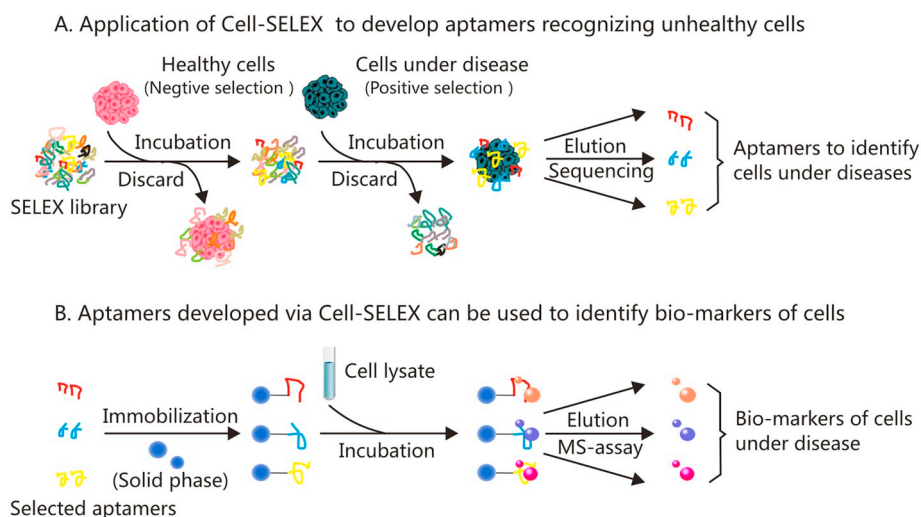
A. Application of Cell-SELEX to develop aptamers recognizing unhealthy cells

B. Aptamers developed via Cell-SELEX can be used to identify bio-markers of cells

Fig. 10. Major steps involved in Cell-SELEX. (A) Cell-SELEX allows to recognize particular cells; (B) Cell-SELEX allows to recognize bio-markers of cells.

(TECS-SELEX) and the un-transfected wild type cells as negative selection controls (Ohuchi et al., 2006). Since the protein targets are prepared in living cells, this method enables the selected aptamer to be used directly for diagnostic and therapeutic applications. Technically, using cells as protein carriers has extra benefits since the negatively charged cell surface membrane could effectively minimize the non-specific binding of DNA libraries to cells by electrostatics and ensuring higher success rate. In recent years, various Cell-SELEX derivatives such as fluorescence-activated cell sorter (FACS)-SELEX (Mayer et al., 2010), 3D Cell-SELEX (Souza et al., 2016) and Cell-internalization SELEX (Thiel et al., 2015) have been developed to select aptamers for different purposes and possess numerous merits.

One of the concerns for TECS-SELEX lies in the observation that regulating any particular gene in a cell could simultaneously affect on average the expression of 20 genes (Coate et al., 2014). However, considering the high efficiency of transient expression mediated by plasmids, the overexpression of the expected gene would be much higher than any other affected genes and therefore ensuring the transfected cells remain valid as a positive protein carrier for SELEX. Compared with the potential co-expression of irrelevant genes in TECS-SELEX, the presence of dead cells is a major pitfall for all types of Cell-SELEX (Sefah et al., 2010). This is because dead cells are likely to lead to nonspecific uptake of library sequences and cause SELEX failure. Although counter SELEX has been commonly used in Cell-SELEX to minimize the effect of non-specific binding (Sefah et al., 2010), as in any other type of SELEX, it can do nothing to sequence-independent nonspecific binding (Fig. 9) caused by dead cells. To solve this problem, microbeads have been used to remove dead cells prior to cell-library incubation. However, a much easier and efficient method is to directly use attached cells rather than cell suspension (used in many of the reported protocols) to conduct Cell-SELEX. By using attached cells, dead or weak cells cannot resist stringent washing and could be removed during the SELEX procedure. And importantly, using attached cells is beneficial as it can not only avoid cell damage during cell suspension preparation, but also minimize the non-specific binding of library sequences to incubation containers, especially plastic containers, which is apt to mediate sequence-independent nonspecific binding (Hong and Sooter, 2015).

Last but not the least, considering the predominant use of proteins as SELEX targets, we would like to address the importance of the origin of protein for aptamer selection. As recorded, due to the different process of post-translational modifications, aptamers obtained against proteins expressed in prokaryotic systems tend to fail to bind to the same target expressed in eukaryotic cells (Mercier et al., 2017). So,

when preparing proper protein targets for SELEX, care should be taken when choosing expression cell systems. For example, to develop protein targeted aptamers for human disease diagnosis or treatment, *E. coli* cells are not suitable.

4. PCR reaction and PCR bias

4.1. Emulsion PCR is a potent method to address the problem of PCR bias

The law of "survival of the fittest" is reflected in SELEX by enriching and amplifying only the library sequences displaying strong binding to a target of interest via PCR. Ideally, to effectively select the best binder from an initial library, after PCR amplification, the proportion of different sequence species in PCR amplicons should faithfully reflect the component constitute of the original template. However, it has long been observed that unlike amplifying homogeneous templates, PCR amplification of complex oligonucleotide libraries with high variety is very complicated (Polz and Cavanaugh, 1998). As each of the sequences in the initial library has different structural properties such as GC content, secondary structures, and melting temperature, their adaption to DNA polymerase, primer, and other PCR conditions could be very different. As a result, apart from the formation of the well-known PCR by-products (PCR parasites) (Fig. 11B) (Tolle et al., 2014), the PCR product tend to be biased toward shorter sequences or sequences adapt better to the applied PCR polymerases (Head et al., 2014; Witt et al., 2017), causing significant loss of sequence information during SELEX process. As demonstrated by a recent study, with a single PCR reaction, the diversity in a library could decrease by up to 50% (Levay et al., 2015). Therefore, without proper control, the SELEX process is very likely to fall into selecting sequences to fit best not to the target of interest but the PCR system. The phenomenon of PCR bias is not observed only in SELEX, it is also a significant challenge for any biochemical reaction involving PCR amplification of heterogeneous templates such as NGS (Head et al., 2014). Unfortunately, although the formation of by-products has been repeatedly reported in previous SELEX work, apart from the commonly performed preparative PCR (or analytical PCR) to determine optimum PCR cycles (Fig. 11A, will be discussed in Section 4.3), this problem is rarely addressed. Recently, a method termed RNA Aptamer Isolation via Dual-cycles SELEX (RAPID-SELEX) was introduced to minimize PCR bias by systematically eliminating unnecessary amplification steps and performing amplifications only when higher numbers of specific sequences or higher pool concentrations are required (Szeto et al., 2013). Obviously, it is not likely to completely control the effects of PCR bias by simply omitting
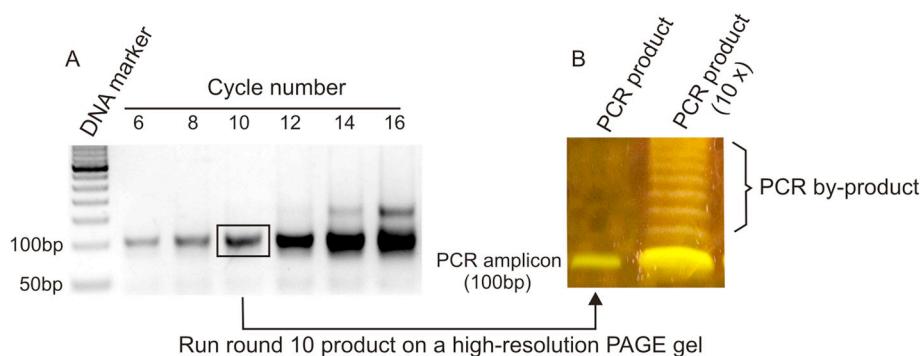
Fig. 11. The commonly used protocol to determine optimal PCR cycle is problematic. (A) The optimized PCR cycles for library amplification is commonly defined by the cycle number at which the most PCR products is created, while no PCR by-product was detected. In this case, cycle 10 is selected to prepare amplification PCR for next round of SELEX; (B) PCR product from cycle 10 was concentrated 10 times and separated on a 12% PAGE gel with the original cycle 10 PCR product as control. Clear PCR parasite bands were detected in the concentrated group, which means the undetected PCR by-product bands on the original PCR products is because of the low sensitivity of detection.

particular steps of SELEX. In the past years, the rapid development of NGS technology lead to the invention of a novel PCR variation termed Emulsion PCR (ePCR) (Kanagal-Shamanna, 2016), by encapsulating each oligonucleotide sequence into an individual PCR droplet surrounded by a hydrophobic organic phase, ePCR is able to significantly reduce the PCR bias and the formation of by-products to a non-detectable level. Nowadays, the ePCR has already emerged as a standard method for sample preparation of NGS, and the related kits are commercially available (Witt et al., 2017). This method may benefit future SELEX in a significant way.

4.2. Concerns with PCR polymerases

PCR reaction does more than just enriching the survived sequences; it has also been used to favor another important prerequisite of aptamer evolution – sequence diversity. For this purpose, both error prone PCR (introducing point mutations to parental sequences) (Pressman et al., 2017) and a more effective non-homologous random recombination (NRR technique enables DNA fragments to randomly recombine to introduce additional diversity) have been developed (Bittker et al., 2002). However, such strategies are hardly used in current SELEX practice. In contrast, high fidelity PCR polymerases have been preferentially applied by the majority of the SELEX research groups. This is mainly because the mutations and complicated procedures introduced during error-prone and NRR PCR amplification inevitably add extra uncertainties and complications to the "black box" SELEX procedure and subsequent data analysis (Blank, 2016). And the up to 10^{15} sequences present in a normal SELEX library, along with current chemical modifications, are generally believed to be sufficient to provide adequate sequence diversity for a successful SELEX. However, it has been noted that error prone PCR and NRR techniques seem promising for identification of functional motifs and improving the binding properties of existing aptamer sequences, by creating a series of structural derivatives of an exist aptamer sequence (Davis et al., 2017).

4.3. What makes an optimum PCR cycle?

The PCR procedure for SELEX commonly includes two steps, i.e., analytical (or preparative) PCR and subsequent amplification PCR (Haghighi et al., 2018). The purpose of analytical PCR is to determine the optimal number of cycles for amplification PCR by conducting PCR with a series of cycle numbers. The optimal number is typically defined as the cycle number displaying the highest yield of PCR product in the expected position without a PCR by-product (one of the main obstacles for SELEX failure (Tolle et al., 2014)) being detected (Shigdar et al., 2013a, b) (Fig. 11A). Originally being estimated as a kind of primer-primer hybridization (Musheev and Krylov, 2006), PCR by-product is a unique product associated with amplifying high diverse templates. Recently, the nature of PCR by-products was studied by Günter Mayer and colleagues (Tolle et al., 2014). According to their results, PCR by-products are initiated by partial binding of primer to complementary

bases appear on random regions of library sequences. The partial binding structure is then extended by the polymerase to yield longer DNA products with higher molecular weights than parental library sequences. Because long stretches of such by-products allow for more efficient annealing during PCR, without proper control, these ever-longer sequences can be preferentially amplified and eventually dominate the library composition (Tolle et al., 2014).

Since the generation of PCR by-products are presumably created by the base pairing combination between primers and random segments of library sequences, the formation of these products seems to be unavoidable considering the vast variety of random sequences in SELEX library. To test the rationale of eliminating parasitic PCR products by the commonly used method of reducing PCR cycles as demonstrated in Fig. 11A, we concentrated PCR products produced with the optimal cycle number. After separating the concentrated products via high-resolution PAGE, clear PCR by-product bands could be detected (Fig. 11B). Similarly, by choosing a more sensitive dye or incubating for a prolonged staining time, non-specific bands could be observed from a gel that did not initially show non-specific bands. This observation suggests that the lack of observed PCR by-products in conventional preparative PCR (with the determined optimal PCR cycle) is only because of the low sensitivity of detection.

Although reducing PCR cycle number can decrease the creation of PCR by-products and PCR bias to a lower level, the amount of PCR products required for individual rounds of SELEX is fixed (0.2 nmol for example), which means more PCR volumes have to be prepared. However, increasing PCR volume proportionally increases the amount of PCR by-product, which not only counteracts the aim of the traditional analytical PCR, but also increases the cost of PCR and subsequent ssDNA preparation or RNA transcription. Consequently, as applied in some recent SELEX protocols, we suggest the criteria for optimal cycle number of preparative PCR should be defined by the cycle number at which the most PCR products corresponding to the expected position is created (measured by either gel imaging or real-time PCR) without considering the presence of PCR by-products (Ruff et al., 2012; Damase et al., 2018).

5. Four common techniques for ssDNA preparation

Although developing DNA aptamers does not require the reverse transcription and transcription steps as RNA aptamers do, the ssDNA preparation procedure associated with DNA aptamer selection makes it not as easy as one would expect. Indeed, except for the single round-selection protocol, generation of high quality ssDNA from double-stranded PCR products is of paramount importance for successful DNA aptamer development (Svobodova et al., 2012). With more and more DNA aptamers being developed in recent years, the question of how to create high quality ssDNA has been comprehensively studied (Table 2).

To develop the first DNA aptamer back in 1992, ssDNAs were prepared by Ellington and colleagues via an asymmetric PCR amplification method (Ellington and Szostak, 1992). The asymmetric PCR is designed

Table 2
Current strategies for ssDNA separation.

Methods	Advantages	Disadvantages	Comments
Asymmetric PCR	•Lower cost (Svobodova et al., 2012)	•Complex steps of native gel separation and gel elution of ssDNA are required (Svobodova et al., 2012) •Time-consuming, low efficient with massive loss of sequence information (Marimuthu Citartan, 2012)	•Preferentially amplify one strand of the parental DNA by adding unequal molar ratio of forward and revers primers
Magnetic separation using streptavidin-coated beads	•Time saving, the whole process takes around 20 min (Shigdar et al., 2013b)	•Dissociation of streptavidin from beads results in pool quality of ssDNA and introduces extra SELEX target (Svobodova et al., 2012) •Tend to accumulate PCR by-products and result in SELEX fail (Shigdar et al., 2013b) •Extra cost required to purify PCR product (Paul et al., 2009)	•The most used Strategy •Biotiylation of the primer corresponding to the undesirable sequence to facilitate streptavidin-based separation
Lambda exonuclease digestion	•Easy to perform (Svobodova et al., 2012)	•Extra step to eliminate exonuclease could compromise the simplicity and yield of this method (Pfeiffer et al., 2018) •Insufficient enzymatic reaction results in pool ssDNA quality (Bergallo et al., 2006) •Tend to accumulate PCR by-products and result in SELEX fail (Svobodova et al., 2012)	•Introducing 5´phosphate group into the primer corresponding to the unwanted DNA strand to allow exonuclease digestion •Extra care required to control the creation of PCR by-product
Denaturing Urea-PAGE gel separation	•Better quality of ssDNA (Svobodova et al., 2012) • High separation resolution (Rahimizadeh et al., 2017) •PCR products can be directly loaded into gel (Svobodova et al., 2012) •Minimal accumulation of PCR by-products (Rahimizadeh et al., 2017)	•With traditional crush-and-soak method, recovering ssDNA from gel is time-consuming (Svobodova et al., 2012)	•Adding poly-nucleotide to one primer to result in the two strands of the PCR products with different lengths •By employing electro-elution, the whole process could be simplified

to preferentially amplify one strand of the parental DNA more than the other by adding an unequal molar ratio of forward and reverse primers. As asymmetric PCR proceeds, the primer with higher concentration can synthesize an excess of ssDNA in each cycle. Theoretically, this is a quite straightforward ssDNA preparation strategy. However, it should be noted that the eventual PCR products comprise not only single but also double stranded DNA as well as free primer sequences. Therefore, the PCR products have to be separated by non-denaturing PAGE gel to separate the different molecular weights of dsDNA, ssDNA and primers, followed by an elution step to extract the expected ssDNA from the gel (Ellington and Szostak, 1992). This extra step, especially the traditional crush-and-soak method used in gel elution of ssDNA, makes the asymmetric PCR method not only time-consuming, but also low efficient with massive loss of sequence information (Marimuthu Citartan et al., 2012). What's more, apart from the requirement to adjust the optimum primer ratios from case to case, the native gel used in this procedure adds extra concern for high quality ssDNA preparation. Although the resolution of PAGE gel with a concentration of 10-15% is high enough to separate oligonucleotide as short as 10 nt, upon loading of the asymmetric PCR product onto the native gel, unlike the dsDNAs and the homogeneous primers which display sharp bands, the position of ssDNA sequences would very likely to be smeary (Wittig and Schagger, 2005). This is caused by the heterogeneous nature of the ssDNA pool. The different intramolecular interactions such as Watson-Crick base-pairings, mismatches, non-Watson-Crick base-pairings, bulges, and loops between different sequence species inevitably result in different structural conformations (Fernandez-Millan et al., 2017). Different to denaturing gel, where the mobility of all sequences was strictly determined by the molecular weight of the particular sequence, in a native gel as used in asymmetric PCR, the mobility of sequences is affected also by other properties of sequences such as electrical charge and tertiary structures. As a result, the accurate separation of the expected ssDNA from native gel could be difficult.

Although having various limitations, asymmetric PCR method had been successfully used for developing some of the early DNA aptamers. Since then, various ssDNA preparation techniques have been explored, including denaturing urea-polyacrylamide gel (Wu et al., 2018),

lambda exonuclease digestion (Pfeiffer et al., 2018) and magnetic separation with streptavidin-coated beads (Shigdar et al., 2013b). Among them, alkaline denaturation of biotinylated PCR products attached to streptavidin-coated magnetic beads is by far the most wildly used strategy (Svobodova et al., 2012). Briefly, during PCR, the primer corresponding to the undesirable sequence is biotinylated, then the biotinylated PCR product is immobilized onto streptavidin beads. After a step of alkaline denaturation, the desired non-biotinylated ssDNA is able to be separated from the biotinylated strand/streptavidin beads and eluted into solution. Aided by the magnetic beads, the whole procedure of this method is very straightforward and typically the process takes only 20 minutes. However, problems associated with this method have been reported recently by various groups. Firstly, during the alkaline treatment, Paul and colleagues observed the dissociation of streptavidin from beads (Paul et al., 2009), which results in the undesired biotinylated strands entering into the elution and detrimentally affecting the quality of the prepared ssDNA pool by re-annealing to the complementary strands. Furthermore, the presence of streptavidin in the aptamer pool provides an extra target during the SELEX process. Indeed, unexpected obtaining of streptavidin-targeted aptamers have been repeatedly reported when such protocols were applied ((Tahiri-Alaoui et al., 2002). The trend of accumulating PCR by-products presents another challenge for the application of this method. As described previously (Tolle et al., 2014), the prolonged PCR by-products, either ladder or non-ladder type, contain more than one biotin conjugated primer sequences. Since the binding of PCR products to streptavidin beads is determined by its biotin portion, with more biotin molecules, streptavidin beads show stronger binding preference to PCR by-products than normal PCR products. Moreover, the long stretches of such by-products allow for more efficient annealing during PCR (Tolle et al., 2014), without an effective procedure to remove them from the reaction, they could accumulate with the progression of SELEX and eventually dominate the PCR products. This problem of accumulating PCR by-products is reflected by the well-recorded phenomenon that the optimal PCR cycle number (determined by maximum cycle without showing PCR by-products) reduces with the progression of SELEX when such a method was used, with typically 12-14 cycles for early rounds

dropping to 6-8 cycles for rounds afterwards (Shigdar et al., 2013b). Furthermore, since any biotin-labelled sequences could equally bind the streptavidin-labelled magnetic beads, before performing ssDNA separation, one needs to remove the biotin-conjugated free primers and primer dimers in advance via devices such as PCR product cleaning columns, which not only increase SELEX cost, but also increases the possibility of losing potential aptamer candidates.

An alternative method to streptavidin bead-based ssDNA separation is the lambda exonuclease digestion of undesired strands (Pfeiffer et al., 2018). Lambda exonuclease is a highly processive exodeoxyribonuclease that selectively digests the 5′-phosphorylated strand of dsDNA. By introducing 5′-phosphate group into the primer corresponding to the unwanted DNA strand, after the PCR reaction, this phosphorylated strand can be removed by lambda exonuclease digestion. Nevertheless, although this technique looks appealing, it is not commonly used. This is partly because the extra step of eliminating exonuclease to prevent the presence of additional target via phenol/chloroform extraction and subsequent ethanol precipitation could significantly compromise the simplicity and yield of this method. As reported, the phenol/chloroform extraction itself could result in loss of 30% nucleic acid sequences (Bergallo et al., 2006). In addition, the application of this method is also challenged by the high costs of lambda exonuclease and the inconsistent ssDNA quality caused by insufficient enzymatic processes (Svobodova et al., 2012). Furthermore, because all sequences without 5' phosphorylation will be collected in the harvest ssDNA pool, a stringent preparative PCR is required to control the creation of PCR by-products (in which the 5' phosphate could absence on both strands or present randomly on any strand) and provides only one defined length of dsDNA (Avci-Adali et al., 2010). However, as discussed in the section of 4.3, as an intrinsic feature of amplifying high diversity template pools, the PCR by-products is inevitable.

The denaturing urea-polyacrylamide gel-based ssDNA separation is another commonly used ssDNA preparation method (Wu et al., 2018). With this method, PCR is performed with a primer that contains a spacer as terminator (e.g., hexaethylene glycol) and an extension of nucleotides (e.g., poly 20A) to result in the two strands of the PCR products with different lengths. After PCR, the two strands of the amplicon are separated by denaturing gel electrophoresis. The gel block containing the desired ssDNA is then collected and the ssDNAs are extracted. Differing from the asymmetric PCR method where a native PAGE gel is employed, the different strand length of the PCR products in this method allows the utilization of a denaturing gel. Theoretically, under a denature condition, all of the components of the PCR product (including primers, unwanted long sequence containing extra oligonucleotides, expected ssDNA sequence, PCR by-product sequence) will all present in single strand forms and the mobility of them will be decided strictly by the molecular weight of individual sequences. This method therefore not only ensures an accurate separation of high-quality ssDNA by a visible manner, but also allows researchers to remove PCR by-products after each round of SELEX, eliminating the accumulation of PCR by-products with the progress of SELEX. The PCR by-products are normally undetectable even after 14 cycles of PCR in later stages of SELEX (Round 10 -12 for example) (Rahimizadeh et al., 2017). Another feature of this method, making it attractive, is that it allows the direct loading of raw PCR products into gels, thus minimizing the loss of ssDNA sequences. In contrast, as discussed previously, for both the lambda exonuclease digestion and magnetic beads-based ssDNA separation, a pre-treatment of the PCR products is required. Without the requirement of fancy equipment, and with various advantages, the traditional denaturing urea-polyacrylamide gel-based ssDNA separation is, however, very time consuming (Svobodova et al., 2012). Similar to the procedure used in the asymmetric PCR method, the classical crush-and-soak method requires up to 12 hours of incubation time to elute ssDNA from the collected gel blocks. Recently, this problem was solved by applying electro-elution via designer dialysis tubes. As reported by

an electro-elution protocol, the whole elution time could be dramatically minimized to as short as 30 min (Rahimizadeh et al., 2017).

6. Enhancing drug-like property of aptamers by Post SELEX modification

Similar with its antibody counterpart, aptamers hold great potential for therapeutic or diagnostic applications. After the binding to their targets, aptamers could serve as either an agonist or an antagonist depending on the intrinsic activity (Mayes et al., 2018). From the perspective of drug development, aptamers do have certain advantages over antibodies, such as low/no immunogenicity, prolonged shelf life, ease of quality control and importantly, the reversible pharmacology effects via complimentary oligonucleotides (Muller et al., 2008). Even so, to qualify as a drug for clinical applications, apart from sufficient targeting capacity and specificity (main concerns during SELEX process), an aptamer also needs to meet other requirements such as reduced produce cost, enhanced serum stability, and sufficient bioavailability. Fortunately, aptamers are developed and synthesized *in vitro*, endowing them with great flexibility to incorporate various chemical modifications for further improving drug-like properties without compromising on aptamer binding specificity and affinity.

6.1. Obtain short aptamer sequences via truncation and site-directed mutagenesis

Aptamers developed by the SELEX process are normally 60-100 nt in length containing a randomized region of 25–50 nt and fixed primer binding region at each terminus (Chen et al., 2017). As discussed in Section 2.2.1.1, fixed primer binding sequences generally do not contribute to the binding properties of aptamers. For this reason, during sequence data analysis, the primer binding sites are normally deleted, leaving only the random region for binding studies. The randomized sequence region of the aptamer could be further shortened by structural analysis of the developed aptamers. As reported, the binding property of an aptamer to its target is contributed mainly by more complex and information-rich structures such as the G-quadruplexes, three/five-way junctions, and hairpin structure (Luo et al., 2010; Tucker et al., 2012). Based on this knowledge, and facilitated by various multiple alignment and structural simulation software such as ClustalW, Mfold, and RNAfold, an existing aptamer can be further shortened by selectively choosing motifs likely to specifically bind to the target. Indeed, in many cases, the truncated shorter sequences do keep the same or even better binding affinity and target specificity when compared with the parent aptamer sequences (Gao et al., 2016b; Nadal et al., 2013; Sung et al., 2014). Site-directed mutagenesis represents a more accurate method to identify the binding motif of a given aptamer and obtain the shortest possible aptamer sequences (Zheng et al., 2015). For this purpose, based on secondary structure prediction, an existing aptamer needs to be truncated and point mutated to create a series of structural derivatives. After comprehensively exploring the relationship between individual truncated sequence and function of the aptamer via binding/biological functional assessment, the minimal required sequence for its binding activity could be identified (Cho and Lee, 2009; Zheng et al., 2015). For example, by introducing modifications in different positions of a thrombin aptamer, and accompanied by functional evaluation, the functional motif of this aptamer to thrombin target was identified (Avino et al., 2012). In addition, similar to previously mentioned error-prone PCR (Section 4.1) and the aptamer affinity maturation techniques (Section 2.3.2.2), this method also provides an option to further improve the binding property of an existing aptamer.

6.2. The stability of aptamers can be further improved by chemical modifications

To introduce aptamers as recognition/detection elements for *in vivo*

applications, the serum stability of aptamer is a major concern. In addition to the introduction of various chemical modifications or non-natural nucleotide analogues to improve the stability of library pools during the SELEX process, such modifications can also be introduced into an already selected aptamer. However, limited by the current technologies to predict the 3D structures of nucleic acids, the effect of introduced chemical modifications on the conformation of aptamers is difficult to predict. As a result, the post-SELEX chemical modification is generally conducted by stepwise exchange of individual bases and subsequent experimental testing. Many post-SELEX chemically modified serum-resistant aptamers have been reported during the past years including 2'-O-methyl, 2'-Fluoro, and LNA (Ni et al., 2017).

6.3. Improve the binding property of selected aptamers

Surprisingly, in many cases, improving nuclease resistance of aptamers by some chemical modification such as 2'-O-methyl also improves the binding affinity of the aptamers (Tucker et al., 1999). The increased binding property presumably comes from the higher thermal stability of the tertiary structure (Vater and Klussmann, 2015). Different from its antibody counterpart which generally displays stable structures in different incubation conditions, the change of incubation condition can dramatically reduce or even eliminate the binding affinity of aptamers. This is because the conformation of a given aptamer is affected heavily by various factors such as temperature, pH, type and concentration of cations. For example, the well characterized thrombin binding aptamer (TBA15) developed by Bock L.C. et al. was found only folding into its functional conformation in the presence of K^+ (Avino et al., 2012). Similarly, aptamers developed in PBS could display no binding property in an *in vivo* setting in serum. We firmly believe that systematic investigations to improve the thermal stability of aptamers could greatly contribute to future aptamer related applications, and progress in nucleic acid chemistry could play an important role in this aspect.

The previously mentioned error-prone PCR (Section 4.1), aptamer affinity maturation technique (Section 2.3.2.2) and site-directed mutagenesis can also be exploited to improve the binding affinity of aptamers. In a recent study, Nonaka and colleagues designed a series of mutated versions of a VEGF-binding DNA aptamer by site-directed mutagenesis using a genetic algorithm and a secondary structure prediction program (Nonaka et al., 2013). Eventually, four new aptamers with binding affinity 16-fold higher than that of the original aptamer were obtained. In another site-mutagenesis study, Bullock et al found the binding affinity of a selected aptamers could be improved by up to 30-fold (Bullock et al., 2000). The construction of bivalent or multivalent aptamers is a relatively straightforward strategy to improve the binding sensitivity and biological function of existing aptamers. Importantly, to develop polyvalent aptamers, one can use the same aptamer or use a combination of different aptamers (Vorobyeva et al., 2016; Hughes et al., 2017). Therefore, a synergistic effect of combined use of different aptamers could be achieved. For example, Mayer and colleagues designed a bivalent aptamer by using two aptamers targeting the different positions of the thrombin molecule. The resulting bivalent aptamer gave a 16.6-fold better inhibition efficiency than the binding of the monovalent ligand (Muller et al., 2008). However, it was observed that the activity of multivalent aptamers is not proportional to the number of monomers. Thus, the most suitable subunit number and linker type must be experimentally tested when constructing such structures (Musumeci and Montesarchio, 2012).

6.4. Modification for sufficient bioavailability

A typical 25 to 40 nt aptamer has a molecular weight of about 8,250–13,200 Da. Due to its small size, after systemic administration, aptamers are rapidly excreted from circulation in minutes via renal clearance. Therefore, after obtaining a serum resistant aptamer, the

plasma half-life and bioavailability of the aptamer in clinical setting is governed by the rate of kidney clearance. The threshold for glomerular filtration is around 10 nm, theoretically, particles with diameter larger than this threshold would avoid the rapid renal clearance and display prolonged circulation time (Wang et al., 2015). Therefore, the clearance rate of an aptamer could be decreased by increasing its molecular size using site-specific addition of various substances such as polyethylene glycol (PEG), lipids (Ni et al., 2017) or by attaching aptamers to the surface of nanoparticles (Jo and Ban, 2016). Among them, the PEGylation (attaching aptamer with different molecular weight of polyethylene glycol) is the most widely used strategy. In fact, this method has been used in some of the clinically tested aptamer drugs entered in clinical trials including the FDA approved aptamer drug Macugen. By attaching a 40 kD PEG, Macugen showed plasma half-life of 9.3h sufficient for most clinical purposes (Drolet et al., 2000).

Obviously, by increasing molecule size, bivalent or multivalent aptamers not only potentially increase the binding property of aptamers, they also show an advantage in terms of bioavailability (Vorobyeva et al., 2016). In short, by combined use of a polyvalent aptamer and hydrophobic groups such as PEG, the drug-like property of the aptamer could be greatly improved. In a previous study, by attaching PEG to a multivalent aptamer consists of four mIgM targeting aptamers, Mallikaratchy and colleagues obtained a chimeric aptamer structure with a particle diameter higher than 25.2 nm, well above the 10 nm threshold of glomerular filtration. As a result, this chimeric structure displayed enhanced binding specificity and desirable pharmacological properties suitable for biomedical applications (Mallikaratchy et al., 2011). To translate aptamers from bench to bedside, the post-SELEX modification is unavoidable. However, Post-SELEX optimization remains a challenging process, due to the incapability to accurately predict the effect of various modifications on the tertiary structure of aptamers. To obtain an aptamer with improved drug-like features such as enhanced affinity, serum stability, and bioavailability, a trial-and-error process is imperative. However, post-SELEX modification does provide an opportunity for aptamer-based drug development.

7. Conclusion

Aptamers are nucleic acid analogue of traditional antibodies and possess various advantages for theranostic purposes. Considering the importance of antibodies in various biomedical applications, especially the current enthusiasm in immuno-oncology therapy associated monoclonal antibodies (such as anti-PD-1/PD-L1 antibody) development (Ventola, 2017), aptamer technology holds great potential to become a new source of lead compounds and play important role in the future biomedical field. However, the lack of high performing aptamers is still a major obstacle for aptamer-based research and translational applications (Zhuo et al., 2017). Fortunately, SELEX is a very conserved process. Despite more than 32 SELEX variations have been reported during the past decades (Darmostuk et al., 2015), only a few selection approaches diverge from the core methodology of the traditional SELEX (Szeto et al., 2013). During the past three decades, with developments in material sciences and analytical techniques, dramatic improvement have made in the key steps of SELEX such as initial library design (e.g. chemical modifications, novel nucleic acids and computer-based library design and bioinformatics), target preparation (e.g. Cell-SELEX), library/target co-incubation (e.g. Capture-SELEX), and PCR amplification (e.g. Emulsion PCR). Researchers nowadays are facing an ever-growing toolkit for aptamer development and new technologies are continuing to improve selection efficiencies and reduce the efforts needed to obtain new aptamers.

Being a comprehensive procedure consisting defined iterative procedures to deal with a wide variety of targets, it should be noted that no single SELEX protocol is suitable for all applications. By carefully studying each of the particular cases, choosing proper techniques for

individual steps of SELEX, and accompanied by rational post-SELEX strategies, we believe that personalized SELEX protocols based on deep understanding of key steps of SELEX will facilitate researchers to address many of the obstacles faced by current aptamer development.

Competing interests

The authors have declared that no competing interest exists.

Acknowledgments

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