Methods 106 (2016) 21–28

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Aptamers: The "evolution" of SELEX

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ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 14 April 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
SELEX
Aptamers

ABSTRACT

It has been more than two decades since the first aptamer molecule was discovered. Since then, aptamer molecules have gain much attention in the scientific field. This increasing traction can be attributed to their many desirable traits, such as 1) their potentials to bind a wide range of molecules, 2) their malleability, and 3) their low cost of production. These traits have made aptamer molecules an ideal platform to pursue in the realm of pharmaceuticals and bio-sensors. Despite the broad applications of aptamers, tedious procedure, high resource consumption, and limited nucleobase repertoire have hindered aptamer in application usage. To address these issues, new innovative methodologies, such as automation and single round SELEX, are being developed to improve the outcomes and rates in which aptamers are discovered.

Contents

1. Introduction

Nucleic acid aptamers (DNA or RNA) are ~ 15–100 nucleotides in length oligonucleotides that are partitioned from a large random pool. These isolated sequences demonstrate high affinity and specificity for a wide range of targets, ranging from ions to complex cellular molecules and whole cells (Fig. 1). Because of their binding abilities, aptamers have great potential in many applications, such as biosensors [2,3], imaging probes [6,7], and pharmaceutical agents [9,10].

Aptamer molecules were first identified in 1990 by two independent laboratories, Gold and Szostak groups [13,14]. Both utilized unique purifications from a combinatorial library of nucleic acid sequences with high affinity for a particular target by repetitive rounds of partitioning and amplifications [13,14], shown in Fig. 2. In essence, selection begins with the generation of a large diverse oligonucleotide library with predesigned primer-binding domains for PCR amplification. The library is introduced to a target of interest; and sequences demonstrating affinity for the target of interest are separated from those unbound sequences. Next, the bound oligonucleotides are collected and PCR amplified for subsequent rounds of enrichment. This process is repeated until the library converges onto a collection of sequences that demonstrate affinity of the target of interest. Finally, the final library is cloned

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http://dx.doi.org/10.1016/j.ymeth.2016.04.020
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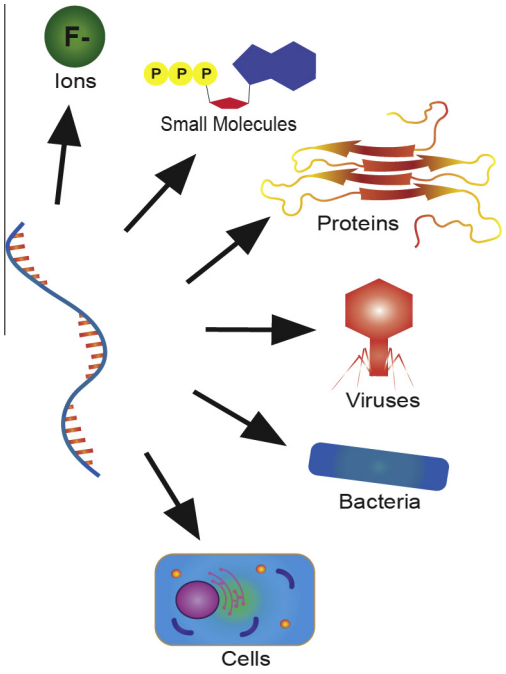

Fig. 1. Versatiliy of aptamers. Variety of target molecules that aptamers have been selected for that demonstrate high specificity and affinity.

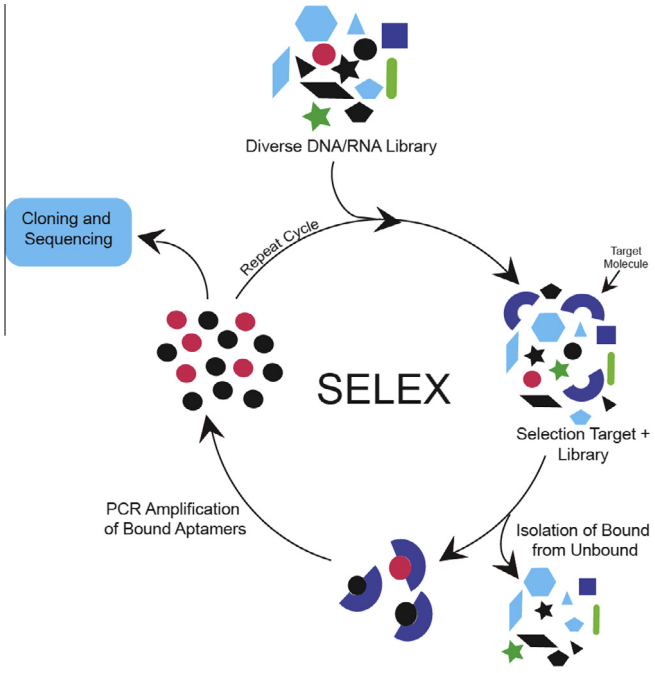

Fig. 2. Schematics of *in vitro* selection. *In vitro* selection begins with the generation of a diverse library of DNA or RNA molecules. The library is then introduced to a target ligand and sequences demonstrating affinity towards the target molecule are isolated from any unbound sequences. The bound sequences are then collected and PCR amplified for subsequent rounds of enrichment. Multiple rounds are performed until the library converges on to a collection of sequences with affinity for the target molecule.

and sequenced. Utilizing this process, Turek et al. isolated RNA aptamer sequences against bacteriophage T4 DNA polymerase and Ellington et al. isolated RNA aptamer sequences against various organic dyes. Although the technique is the same, the two unaffiliated laboratories coined this process different terms—SELEX (Systematic Evolution of Ligands by Exponential Enrichment) from Gold laboratory and *in vitro* selection from Szostak laboratory [13,14].

Since their discovery, aptamers have demonstrated to have many desirable properties. For example, once an aptamer molecule is obtained and sequenced, large amount of it can be synthesized via phosphoramidite chemistry at a low cost [1,20,21]. The ease of production only marks the beginning of its advantages over other competing technologies, such as antibodies. Unlike the *in vivo* production of antibodies, aptamers are isolated via an *in vitro* method; thus, no animal or cell lines are used in their isolation or production [1,21–23]. In addition, the process of aptamer selection can be manipulated to obtain aptamers that bind at a specific region of a target and function under an array of conditions [1,21]. During the *in vivo* generation of antibodies, the antigenic sites are dictated by the animal's immune system, and their optimal functionality is restricted to physiological conditions. This limits the extents in which antibody technologies can be applied [1,21]. In contrast to antibodies, aptamers are much easier to conjugate to molecules, such as fluorophores and quantum dots [22,24,25]. Aptamers also have a temperature stability advantage compared to antibodies. High temperature usually renders antibodies permanently denatured and useless, while aptamer molecules can still recover their native active conformation, allowing them to survive multiple usages [1,21]. Furthermore, aptamers have shown to exhibit low to no toxicity or immunological effects, which are highly desirable for pharmaceutical usage [23,26]. Though aptamers contain various advantageous traits, their main limitation is their sensitivity to nucleases (especially with RNA aptamers) which can be detrimental to an *ex vivo* and *in vivo* applications. However, their stability against nucleases can be improved via various chemical modifications [26–28]. As demonstrated by many, the malleability of aptamers holds much potential.

2. Challenges in the aptamer field

Only a few aptamer-based technologies and pharmaceutical agents are used today. Unfortunately, there is no simple answer as to why this is. In a report by Geoffray Baird, the major issues in the aptamer field are described as the "thrombin problem" [23,29]. Essentially, the major efforts have been focused on the known aptamers, such as the thrombin aptamer, for novel design strategies (Table 1) rather than expending the efforts and energies in isolating new aptamers for practical usage [23,29]. At a glance, a quick search on PubMed leads to total hits of 340 articles on the thrombin aptamer, while only 150 hits for the vascular endothelial growth factor (VEGF) aptamer (a FDA approved drug, Macugen, for neovascular age-related macular degeneration [9,10]) and 22 hits for a prostate specific antigen (PSA) aptamer. Other reasons that contribute to the problems in the aptamer field includes 1) the process of isolating new aptamers is currently repetitious, tedious, and

Table 1
Thrombin aptamer platforms. There are various platforms that have utilized the thrombin aptamer as proof of concept.

Thrombin based biosensors	
Systems	References
Electrochemical sandwich assay coupled to magnetic beads	[1]
Gold nanoparticles enhanced SPR aptasensor	[4]
Label-free electrochemical biosensor	[5]
Aptamer-based surface enhanced resonance Raman scattering (SERRS) sensor	[8]
Molecular beacons	[11]
Catalytic molecular beacons	[12]
Cyclic enzymatic signal amplification (CESA)	[15]
Single-walled carbon nanotube (SWCTs) with aptamer	[16]
Aptamer-aptamer proximity based assay	[17–19]

time intensive because of suboptimal SELEX methods [30,31], 2) the high costs associated with SELEX [30], 3) limited abilities of the polymerases to accommodate for modified and unnatural nucleic acids [31–33], and 4) relatively short half-life *in vivo* due to nuclease degradation and renal clearance [27,34–36]. Despite these issues, aptamer molecules hold much promise as detection and pharmaceutical agent that deserves to be explored.

3. Optimizing *in vitro* selection methods

3.1. Automated SELEX

Automated SELEX methods are characterized by the ability to perform multiple SELEX cycles without supervision, intervention or manual manipulation. Advantages of this method include: 1) increase the number of selection rounds per day and 2) multiple selections can be done in parallel. Automated SELEX method designs hinge on either a larger scale liquid-handler based design (that relies on a small volume liquid-handler robotics platform with various modifications and attachments to complete each step), or a microfluidic centered paradigm [37]. Liquid-handler based automated SELEX platforms have been widely reported. Cox et al. utilized a Biomek 2000 platform modified with a thermocycler and a filtration module to isolate RNA aptamers [38,39]. This platform has been cited as the basis for multiple subsequent automated selection attempts for such targets as amyloid fibrils [40], the virus causing foot and mouth disease [41] and PSA [42].

Eulberg et al. have also designed a liquid-handler based automation method, building upon Cox et al. They utilized a RoboAmp 4200 handler, modified with a microwell lid manipulation system, a vacuum manifold with ultrafiltration cassettes (for RNA purification), as well as a fluorescence detection module to monitor PCR cycling [43]. An online monitoring tool was also fabricated for ease of progress tracking. This method was able to isolate a new RNA aptamer against substance P (a neuropeptide), with a K_d of 40 nM, compared to the previous aptamer at 190 nM [43]. Eulberg's method still necessitated performing the initial selection rounds manually to ensure diversity in the starting library since the system is limited on the amount of material that can be handled in the microtiter plates by the robot [43]. Only two selection rounds could be completed per day compared to approximately 6 per day for Cox et al., primarily due to differences in incubation times.

Microfluidic-based methods have also been fruitful in automating SELEX. A microline approach designed by Hybarger et al. utilized a fluidics platform similar in appearance to high pressure liquid chromatography systems, including pressurized fluid lines of 75–100 μm diameters, and fast-switching selection and routing valves [44]. Selection reagents are preloaded into an array of storage lines with input library loaded into a sample loop. PCR was performed in a microline embedded in a modified thermocycler. This platform benefits from a fully enclosed system, simplified liquid handling, and lowered costs in consumables and reagents. Hybarger et al. were able to isolate a sequence with this platform identical to one isolated in tandem using standard SELEX [44]. Since then, other aptamers have been isolated using this method [45,46]. Automating the selection method had demonstrated to decrease the time required to accomplish multiple rounds of SELEX in a single day. With the development of microfluidic system, selections can be miniaturized and potentially less costly.

3.2. Modified and unnatural nucleobases

With four natural occurring nucleobases, a possible of 4^n (n equals to the number of nucleobases) number of oligonucleotides can be produced. This makes up an enormous, highly diverse DNA library for SELEX. With such a large library, one would think that an aptamer can be generated for any target imaginable. Unfortunately, previous findings have shown that not every molecule is compatible in binding a negatively charged nucleic acid molecule [47]. In order to increase the probabilities in generating aptamers for these elusive molecules, researchers looked toward expanding the genetic code beyond the four natural nitrogenous bases. In this section, the expansion of the nitrogenous bases repertoire will be focused.

One method in increasing the nucleoside repertoire is via the use of modified natural nucleobases [47,48]. For example, 2′-deoxyuridine triphosphates can be synthesized with various functional groups (such as benzyl, naphthyl, tryptamino, isobutyl, etc.) at the 5-position via amide linkage [48]. The attachments help with the stabilities of aptamers by improving 3-diminsional structures [47,48]. In addition, the different functional groups can improve the interaction between the aptamer and its target molecule. For instance, the addition of aromatic and aliphatic functional groups can help increase the hydrophobicity of DNA polymers, which provides additional interaction between the aptamer and some more hydrophobic molecules [47,48].

The SELEX methodology remains the same. However, a starting library with modified nucleotides is generated by reverse transcription from a template library. Unfortunately, the incorporation of modified natural nucleotides comes at a cost of polymerase fidelity and processivity [47,48]. Vaught et al. discovered that KOD XL (*Pyrococcus* species GB-D) and D. Vent (*Thermococcus kodakaraensis* KODl) polymerases, along with time manipulation, solved the problem [47,48]. Using this method, Gold et al. found that aptamers containing functional groups with hydrophobic characteristics typically yielded highest binding affinity SOMAmers (Slow Off-rate Modified Aptamers) [47]. In a comparative experiment, it was found that modified natural nucleotide aptamers are a step up from the traditional unmodified aptamers. Gold et al. demonstrated that the modified nucleotide aptamers were capable of generating high affinity aptamers for human proteins that had repeatedly failed using SELEX with conventional DNA polymers [47].

Another method of expanding the genetic alphabet is through the use of unnatural nucleobases, as shown in Fig. 3. Unlike the modified natural nucleobases, which can form hydrogen bonds with the naturally occurring nitrogenous bases, the unnatural nucleotides form a third base-pairing. To prove the concept, Kimoto et al. chose 7-(2-thienyl) imidazo [4,5-b] pyridine (Ds) and 2-nitro-4-propynylpyrrole (Px) in a SELEX experiment where he and his colleagues generated an aptamer containing adenine (A), guanine (G), thymine (T), cytosine (C), and 7-(2-thienyl) imidazo [4,5-b] pyridine (Ds) [49]. The nucleoside chosen in this case (Ds) is a highly hydrophobic unnatural base. Since Ds. and Px are complements of each other, amplification with unnatural nucleotide was possible. In their study, it was demonstrated that the addition of few Ds. bases could increase the structural diversity of DNA molecules and improve the performance and property of aptamers [49]. Like the modified nucleotide aptamers, the addition of the unnatural nucleotides is mainly to improve the hydrophobicity of DNA polymers. Utilizing this methodology, Kimoto et al. generated aptamers for VGF-165 and INF-γ [49]. In a functional comparison analysis between the unnatural nucleotide containing aptamers were found to be superior to their natural counterparts [49].

The expansion of the genetic code does bring along some limitations. For example, the locations of the Ds. nucleotides were fixed and a tagging system was needed to keep track of their location and how many were in the sequence [49]. This means that the sequences are not completely randomized; thus, the starting library diversity is limited. An additional challenge is that since

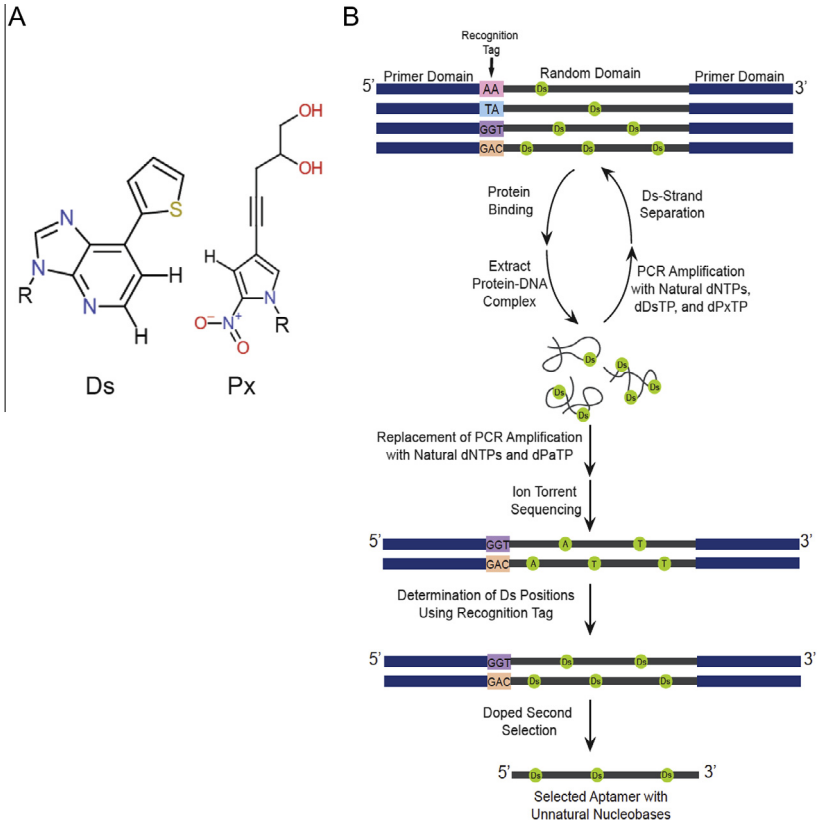

Fig. 3. Schematic on unnatural nucleobases selection. A) Chemical structures of Ds. and Px unnatural nucleobases ulitized by Kimoto et al. in their selection [46]. **B)** Selection scheme utilizing unnatural nucleobases. Selection begins by mixing 22 different synthetic DNA sublibraries to generate the selection pool. Each sublibrary contains sequences with 1–3 Ds. bases at predetermined positions. The selection methodology remains the same. However, Ds-Px base-pairs along with the natural nucleotides are used to perform asymetric PCR for subsquent rounds and sequencing required the replacement of all unnatural nucleotides [46].

these nucleotides are not naturally occurring, the biological system will not recognize them; hence, amplification is limited and sequencing is not possible [49]. Additional steps are required to clone and sequence the aptamers.

Another recent method in expanding the genomic repertoire is through the use of an artificially expanded genetic information system (AEGIS) [50–55]. In the work of Sefah et al., AEGIS was applied in an aptamer selection scheme. In principle, the starting library of an AEGIS-SELEX is composed of the four naturally occurring nucleotides and two artificial nucleotides. These synthetic nucleotides are [2-amino-8-(1′-β-D-2-deoxyribofuranosyl)-imidazo[1,2-a]-1,3,5-triazin-4(8H)one (P) and 6-amino-5-nitro-3-(1′-β-D-2′-deoxyribofur-anosyl)-2(1H)-pyridone (Z) [52]. Thus, this generates a GACTZP xNA biopolymer with three base-pairing sets (A:T, G:C, and Z:P). One of the features of AEGIS is that these artificial nucleotides retain Watson-Crick pairing rules of complementarity (steric complementarity and hydrogen bonding) [50–55]. Essentially, AEGIS gives rise to a system that allows for base-pairing, amplification, and evolution like natural genetic polymers while capable of carrying higher informational density and provide more functional groups [50–55]. To test this method, a standard protocol of cell-SELEX was applied. After 12 rounds of selection, an AEGIS-aptamer was found to have a K_d of 30 nM for a breast cancer cell-line, MDA-MB-231, which has been known to be a challenging and medically interesting target [52,56,57]. However, like the aforementioned methods, AEGIS still requires a specific polymerase for amplification and specialized techniques for sequencing [53,54]. Overall, this helps expand the genomic alphabet and increase the diversity of aptamers.

Incorporating modified nucleotides in SELEX definitely brings along great benefits. It helps with expanding the diversity of the DNA polymers for more versatility in binding with different target molecules. As some studies have demonstrated, the modified nucleotides also improved the kinetics of binding between aptamer and its target. However, the major disadvantages of both modified and unnatural nucleobases SELEX methods are the requirement of complicated chemistry for nucleotide synthesis and specialized polymerases and amplification techniques [49]. Additionally, modified and unnatural nucleobases interrupt cloning and sequencing of aptamers. Despite those complications, modified and unnatural nucleobases SELEX have shown to outperform conventional aptamers and should be further explored.

3.3. One round SELEX

Although SELEX is simple and straight forward, the process itself is tedious and repetitious. Each round of selection takes up to days to accomplish. Not to mention, the large amount of reagents and target molecules wasted performing 5–20 cycles of SELEX and amplification [30,58,59]. These limitations have led researchers to strive for improvements in SELEX efficiency. One way to do so is performing a single round of selection to achieve desirable aptamers without the need for subsequent rounds of enrichment. One of the first occurrences in single round selection was observed in a study done by Berexovski et al. utilizing an electrophoretic method known as Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) [58]. In essence, gel-free capillary electrophoresis is used to separate free and ligand-bound DNA molecules. First, the DNA library and desired target molecules are mixed until equilibrium is reached. This is followed by gel free capillary electrophoresis under nonequilibrium conditions to partition the equilibrium DNA-target mixture. Since

the migration pattern depends on the "size to charge" ratio of molecules and all the oligonucleotides have the same size, all the DNA molecules essentially migrate at the same rate [58]. Thus, the DNA-target complex is expected to have a different migration time compared to the free DNA, and vice versa for target molecules alone. Based on this principle, DNA-target complexes can be partitioned from the free DNA and free target molecules. Finally, fractions containing the complex are collected and amplified. Using this methodology, a farnesyltransferase (PFTase) aptamer was identified in a single round of selection with 1 nM K_d [58]. This was the first documented study where strong binding aptamers were identified via a single round of SELEX [58]. Although utilizing capillary electrophoresis in SELEX has been well established [58,60–63], a disadvantage of this methodology lies in the size limit of the target molecules. The mobility of the DNA-target complex should migrate at different rates compared to that of the free sequences in order for fraction collections [37]. Small molecules might not allow for a significant migration between bound and unbound aptamers for proper fractions to be collected.

Microfluidic techniques have also achieved single round selection. Lou et al. utilized magnetic bead covered proteins integrated with microfluidics technology to isolate high-affinity aptamers via a single round of SELEX, or M-SELEX [30]. The M-SELEX approach begins by incubating the DNA library with the magnetic bead covered proteins. The mixture is separated using a continuous flow magnetic activated chip-based separation (CMACS) device. The device is coupled with magnets strategically located to aid with the separation of desired aptamers. This methodology is extremely fast and simple to perform.

Another single round SELEX came from a study done by Liu et al. utilizing DNase I to digest unbound and loosely bound DNA sequences [59] (shown in Fig. 4). The essence of this methodology revolves around the concept that DNAs that are tightly bound to proteins can be protected from DNase I digestion [59,64,65]. This procedure requires purified proteins and serum proteins to be blotted onto two individual polyvinylidene fluoride membranes. In this selection, the serum protein membrane acts as a counter selection to remove any nonspecific aptamer sequences. Both membranes are then co-incubated in a well with the random DNA library. The removal of the desired target membrane isolates the bound from any non-specific DNA sequences that are bound to the serum membrane. Upon transferring, the target membrane is washed with an increasing concentration of urea to remove any weak binding sequence. Finally, to achieve highly specific aptamer sequences after a single round of selection, the target-membrane is treated with DNase I to remove any unbound and weakly bound sequences, thus leaving only strong binding aptamers. Using this methodology, Liu et al. isolated two aptamers, A-18 and A-38, against Hepatitis B virus core protein (HBcAg) with K_d values of 0.14 nM and 0.16 nM, respectively [59].

Achieving aptamers in a single round of selection definitely increases likelihood of more investigators to identify aptamers for more relevant target molecules. The short selection scheme undeniably reduces the time required to obtain aptamer

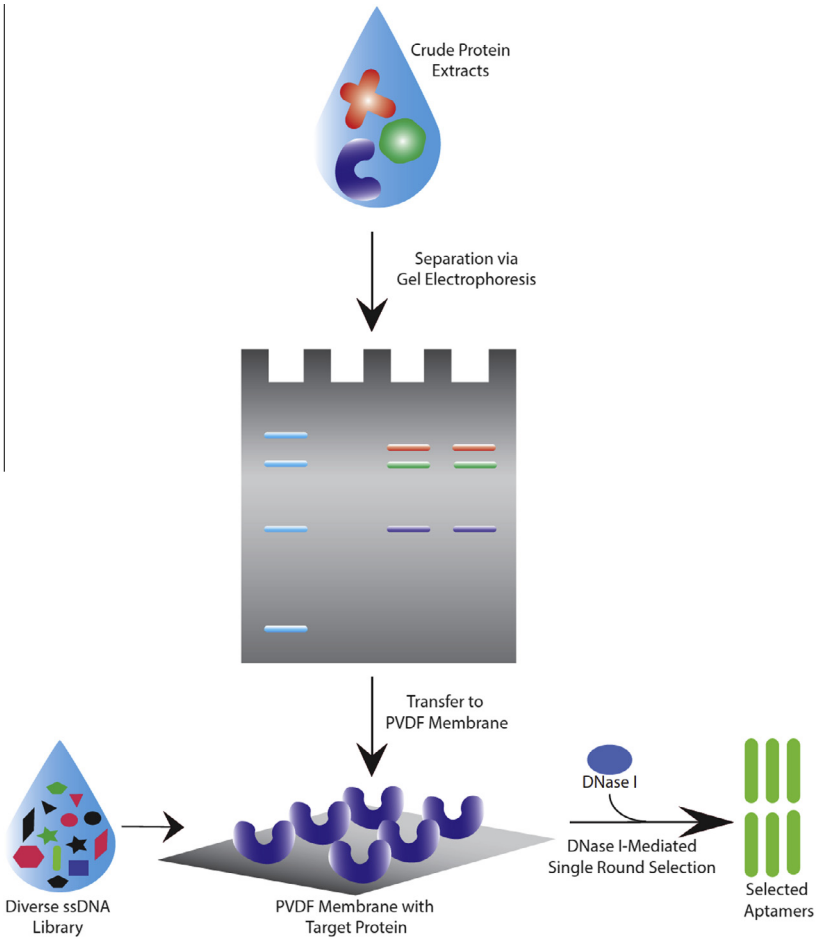

Fig. 4. Schematic on DNase I mediated single rounded *in vitro* selection. Target proteins are purified and blotted on to a polyvinylidene fluoride (PVDF) membrane. A diverse ssDNA library is then introduced to the PVDF membrane with target moluecles. After incubation, DNase I treatment is used to digest any unbound or weakly bounded sequences to achieve single-round selection [48].

sequences. This technique coupled with recent advances in next generation sequencing (NGS) can quickly allow one to examine the selected pool [66]. However, these particular selection schemes eliminate the important trait in SELEX: evolution. Performing a single round of selection removes the element of enrichment via subsequent rounds. Enrichment can potentially improve the overall fitness of a giving library via insertion of mutations during polymerase amplification. Subsequent rounds of selection also allow one to increase the stringency of the selection condition to further isolate the optimal fit sequences from a given diverse library. The drawback of a single round selection is that the fit sequences are selected from a given library without room for improvements via multiple rounds of selection (mutations and increase stringency of the selection conditions).

4. Discussion

Aptamers are extremely versatile molecules that demonstrates to have high applicable potentials. Although many different types of aptamers have been identified via the variation of the SELEX technique, the main steps of SELEX are conserved, which includes: 1) selection of ligand-specific sequences that bind to a target; 2) separation of aptamer vs. non-aptamer sequences via affinity methods; 3) amplification of functional aptamer sequences [58]. However, the aptamer field still contains many hurdles before it can be fully adapted in the scientific community and its potentials are recognized. Many researchers attempted to manipulate the selection scheme to improve the rate in which aptamers can be achieved as above mentioned. A potential trait of SELEX that makes it worthy of pursuing is that it enables the isolation of functional DNA and RNA molecules (binding, ligation, catalysis, etc.) from enormous, randomized pools without any prior knowledge of target molecules or binding interaction with its ligand [67,68].

In an ideal SELEX scenario, each cycle should contain rapid positive and negative selection processes and efficient and precise partitioning of unbound from bound aptamers, as well as, efficient separation of bound aptamers from its target molecules [58]. Amplification of remaining binding aptamers should be quick, followed by efficient purification of the amplified aptamer pool so that it may be fed back into the beginning of the system. Furthermore, SELEX should have the abilities to select for aptamers with predefined binding specificity. Unfortunately, SELEX, like any other techniques, is not without limitations.

The inefficiency of SELEX lies mainly with the high reagent consumption and time demands [30,31]. However, the methodologies in pushing for shorter cycles of SELEX, as reviewed here, have made SELEX easier and more affordable to pursue. The idea of obtaining aptamers after a single round of selection is fantastic; however, by doing so, a SELEX factor, evolution, will be eliminated. At which point, the selection method is no different from a molecule screening protocol, except the SELEX-library is a variable and will require sequencing to elucidate the selected molecules.

Aptamer selections can easily be compared to a Darwinian population dynamic model or Darwinian natural selection. The huge diversity in the starting DNA or RNA pool represents the genes in a common ancestor. As different selection pressures (i.e. environment) are introduced to the population, certain traits are more favored than others. Over time those traits are selected and evolved to best suit the population of the species. Essentially, SELEX is the same way. A selection pressure (i.e. selection scheme, buffer conditions, desired target) is introduced to the diverse population and sequences that function under those conditions get selected and amplified. During amplification, mutations can be introduced (or induced intentionally via error prone PCR [69,70]) to the sequences where it can be further enhanced. Evolution in SELEX is very crucial in obtaining a family of highly specific aptamers for their targets. One study of evolution in SELEX was done by Famulok, where he reported the evolution of an L-arginine specific RNA aptamer from an initial L-citrulline specific RNA aptamer [71]. In his report, he noted that minor alterations in the sequence motif could lead to changes in substrate specificity and affinity [71]. Other reports relating to evolution in SELEX focused more on evolving or obtaining a secondary functionality among aptamer species, such as deoxyribozymes and ribozymes. The studies demonstrated that these aptamer species can gain or evolve to obtain a secondary function, such as structure switching [6,72], cleavage activity [67], or ligation [73], via multiple rounds of selection. For example, Chiuman et al. showed that DNAzyme sequences that were weaved out in earlier rounds, or abandoned, can be evolved to be robust DNAzymes via multiple rounds of re-selection [74]. Evolution definitely plays an important role between subsequent rounds of selection. Presumably, polymerase amplifications between subsequent rounds insert mutations to the selection library, hence the enrichment. Enrichment can provide better fitness to a given selection library after few rounds of amplification. However, one can see how this trait is an important and useful tool but not a necessity, especially with some of the emerging minimum round selections.

5. Future directions

With all these new techniques, the number of aptamer sequences will continue to grow exponentially. More recently, databases dedicated to cataloging identified sequences of aptamers and the characteristics of the identified aptamers (structural information, selection conditions, and binding affinities) have been developed [75–77]. These comprehensive databases can provide insights on better library and selection designs. With the ever growing sequence library of identified aptamers, a possible future direction for the aptamer field will be performing *in vitro* selection utilizing computational methods. The next step in the innovation of SELEX will take advantage of the aptamer database which will provide structural data from available aptamer-ligand structures. Using free energy calculations and understanding binding energy between molecules could provide the means for performing SELEX computationally without the expenses and the tedious routines of performing experimental *in vitro* selection. Recently, computational aptamer selection, or *in silico* selection, have been explored using various program [78–86]. One of the early works in this field was done by Chushak et al. Using computational methods (structural predictions and docking), a pool composed of 994 random and 6 known sequences (well characterized aptamers) was tested against small molecule ligands. From this approach, known aptamer sequences were found to ranked in the top 5% of the best structures with optimal binding affinities for its respective ligand [79]. Of course, computational techniques will only provide possible constructs for sequences that bind to a target of interest. Also, *in silico* selection is potentially limited by the abilities to mimic highly specialized selection conditions (bio-fluids); and its ability to predict de novo ligand-aptamer interactions relies strictly on well-characterized aptamers. Thus, experimental measures will still be needed for confirmation of the selected sequences. Even if the screened sequences do not demonstrate high affinity for a particular target of interest, computational techniques will reduce the diversity of sequences that can then be further evolved via SELEX to have high affinity and specificity. Consequentially, computational techniques can provide a starting point for SELEX with predetermined library diversity. Especially, combining computational methods with microarrays could provide a synergetic tool for aptamer selection [79].

References

[1] S. Centi, S. Tombelli, M. Minunni, M. Mascini, Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads, Anal. Chem. 79 (2007) 1466–1473.
[2] C. Yang, S. Jockusch, M. Vicens, N.J. Turro, W. Tan, Light-switching excimer probes for rapid protein monitoring in complex biological fluids, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 17278–17283.
[3] W. Zhao, W. Chiuman, M.A. Brook, Y. Li, Simple and rapid colorimetric biosensors based on DNA aptamer and noncrosslinking gold nanoparticle aggregation, ChemBioChem (2007).
[4] Y. Bai, F. Feng, L. Zhao, C. Wang, H. Wang, M. Tian, et al., Aptamer/thrombin/aptamer-AuNPs sandwich enhanced surface plasmon resonance sensor for the detection of subnanomolar thrombin, Biosens. Bioelectron. 47 (2013) 265–270.
[5] Y. Xiao, A.A. Lubin, A.J. Heeger, K.W. Plaxco, Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor, Angew. Chem. Int. Ed. Engl. 44 (2005) 5456–5459.
[6] R. Nutiu, Y. Li, Structure-switching signaling aptamers: Transducing molecular recognition into fluorescence signaling, Chemistry (2004).
[7] R. Yamamoto, P.K.R. Kumar, Molecular beacon aptamer fluoresces in the presence of Tat protein of HIV-1, Genes Cells (2000).
[8] H. Cho, B.R. Baker, S. Wachsmann-Hogiu, C.V. Pagba, T.A. Laurence, S.M. Lane, et al., Aptamer-based SERRS sensor for thrombin detection, Nano Lett. 8 (2008) 4386–4390.
[9] E. Cunningham, A. Adamis, M. Altaweel, L. Aiello, N. Bressler, D. D'Amico, et al., A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular edema, Ophthalmology 112 (2005) 1747–1757.
[10] E.W.M. Ng, D.T. Shima, P. Calias, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease, Nat. Rev. Drug Discovery 5 (2006) 123–132.
[11] N. Hamaguchi, A. Ellington, M. Stanton, Aptamer beacons for the direct detection of proteins, Anal. Biochem. 294 (2001) 126–131.
[12] J.J. Li, X. Fang, W. Tan, Molecular aptamer beacons for real-time protein recognition, Biochem. Biophys. Res. Commun. 292 (2002) 31–40.
[13] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.
[14] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science (New York, N.Y.) 249 (1990) 505–510.
[15] J. Li, H.-E.E. Fu, L.-J.J. Wu, A.-X.X. Zheng, G.-N.N. Chen, H.-H.H. Yang, General colorimetric detection of proteins and small molecules based on cyclic enzymatic signal amplification and hairpin aptamer probe, Anal. Chem. 84 (2012) 5309–5315.
[16] H. Li, Y. Zhang, Y. Luo, X. Sun, Nano C60: a novel, effective, fluorescent sensing platform for biomolecular detection, Small 7 (2011) 1562–1568.
[17] S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, Protein detection using proximity-dependent DNA ligation assays, Nat. Biotechnol. 20 (2002) 473–477.
[18] D.D.A. Giusto, W.A. Wlassoff, J.J. Gooding, Proximity extension of circular DNA aptamers with real-time protein detection, Nucleic Acids Res. 33 (2005).
[19] E. Heyduk, T. Heyduk, Nucleic acid-based fluorescence sensors for detecting proteins, Anal. Chem. 77 (2005) 1147–1156.
[20] M.M. Ngundi, N.V. Kulagina, G.P. Anderson, C.R. Taitt, Nonantibody-based recognition: alternative molecules for detection of pathogens, Expert Rev. Proteomics 3 (2006) 511–524.
[21] S. Tombelli, M. Minunni, M. Mascini, Aptamers-based assays for diagnostics, environmental and food analysis, Biomol. Eng. 24 (2007) 191–200.
[22] R. Stoltenburg, C. Reinemann, B. Strehlitz, FluMag-SELEX as an advantageous method for DNA aptamer selection, Anal. Bioanal. Chem. 383 (2005) 83–91.
[23] G.S. Baird, Where are all the aptamers?, Am J. Clin. Pathol. 134 (2010) 529–531.
[24] J. Liu, Z. Cao, Y. Lu, Functional nucleic acid sensors, Chem. Rev. 109 (2009) 1948–1998.
[25] A. Nitsche, A. Kurth, A. Dunkhorst, O. Pänke, H. Sielaff, W. Junge, et al., One-step selection of Vaccinia virus-binding DNA aptamers by MonoLEX, BMC Biotechnol. 7 (2007) 48.
[26] B. Wlotzka, S. Leva, B. Eschgfäller, J. Burmeister, F. Kleinjung, C. Kaduk, et al., In vivo properties of an anti-GnRH Spiegelmer: an example of an oligonucleotide-based therapeutic substance class, Proc. Natl. Acad. Sci. 99 (2002) 8898–8902.
[27] W.A. Pieken, D.B. Olsen, F. Benseler, H. Aurup, F. Eckstein, Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes, Science (New York, N.Y.) 253 (1991) 314–317.
[28] P.E. Burmeister, S.D. Lewis, R.F. Silva, J.R. Preiss, L.R. Horwitz, P.S. Pendergrast, et al., Direct in vitro selection of a 2'-O-methyl aptamer to VEGF, Chem. Biol. 12 (2005) 25–33.
[29] H. Lin, W. Zhang, S. Jia, Z. Guan, C.J. Yang, Z. Zhu, Microfluidic approaches to rapid and efficient aptamer selection, Biomicrofluidics 8 (2014) 41501.
[30] X. Lou, J. Qian, Y. Xiao, L. Viel, A.E. Gerdon, E.T. Lagally, et al., Micromagnetic selection of aptamers in microfluidic channels, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 2989–2994.
[31] M.T. Bowser, SELEX: just another separation?, Analyst 130 (2005) 128–130
[32] S. Klußmann, A. Nolte, R. Bald, V.A. Erdmann, J.P. Fürste, Mirror-image RNA that binds D-adenosine, Nat. Biotechnol. 14 (1996) 1112–1115.
[33] A. Nolte, S. Klussmann, R. Bald, V.A. Erdmann, J.P. Fürste, Mirror-design of L-oligonucleotide ligands binding to L-arginine, Nat. Biotechnol. 14 (1996) 1116–1119.
[34] J.M. Healy, S.D. Lewis, M. Kurz, R.M. Boomer, K.M. Thompson, C. Wilson, et al., Pharmacokinetics and biodistribution of novel aptamer compositions, Pharm. Res. 21 (2004) 2234–2246.
[35] E.W.M. Ng, D.T. Shima, P. Calias, E.T. Cunningham, D.R. Guyer, A.P. Adamis, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease, Nat. Rev. Drug Discovery 5 (2006) 123–132.
[36] L.C. Griffin, G.F. Tidmarsh, L.C. Bock, J.J. Toole, L.L. Leung, In vivo anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracorporeal circuits, Blood 81 (1993) 3271–3276.
[37] R.K. Mosing, M.T. Bowser, Microfluidic selection and applications of aptamers, J. Sep. Sci. 30 (2007) 1420–1426.
[38] C.J. Cox, P. Rudolph, A.D. Ellington, Automated RNA Selection, Biotechnol. Prog. 14 (1998).
[39] J.C. Cox, A.D. Ellington, Automated selection of anti-protein aptamers, Bioorg. Med. Chem. 9 (2001) 2525–2531.
[40] D.H.J. Bunka, B.J. Mantle, I.J. Morten, G.A. Tennent, S.E. Radford, P.G. Stockley, Production and characterization of RNA aptamers specific for amyloid fibril epitopes, J. Biol. Chem. 282 (2007) 34500–34509.
[41] M. Ellingham, D. Bunka, D.J. Rowlands, N.J. Stonehouse, Selection and characterization of RNA aptamers to the RNA-dependent RNA polymerase from foot-and-mouth disease virus, RNA 12 (2006) 1970–1979.
[42] M. Svobodova, D.H.J. Bunka, P. Nadal, P.G. Stockley, C.K. O'Sullivan, Selection of 2′F-modified RNA aptamers against prostate-specific antigen and their evaluation for diagnostic and therapeutic applications, Anal. Bioanal. Chem. 405 (2013).
[43] D. Eulberg, K. Buchner, C. Maasch, S. Klussmann, Development of an automated in vitro selection protocol to obtain RNA-based aptamers: identification of a biostable substance P antagonist, Nucleic Acids Res. 33 (2005).
[44] G. Hybarger, J. Bynum, R.F. Williams, J.J. Valdes, J.P. Chambers, A microfluidic SELEX prototype, Anal. Bioanal. Chem. 384 (2006) 191–198.
[45] H.-C.C. Lai, C.-H.H. Wang, T.-M.M. Liou, G.-B.B. Lee, Influenza A virus-specific aptamers screened by using an integrated microfluidic system, Lab Chip 14 (2014) 2002–2013.
[46] H.-I.I. Lin, C.-C.C. Wu, C.-H.H. Yang, K.-W.W. Chang, G.-B.B. Lee, S.-C.C. Shiesh, Selection of aptamers specific for glycated hemoglobin and total hemoglobin using on-chip SELEX, Lab Chip 15 (2015) 486–494.
[47] D.R. Davies, A.D. Gelinas, C. Zhang, J.C. Rohloff, J.D. Carter, D. O'Connell, et al., Unique motifs and hydrophobic interactions shape the binding of modified DNA ligands to protein targets, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) 19971–19976.
[48] J.D. Vaught, C. Bock, J. Carter, T. Fitzwater, M. Otis, D. Schneider, et al., Expanding the chemistry of DNA for in vitro selection, J. Am. Chem. Soc. 132 (2010) 4141–4151.
[49] M. Kimoto, R. Yamashige, K.-I. Matsunaga, S. Yokoyama, I. Hirao, Generation of high-affinity DNA aptamers using an expanded genetic alphabet, Nat. Biotechnol. 31 (2013) 453–457.
[50] S.A. Benner, Understanding nucleic acids using synthetic chemistry, Acc. Chem. Res. 37 (2004) 784–797.
[51] M.M. Georgiadis, I. Singh, W.F. Kellett, Structural basis for a six nucleotide genetic alphabet, J. Am. Chem. Soc. 137 (2015) 6947–6955.
[52] K. Sefah, Z. Yang, K.M. Bradley, S. Hoshika, E. Jiménez, L. Zhang, et al., In vitro selection with artificial expanded genetic information systems, Proc. Natl. Acad. Sci. 111 (2014) 1449–1454.
[53] Z. Yang, F. Chen, B.J. Alvarado, S.A. Benner, Amplification, mutation, and sequencing of a six-letter synthetic genetic system, J. Am. Chem. Soc. 133 (2011) 15105–15112.
[54] Z. Yang, F. Chen, S.G. Chamberlin, S.A. Benner, Expanded genetic alphabets in the polymerase chain reaction, Angew. Chem. Int. Ed. 49 (2010) 177–180.
[55] L. Zhang, Z. Yang, K. Sefah, K.M. Bradley, Evolution of functional six-nucleotide DNA, J. Am. Soc. 137 (2015) 6734–6737.
[56] K. Sefah, K.-M.M. Bae, J.A. Phillips, D.W. Siemann, Z. Su, S. McClellan, et al., Cell-based selection provides novel molecular probes for cancer stem cells, Int. J. Cancer 132 (2013) 2578–2588.
[57] K. Zhang, K. Sefah, L. Tang, Z. Zhao, G. Zhu, M. Ye, et al., A novel aptamer developed for breast cancer cell internalization, ChemMedChem 7 (2012) 79–84.
[58] M. Berezovski, A. Drabovich, S.M. Krylova, M. Musheev, V. Okhonin, A. Petrov, et al., Nonequilibrium capillary electrophoresis of equilibrium mixtures: a universal tool for development of aptamers, J. Am. Chem. Soc. 127 (2005) 3165–3171.
[59] Y. Liu, C. Wang, F. Li, S. Shen, D.L. Tyrrell, X.C. Le, et al., DNase-mediated single-cycle selection of aptamers for proteins blotted on a membrane, Anal. Chem. 84 (2012) 7603–7606.
[60] A. Drabovich, M. Berezovski, S.N. Krylov, Selection of smart aptamers by equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM), J. Am. Chem. Soc. 127 (2005) 11224–11225.
[61] S.D. Mendonsa, M.T. Bowser, In vitro evolution of functional DNA using capillary electrophoresis, J. Am. Chem. Soc. 126 (2004) 20–21.
[62] S.D. Mendonsa, M.T. Bowser, In vitro selection of high-affinity DNA ligands for human IgE using capillary electrophoresis, Anal. Chem. 76 (2004) 5387–5392.

[63] S.D. Mendonsa, M.T. Bowser, In vitro selection of aptamers with affinity for neuropeptide Y using capillary electrophoresis, J. Am. Chem. Soc. 127 (2005) 9382–9383.
[64] D.J. Galas, A. Schmitz, DNAse footprinting: a simple method for the detection of protein-DNA binding specificity, Nucleic Acids Res. 5 (1978) 3157–3170.
[65] S.E. Smith, A.G. Papavassiliou, A coupled Southwestern–DNase I footprinting assay, Nucleic Acids Res. 20 (1992) 5239–5240.
[66] T. Schütze, B. Wilhelm, N. Greiner, H. Braun, F. Peter, M. Mörl, et al., Probing the SELEX process with next-generation sequencing, PLoS ONE 6 (2011).
[67] M.M. Ali, S.D. Aguirre, H. Lazim, Y. Li, Fluorogenic DNAzyme probes as bacterial indicators, Angew. Chem. Int. Ed. (2011).
[68] S.J. Klug, M. Famulok, All you wanted to know about SELEX, Mol. Biol. Rep. 20 (1994) 97–107.
[69] A.A. Beaudry, G.F. Joyce, Directed evolution of an RNA enzyme, Science (1992).
[70] N. Lehman, G.F. Joyce, Evolution in vitro of an RNA enzyme with altered metal dependence, Nature Publishing Group (1993).
[71] M. Famulok, Molecular recognition of amino acids by RNA-aptamers: an L-citrulline binding RNA motif and its evolution into an L-arginine binder, J. Am. Chem. Soc. (1994).
[72] Z. Liu, S.H. Mei, J.D. Brennan, Y. Li, Assemblage of signaling DNA enzymes with intriguing metal-ion specificities and pH dependences, J. Am. Chem. Soc. 125 (2003) 7539–7545.
[73] A.K. Sharma, J.M. Heemstra, Small-molecule-dependent split aptamer ligation, J. Am. Chem. Soc. 133 (2011) 12426–12429.
[74] W. Chiuman, Y. Li, Revitalization of six abandoned catalytic DNA species reveals a common three-way junction framework and diverse catalytic cores, J. Mol. Biol. 357 (2006) 748–754.
[75] J.F. Lee, J.R. Hesselberth, L. Meyers, A.D. Ellington, Aptamer database, Nucleic Acids Res. 32 (2004).
[76] J. Cruz-Toledo, M. McKeague, X. Zhang, A. Giamberardino, E. McConnell, T. Francis, et al., Aptamer base: a collaborative knowledge base to describe aptamers and SELEX experiments, Database 2012 (2012).
[77] M. McKeague, E.M. McConnell, J. Cruz-Toledo, E.D. Bernard, A. Pach, E. Mastronardi, et al., Analysis of in vitro aptamer selection parameters, J. Mol. Evol. 81 (2015) 150–161.
[78] R. Ahirwar, S. Nahar, S. Aggarwal, S. Ramachandran, S. Maiti, P. Nahar, In silico selection of an aptamer to estrogen receptor alpha using computational docking employing estrogen response elements as aptamer-alike molecules, Sci. Rep. 6 (2016) 21285.
[79] Y. Chushak, M.O. Stone, In silico selection of RNA aptamers, Nucleic Acids Res. 37 (2009).
[80] R. Das, J. Karanicolas, D. Baker, Atomic accuracy in predicting and designing noncanonical RNA structure, Nat. Methods 7 (2010) 291–294.
[81] C. Detering, G. Varani, Validation of automated docking programs for docking and database screening against RNA drug targets, J. Med. Chem. (2004).
[82] J. Hoinka, E. Zotenko, A. Friedman, Z.E. Sauna, Identification of sequence-structure RNA binding motifs for SELEX-derived aptamers, Bioinformatics 28 (2012) 1215–1223.
[83] P. Jiang, S. Meyer, Z. Hou, N.E. Propson, H.T. Soh, MPBind: a Meta-motif-based statistical framework and pipeline to Predict Binding potential of SELEX-derived aptamers, Bioinformatics (2014).
[84] R.R. Rahrig, N.B. Leontis, C.L. Zirbel, R3D Align: global pairwise alignment of RNA 3D structures using local superpositions, Bioinformatics (2010).
[85] M. Sarver, C.L. Zirbel, J. Stombaugh, A. Mokdad, FR3D: finding local and composite recurrent structural motifs in RNA 3D structures, J. Math. Biol. 56 (2008) 215–252.
[86] S. Zhang, K. Kumar, X. Jiang, DOVIS: an implementation for high-throughput virtual screening using AutoDock, BMC Bioinformatics (2008).